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Dectin-1 expression by macrophages and related antifungal mechanisms in a murine model of *Sporothrix schenckii sensu stricto* systemic infection

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ABSTRACT

The available information about the role of Dectin-1 in sporotrichosis is scarce. Hence, we aimed to assess Dectin-1 expression by macrophages and the activation of some related antifungal mechanisms during the *Sporothrix schenckii sensu stricto* infection as a first attempt to elucidate the role of this receptor in sporotrichosis. Balb/c mice were intraperitoneally infected with *S. schenckii sensu stricto* yeast ATCC 16345 and euthanized on days 5, 10 and 15 post-infection, when the following parameters were evaluated: fungal burden in spleen, Dectin-1 expression and nitric oxide (NO) production by peritoneal macrophages, as well as IL-1β, TNF-α and IL-10 *ex vivo* secretion by these same cells. Peritoneal macrophages were *ex vivo* challenged with either the alkali-insoluble fraction (F1) extracted from the *S. schenckii* cell wall, a commercially available purified β-1,3-glucan or whole heat-killed *S. schenckii* yeasts (HKss). Additionally, a Dectin-1 antibody-mediated blockade assay was performed on day 10 post-infection to assess the participation of this receptor in cytokine secretion. Our results showed that Dectin-1 expression by peritoneal macrophages was augmented on days 10 and 15 post-infection alongside elevated NO production and *ex vivo* secretion of IL-10, TNF-α and IL-1β. The antibody-mediated blockade of Dectin-1 inhibited cytokine production in both infected and non-infected mice, mainly after β-1,3-glucan stimulation. Our results suggest a role for Dectin-1 in triggering the immune response during *S. schenckii* infection.

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1. Introduction

Sporotrichosis is an emergent subcutaneous mycosis affecting humans and other animals worldwide, but mainly in tropical and subtropical regions. This disease is caused by the traumatic inoculation of dimorphic fungi from the *Sporothrix* genus, including *Sporothrix brasiliensis, Sporothrix globosa, Sporothrix mexicana, Sporothrix luriei, Sporothrix albicans, and Sporothrix schenckii sensu stricto* [1,2]. These organisms are commonly found in the soil, surviving in different environmental conditions while developing resistance mechanisms against the host immune system [3,4]. Sporotrichosis is clinically characterized by cutaneous and subcutaneous lesions with regional lymphocutaneous dissemination. Systemic infections may also occur, mainly in immunosuppressed individuals [5].

Early activation of the immune system largely depends on pattern recognition receptors (PRRs), many of which involved in the recognition of fungal pathogens [6,7]. Several studies performed in our lab evidenced a role for different PRRs, including the Toll-like receptors (TLRs) 2 [8] and 4 [9,10] and the NOD-like receptor (NLR) family member NLRP3 [11], as important innate components triggered during the *S. schenckii* infection. β-Glucans are a heterogeneous group of natural polysaccharides widely localized in the fungal cell wall and possessing immune-modulating activities through their ability to bind Dectin-1, a

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member of the C-type lectin receptor family [12]. This interaction is involved in the immune recognition of several pathogenic fungi, including *Candida albicans, Aspergillus fumigatus, Pneumocystis carinii, Cryptococcus neoformans, Coccidioides* spp. and *Paracoccidiodes brasiliensis* [13–15], leading to activation of phagocytosis and production of inflammatory mediators such as H_2O_2, NO and cytokines [16,17]. Furthermore, β-1,3-glucan and other Dectin-1 ligands have been successfully used as adjuvants for various experimental antifungal vaccines [18]. Some studies showed that the *S. schenckii* infection triggers a series of mediators commonly associated to Dectin-1 activation [11,19].

A study using rats co-infected with *Taenia taeniaeformis* and *S. schenckii* or infected with *S. schenckii* alone, revealed a high Dectin-1 expression in the cutaneous lesions of co-infected rats only, as detected by immunohistochemical staining. The authors concluded that *S. schenckii* has a different molecular pattern and thus evokes anti-infection mechanisms other than Dectin-1 [20]. Regarding other immune mechanisms known to have a role in the host immune response to *S. schenckii*, a recent study evidenced that the Th17 response is important for an optimal control of the S. schenckii infection in mice [21], whereas other studies have shown that the combination of itraconazole with β-1,3-glucan promoted an earlier regression of sporotrichosis lesions in comparison with the antifungal therapy alone [22,23].

The available information on the role played by Dectin-1 in sporotrichosis is scarce. Therefore, here we aimed to assess Dectin-1 expression by macrophages and the activation of some related antifungal mechanisms during the *S. schenckii sensu stricto* infection to better understand the role of this receptor in a murine model of systemic infection by this pathogen.

2. Materials and methods

2.1. Microorganism and culture conditions

Sporothrix schenckii sensu stricto ATCC 16345 was kindly provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). For mice infection and heat-killed *S. schenckii* yeast (HKss) preparation, a piece of mycelium grown on Mycosel™ (BD Biosciences) agar tubes was transferred to brain-heart infusion broth (BHI, Difco) and then cultured for 7 days at 37 °C in order to achieve a near 100% mycelium-to-yeast conversion during the logarithmic phase of growth. Before use, the culture was filtered through sterile gauze to remove mycelium fragments.

2.2. Heat-killed S. schenckii preparation

Yeasts were harvested from the BHI culture by centrifugation, washed twice and adjusted to 2.5×10^8 yeasts/mL in sterile phosphate buffered saline (PBS), pH 7.4. Aliquots of this suspension were incubated for 1 h in a 60 °C water bath and then stored at 2–8 °C until use. The efficiency of the heat-killing process was assessed by plating aliquots on Mycosel™ agar and checking for colony forming units (CFU) growth before use [21].

2.3. Alkali-insoluble fraction (F1)

The alkali-insoluble fraction was isolated as described previously [24]. Briefly, yeast cell walls were suspended in 1 N NaOH and gently stirred for 1 h at room temperature. After centrifugation at 5000×*g* for 10 min, the supernatant was collected and the procedure was repeated four times. The alkali-insoluble sediment was washed with water until it reached pH 7.0 and was then washed with ethanol, acetone and diethyl ether, in that order. The resulting white powder was called the alkali-insoluble fraction (F1).

2.4. Animals and experimental design

Male Balb/c mice, 5–7 weeks old, were purchased from "Centro Multidisciplinary para Investigação Biológica na Área da Ciência de Animais de Laboratório" (CEMIB), UNICAMP University (Brazil). Five mice per group were housed in microisolator cages and maintained in specific pathogen-free (SPF) conditions. Animals were intraperitoneally (i.p.) inoculated with 10^6 *S. schenckii* yeasts in PBS or with an equal volume of PBS alone. The animals were euthanized on days 5, 10 and 15 post-infection. All animal procedures were performed according to the guidelines of the Brazilian College of Animal Experimentation (COBEA) and were approved by the research ethics committee of Araraquara's School of Pharmaceutical Sciences from UNESP University (CEUA/FCF/CAr n° 02/2015).

2.5. Peritoneal exudate cells

Thioglycollate-elicited peritoneal exudate cells (PECs) were harvested from mice 3 days after the i.p. inoculation with 3% sodium thioglycollate, which was performed by washing the peritoneal cavity with 5.0 mL of cold PBS. The cells were washed twice by centrifugation at 300×*g* for 5 min at 4 °C with PBS and then resuspended in complete RPMI medium (cRPMI). PECs were counted in a Neubauer chamber and adjusted to 5×10^6 macrophages/mL.

2.6. Assessment of the systemic fungal load

Spleens were aseptically removed, macerated in PBS and passed through a 100 µm cell strainer. The systemic fungal load was determined by counting the CFU grown on Mycosel™ agar plates after the spread-plating of a previously determined dilution of the spleen macerate.

2.7. Flow cytometry

In all staining procedures, only freshly isolated, unstimulated PECs were used. Briefly, the cells were extracellularly labeled with the following monoclonal antibodies (mAb): anti-CD11b FITC (clone M1/70), anti-F4/80 APC (clone BM8), and anti-Dectin-1 PE (clone bg1fpj), all from eBioscience. Unspecific staining was controlled by pre-incubating all samples with anti-CD16/CD32. Alternatively, the cells were stained for CD11b and F4/80 only and incubated with the PE isotype control. Events were acquired using a BD Accuri C6 flow cytometer (BD Biosciences).

2.8. Nitric oxide production

Nitric oxide (NO) production by PECs was assessed by the Griess method [25]. NO concentrations were calculated from a previously established standard curve using known concentrations of sodium nitrite. Absorbance was read in a UV/visible microplate spectrophotometer (Multiskan Ascent, Labsystems) at 540 nm.

2.9. Ex vivo secretion of cytokines

PECs were cultured for 24 h at 37 °C and 5% CO_2 on flat-bottom 48-well tissue culture plates in the presence of 1 mL of cRPMI containing the following stimuli: β-1,3-glucan from *Saccharomyces cerevisiae* (Sigma, 100 µg/mL), F1 (100 µg/mL), HKss ($2,5 \times 10^7$/mL). LPS (10 µg/mL) or cRPMI alone were used as the positive or negative control, respectively. Cytokines were measured by ELISA (eBioscience) according to the manufacturer's instructions.

2.10. Dectin-1 antibody-mediated blockade assays

In an independent experiment, PECs from *S. schenckii*-infected and non-infected mice were used for a Dectin-1 antibody-mediated blockade assay aiming to evaluate the impact of Dectin-1 engagement in macrophage activation on day 10 post-infection. The PECs were seeded at 1×10^6 cells/mL in a 24-well plate. After a 2-h preincubation, the cells were incubated with 10 μg/mL of an anti-Dectin-1 mAb (eBiosciense) for 30 min at 37 °C and then added with 1 mL of cRPMI containing the following stimuli for another 24 h: β-1,3-glucan (100 μg/mL), F1 (100 μg/mL), HKss (2,5 $\times$ 10^7/mL), LPS (10 μg/mL), or cRPMI only. The supernatants were collected and used to determine the concentration of IL-10, TNF-α and IL-1β by ELISA.

2.11. Role of Dectin-1 in the phagocytosis of S. schenckii yeast

Peritoneal exudate was collected as described above. The macrophage monolayers from infected and un-infected mice were incubated for 1 h for adherence. They were then treated with 10 μg/mL of anti-dectin-1. After 1 h of incubation at 37 °C, they were incubated with live *S. schenckii* yeast, resulting in a 1: 5 ratio of *S. schenckii*-to-macrophages. Control with cytochalasin D final concentration, 10 μM, Sigma-Aldrich, St. Louis, MO (to prevent internalization) or vehicle control were included. All assays were done with five wells per condition. After incubation, the culture medium was carefully removed and the monolayers were washed with 1 mL of distilled water to lyse macrophages. One hundred microliters of cell homogenates were assayed for the presence of viable yeasts in Petri dishes containing Mycosel agar, incubated at 30 °C for 5 days. The colony forming units (CFU) per well (1 mL) were determined by counting the number of fungi growing on the culture dish. Performed with five wells per condition. The occurrence of effective phagocytosis of *S. schenckii* yeast by macrophages was controlled in parallel by incubation under the same conditions on glass slides and microscope.

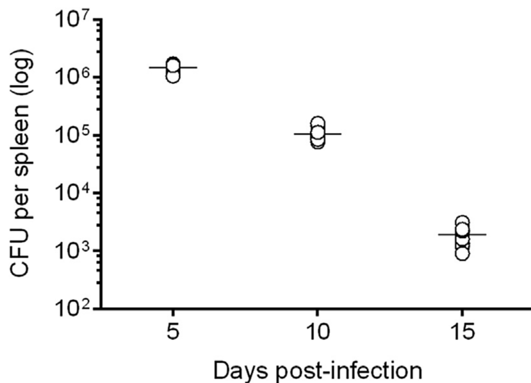

Fig. 1. Assessment of spleen fungal load. Mice were i.p. inoculated with 10^6 *S. schenckii* yeasts. On the indicated days, mice were euthanized and the spleens were removed for assessment of the fungal load in the spleen.

2.12. Statistical analysis

Statistical analysis was performed in GraphPad Prism ver. 6.01 using one- or two-way analysis of variance (ANOVA) with Tukey or Sidak's multiple comparisons test, respectively. Student's t-test was also used where indicated. The confidence interval was set at 95% for all tests.

3. Results

3.1. Sporothrix schenckii systemic infection model

According to previous studies, the days 5, 10 and 15 post-infection corresponded to the peak of animal susceptibility, the ongoing elimination of the fungus, and the final clearance of the infection, respectively [21]. This result confirmed that model was adequately reproduced here (Fig. 1).

3.2. Dectin-1 expression by PECs

The frequency of Dectin-1-expressing cells, as well as the expression level of this receptor, was significantly increased among peritoneal macrophages on days 10 and 15 post-infection when compared to non-infected mice (Fig. 2). The presented results indicate that Dectin-1 expression is upregulated during the *S. schenckii* infection, suggesting a role for Dectin-1-triggered immune responses in controlling this infection.

3.3. NO production

NO production by peritoneal macrophages from *S. schenckii*-infected mice was increased upon exposure to all the stimuli, but especially F1 and HKss. The purified β-1,3-glucan-induced NO production, albeit significant, was comparatively low during the entire experimental period, suggesting that NO production during the *S. schenckii* infection is triggered not only by recognition of βglucan but also by other components of the fungal cell wall (Fig. 3).

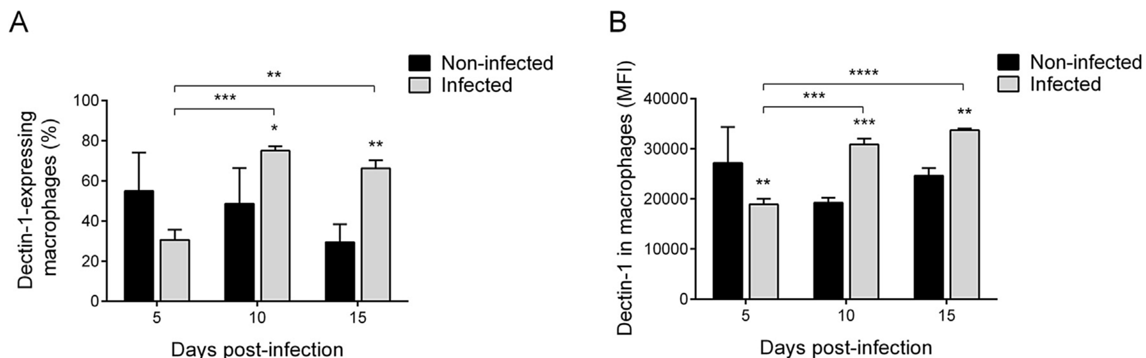

Fig. 2. Dectin-1 expression by peritoneal macrophages. (A) Frequency of Dectin-1-expressing macrophages (CD11b + F4/80 + cells). (B) Median fluorescence intensity (MFI) of the Dectin-1 staining on macrophages. The results are presented as the mean ± SD of five mice. * ($P < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) for comparisons between “infected” and “non-infected” mice at each respective time point.

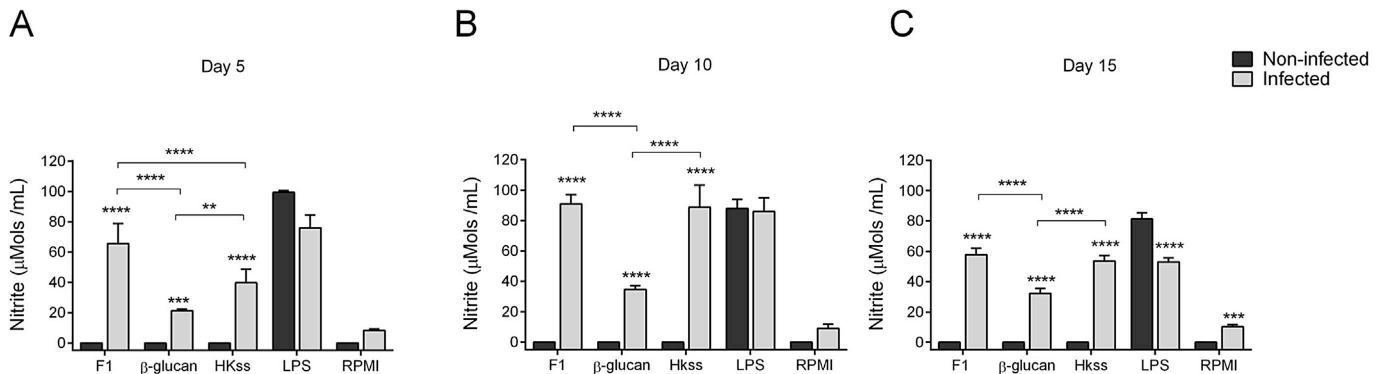

Fig. 3. NO production by PECs. NO production was measured in PEC supernatants by the Griess assay on days 5, 10 and 15 post-infection. The results are presented as the mean ± SD of five mice. ** (P < 0.01), *** (p < 0.001) and **** (p < 0.0001) for comparisons between "infected" and "non-infected" mice treated with the same stimulus or as indicated.

3.4. Cytokine detection in culture supernatant

Except for β-1,3-glucan, all the other stimuli were able to induce, to a greater or lesser extent, the secretion of IL-10, TNF-α and IL-1β during the entire studied period. When challenged with β-1,3-glucan, peritoneal macrophages from *S. schenckii*-infected mice were able to secrete significant amounts of IL-10 only on days 5 and 10 post-infection (Fig. 4A and B), and of IL-1β only on days 10 and 15 post-infection (Fig. 4H and I). On the other hand, the β-1,3-glucan-induced TNF-α secretion was significant only on day 10 post-infection (Fig. 4E), although the mean secretion on day 15 post-infection was increased more than 4 times as compared to unstimulated cells from infected mice or β-1,3-glucan-stimulated cells from non-infected ones. Except for the secretion of IL-10 on day 5 post-infection and of IL-1β on day 15 post-infection, the same previous pattern was observed for F1 and HKss, which were more efficient stimuli than β-1,3-glucan as inducers of the *ex vivo* secretion of IL-10, IL-1β and TNF-α by macrophages from infected mice. Furthermore, contrary to IL-10 and TNF-α, IL-1β secretion, although still significant, was clearly reduced on day 15 post-infection.

3.5. Dectin-1 antibody-mediated blockade assay

The antibody-mediated blockade of Dectin-1 clearly inhibited the secretion of IL-10, IL-1β and TNF-α in response to HKss, F1 and β-1,3-glucan by peritoneal macrophages from *S. schenckii*-infected mice. On the other hand, TNF-α secretion only was inhibited, and solely in response to β-1,3-glucan stimulation, when using macrophages from non-infected mice (Fig. 5). It is noteworthy that, in general, the strongest inhibition was reached upon β-1,3-glucan stimulation, given its role as a specific Dectin-1 ligand, suggesting HKss and F1 may be engaging other receptors in addition to Dectin-1. These results indicate that Dectin-1 plays an important role in macrophage activation during *S. schenckii* infection.

3.6. Role of Dectin-1 in the phagocytosis of S. schencki yeast

The absolute amounts of phagocytized S. schenckii yeast (calculated as the difference between the CFU of wells without and with cytochalasin D) was determined. As expected, a higher count of intracellular CFU was observed in infected mice in comparison with non-infected. However, it was also observed a reduction of phagocytized yeasts in the wells with anti-Dectin-1 antibodies of both groups. Fig. 6 depict the reduction of intracellular CFU by Dectin1 blocking expressed as absolute count (Fig. 6A) and in percent (Fig. 6b). The presence of intracellular yeast was microscopically confirmed (Suplementary Fig. 2).

4. Discussion

Dectin-1 is a cell surface innate immune receptor that plays an important role in immunity against fungal pathogens. Netea et al. [26] showed that *C. albicans* induces cytokine production by binding to different macrophage receptors, each one recognizing a different structure on the *Candida* cell wall. Mannan polymers are recognized by the mannose receptor, while β-1,3-glucan are recognized by Dectin-1 and TLR-2, showing that the collaborative recognition of distinct fungal components by different classes of innate immune receptors is critical for inflammatory response development [27].

The engagement of Dectin-1 by β-1,3-glucan leads to activation of phagocytosis and stimulates production of fungicidal and pro- or anti-inflammatory mediators, including reactive oxygen species (ROS), cytokines such as IL-1 β, TNF-α, IL-10, and chemokines [15–17]. These early innate immunity events play a role in determining the development of subsequent antifungal adaptive immune responses, such as the polarization of naïve Th cells into effector IL-17 + Th cells [28–30].

The fungi cell wall polysaccharides include glucose, mannose, galactose, glucans, galactomannans, and chitin polymers. The alkali-insoluble glucans, which compose the F1 fraction, contain β(1,3), β(1,6) and β(1,4) linkages in a proportion of 66%, 29% and 5%, respectively [24,31]. Here, we show that the frequency of Dectin-1-expressing cells, as well as the expression level of this receptor, was increased among peritoneal macrophages on days 10 and 15 post-infection, suggesting this receptor may be participating in *S. schenckii* recognition. This result contradicts a previous study where the authors suggested that Dectin-1 does not participate in *S. schenckii* recognition [20]. Differences in experimental design and animal model (rats versus mice), may account for this.

The secretion of IL-10 during the resolution stage of the infection (day 15 post-infection) and of TNF-α and IL-1β during the peak of animal susceptibility (day 5 post-infection) do not appear to depend on β-1,3-glucan recognition but on additional components of the *S. schenckii* cell wall present in the F1 fraction used by us. Moreover, it is possible to suggest that secretion of IL-10 during the peak of animal susceptibility and of IL-1β during the resolution stage of the infection depend on the recognition of *S. schenckii* β-1,3-glucans since our results were virtually identical across all the three stimuli. In any case, it is important to consider that, although we used an equal amount of purified β-1,3-glucan and F1 plus the fact that most glucans present in F1 are of the β(1,3) type, there are still enough differences between the preparations to justify at least

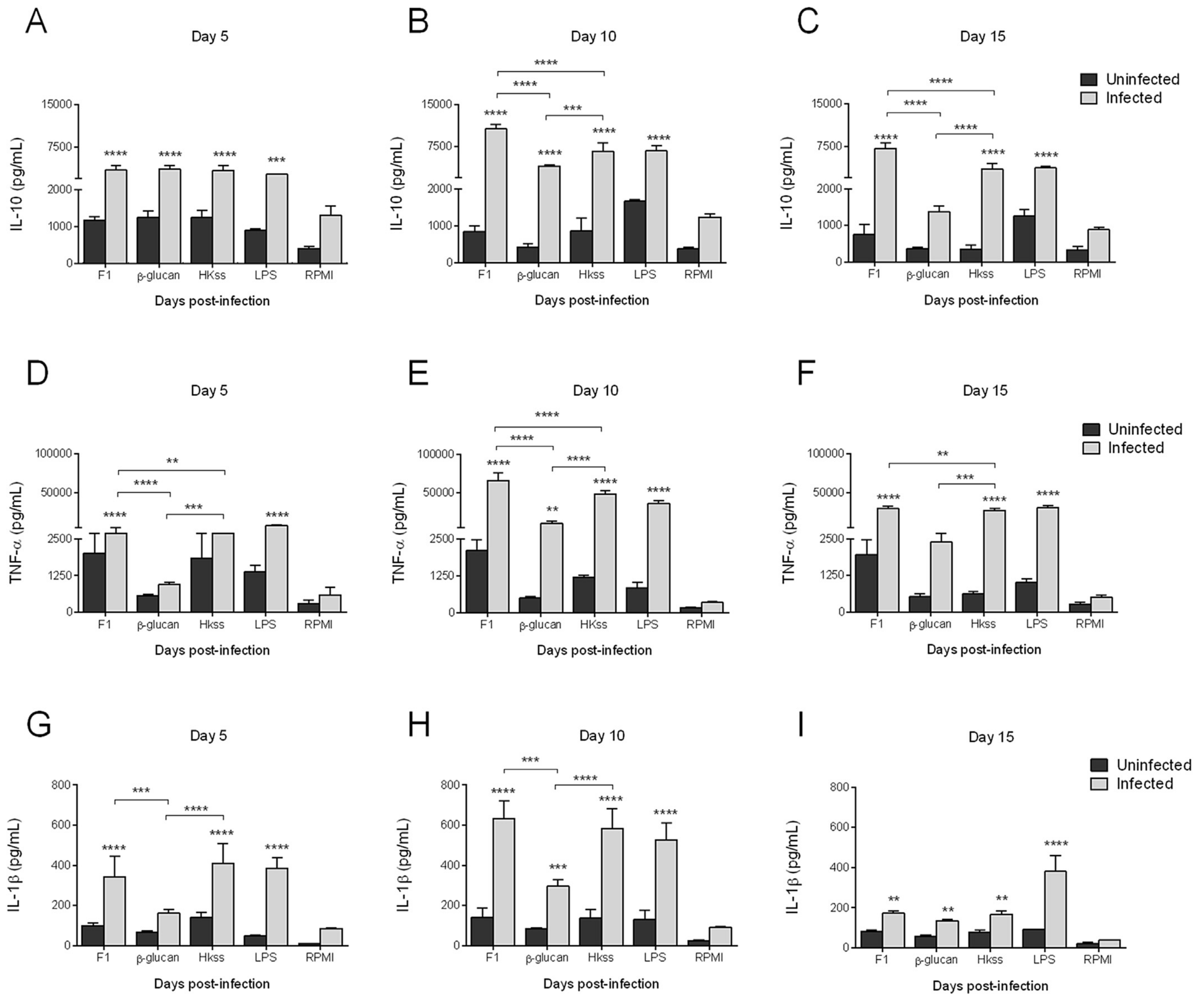

Fig. 4. *Ex vivo* cytokine secretion. Cytokines were measured in PEC supernatants by ELISA on days 5, 10 and 15 post-infection. (A–C) IL-10. (D–F) TNF-α. (G–I) IL-1β. The results are presented as the mean ± SD of five mice. ** ($P < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$) for comparisons between "infected" and "non-infected" mice treated with the same stimulus or as indicated.

some of the observed differences, namely: the presence of β(1,6) and β(1,4) glucans, the lower concentration of β-1,3-glucan and the presence of other non-glucan components in F1. Future studies will clearly benefit from using more purified preparations containing specific components of the *S. schenckii* cell wall.

To confirm the role of Dectin-1 in the proinflammatory response triggered by *S. schenckii*, peritoneal macrophages from infected and non-infected mice were challenged with different stimuli, including β-1,3-glucan, in the presence or absence of an anti–Dectin-1 mAb. When Dectin-1 was blocked, secretion of the proinflammatory cytokines TNF-α and IL-1β and the anti-inflammatory cytokine IL-10 by HKss, F1 or β-1,3-glucan were strongly suppressed. This indicates that Dectin-1 is deeply involved in macrophage activation upon exposure to *S. schenckii*, although, as already discussed, other PRRs also participate.

Phagocytosis is an important innate mechanism in sporotrichosis. However, once S. schenckii yeast have been phagocytosed, they are able to survive within the phagocytic cells. Survival of virulent *S. schenckii* yeast cells after phagocytosis relies on diverse detoxification including the ergosterol peroxide synthesis [32]. Binding of β-1–3 glucan by dectin-1 has been shown to be essential for phagocytosis of *C. albicans* [33] and *Aspergillus fumigatus* conidia [34]. These findings prompted us to analyse whether dectin-1 is also involved in the phagocytosis of *S. schenckii* yeast. At a concentration of 10 μg/mL of anti-dectin-1 antibody reduced the phagocytic uptake of yeast by approximately 60% in infected and non-infected mice. And this reduction suggest that Dectin-1 is involved, together with other receptors in the phagocytic process on this infection.

According to recent studies, the innate immunity can be "trained", thereby acquiring a higher capacity to respond to invasive infections [35,36]. It has been reported that exposure to a small load of *C. albicans* was able to induce protection against reinfection with this fungus in mice deficient in functional T and B cells [37]. These authors also described an *in vitro* assay where monocytes were pre-incubated with heat-killed *C. albicans* and then exposed to different secondary stimuli, including β-1,3-glucan, leading to enhanced cytokine production. It would be interesting to assess if a

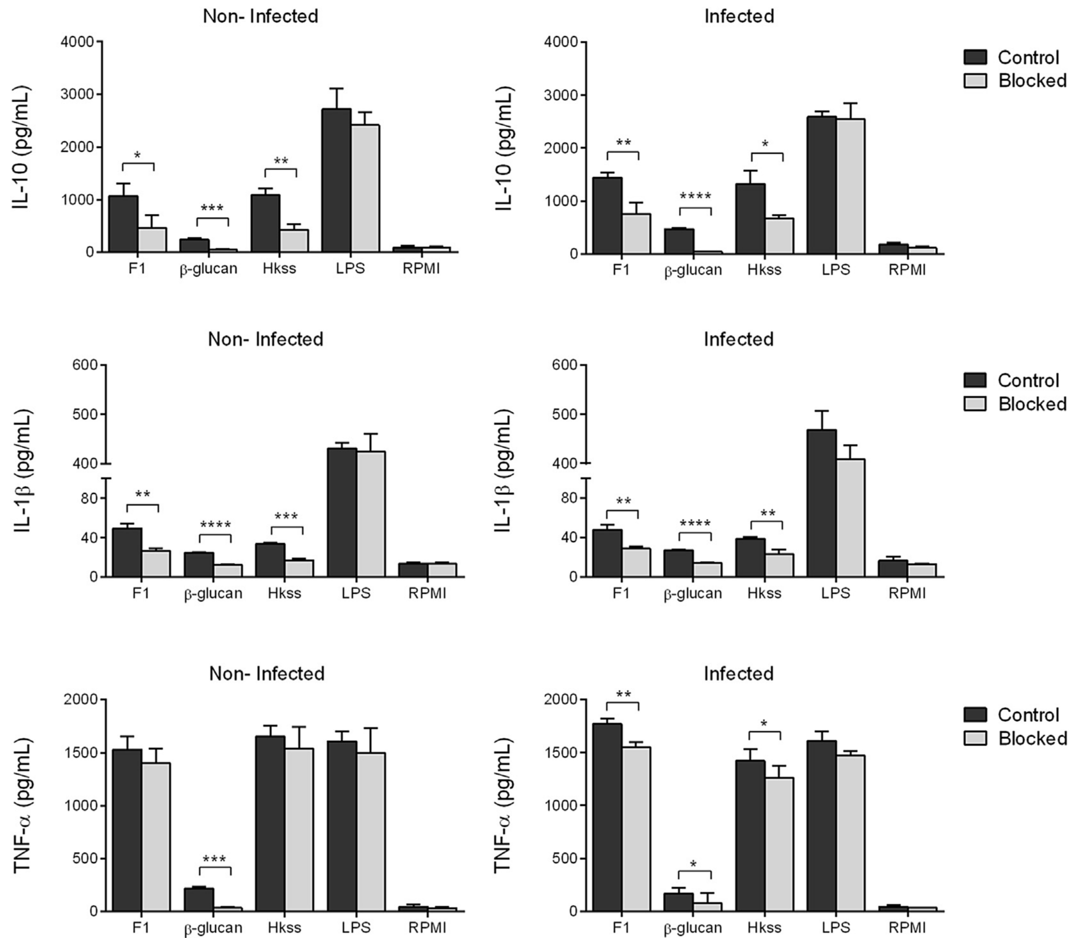

Fig. 5. Dectin-1 triggers cytokine production in response to different *S schenckii* components. PECs from either infected or non-infected mice were incubated with 10 μg/mL of an anti-Dectin-1 mAb and then challenged with the indicated stimulus for 24 h. The supernatant concentration of IL-10, TNF-α and IL-1β was measured by ELISA. ** ($P < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$) for comparisons between "blocked" and "non-blocked (control)" cells.

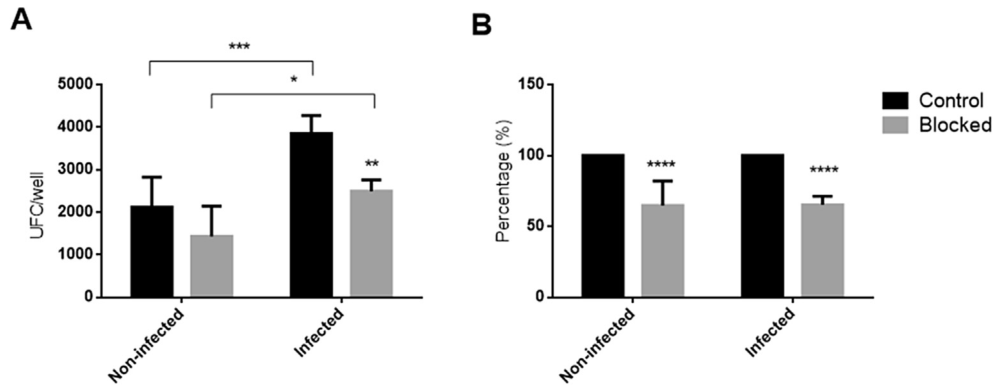

Fig. 6. Dectin-1 is involved in *S schenckii* phagocytosis. The macrophage monolayers from infected and un-infected mice were incubated for 1 h for adherence and treated with 10 μg/mL of anti-dectin-1. After 1 h of incubation at 37 °C, they were incubated with live *S. schenckii* yeast, resulting in a 1:5 ratio of *S. schenckii*-to-macrophages. Control with cytochalasin D (10 μM) or vehicle control were included. All assays were done with five wells per condition. After incubation, monolayers were washed with 1 mL of distilled water to lyse macrophages. One hundred microliters of cell homogenates were plated for the presence of viable yeasts in Petri dishes containing Mycosel agar, incubated at 30 °C for 5 days. The colony forming units (CFU) per well (1 mL) were determined by counting the number of fungi growing on the culture dish. ** ($P < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$) for comparisons between "blocked" and "non-blocked (control)" cells.

trained state can be evoked in response to *S. schenckii* infection in a Dectin-1-dependent manner. Efforts in this area would be very useful for the development of immunomodulatory drugs and vaccines against sporotrichosis, an area of great current interest [38–40].

5. Conclusion

In summary, our data greatly contributes to understand the way in which *S. schenckii* is recognized by and stimulates cells of the innate immune system and for understanding the pathophysiology

of sporotrichosis. On one hand, we demonstrated that recognition of β-1,3-glucan and other fungal components by Dectin-1 increases the cytokine release induced by the *S. schenckii* infection. To the best of our knowledge, this is the first attempt to determine the role of Dectin-1 in the development of immune response against *S. schenckii*. Further studies using Dectin-1 knockout animals and assessing the activation of related signaling pathways will help to deepen our understanding of these mechanisms.

Conflicts of interest

The authors declare no commercial or financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2017.06.025.

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