

Protection against *Paracoccidioides brasiliensis* infection conferred by the prophylactic administration of native and recombinant ArtinM

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We determined the prophylactic effect of both the d-mannose-binding lectin ArtinM extracted from the seeds of *Artocarpus integrifolia* (jackfruit) and its recombinant counterpart during the course of experimental paracoccidioidomycosis induced in BALB/c mice. Four experimental protocols of prophylaxis were employed to evaluate the most protective regimen of ArtinM administration. It was demonstrated that the best effect was obtained by administration of two ArtinM doses on days 10 and 3 before the challenge with *Paracoccidioides brasiliensis*. By following this protocol, the lungs of mice that received native or recombinant ArtinM exhibited reduced fungal burden and granuloma incidence. In addition, the protocol augmented contents of IL-12, IFN- γ , TNF- α and NO. On the other hand, the control group consisting of untreated infected mice had higher pulmonary levels of IL-4 and IL-10. In conclusion, prophylaxis with ArtinM significantly reproduces the effect of its therapeutic administration, i.e. it confers resistance to *P. brasiliensis* infection in mouse models by promoting IL-12 production and favours Th1-immunity.

Keywords paracoccidioidomycosis, *Paracoccidioides brasiliensis*, prophylaxis, ArtinM Lectin

Introduction

Paracoccidioidomycosis (PCM), caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, is the most prevalent human systemic mycosis in Latin America. It is endemic in Brazil, Argentina, Venezuela, and Colombia. The infection begins after inhalation of airborne conidia which convert to yeast cells in the lungs [1]. When the yeast cells are not eliminated, PCM may develop into multiple

clinical manifestations, ranging from asymptomatic pulmonary infection to severe and disseminated disease [2,3]. The cell-mediated immunity represents the main mechanism of defense in PCM [1], while a high humoral response correlates with increased disease dissemination [4]. Therefore, resistance to the disease is linked to predominant IFN- γ production by T helper type 1 (Th1) lymphocytes, and susceptibility is associated with impairment of cellular immune responses and activation of B cells [5,6]. The differentiation of Th1 and Th2 cells is driven by IL-12 [7,8] and IL-4 [9,10] cytokines, respectively. Indeed, an early secretion of IL-12 followed by a sustained secretion of IFN- γ is required to achieve protection against *P. brasiliensis* infection [11]. IL-12 production by phagocytes is most often initiated by the interaction of surface Toll-like receptors (TLRs) on the cell surface with

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pathogen-associated molecular patterns (PAMPs). The most physiologically important IL-12 target cells are T lymphocytes, in which IL-12 induces proliferation and differentiation to cells that produce type-1 cytokines, particularly IFN- γ . IL-12 is a highly efficient IFN- γ inducer, acting at low concentrations, and it also acts in synergy with other activating stimuli (reviewed by [12]). This sequence of events initiates and amplifies the innate and adaptive Th1-cell immunity.

We have previously shown that ArtinM, a D-mannose-binding lectin from jackfruit (*Artocarpus integrifolia*), induces IL-12 production via recognition of TLR2 glycans on either macrophages or dendritic cells, thereby switching the cytokine profile in mice from Th2 (IL-4) to Th1 (IFN- γ) [13,14]. As a consequence, ArtinM induces a protective Th1-type response against intracellular pathogens, such as *Leishmania* [14,15] and *P. brasiliensis* [13]. In the case of *Leishmania* infection, the effect of ArtinM was evaluated when it was prophylactically administered, whereas in the study of *P. brasiliensis* infection, ArtinM was therapeutically employed. In the present work, we have investigated the effect exerted on experimental PCM by native and recombinant ArtinM when they are administered in a prophylactic regimen.

Materials and methods

Mice

The animals used for this study were 6–8-week-old male BALB/c mice. They were bred and maintained under standard conditions in the animal house of the Ribeirão Preto School of Medicine, São Paulo University, Ribeirão Preto, SP, Brazil. Each experimental group consisted of five mice and the assays were done in duplicates. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Fungal isolate

The virulent *P. brasiliensis* isolate (Pb18) used in this study was maintained on Fava-Netto's semisolid medium at 36°C for 7 days. To maintain virulence, consecutive passages in mice were accomplished by intravenous infection, and the yeast recovered from the lung tissue reinoculated on the indicated culture medium. The viability of the yeast cells, determined by fluorescein diacetate and ethidium bromide staining [16], was always greater than 90%.

ArtinM affinity-purification

Native ArtinM from jackfruit seeds (*A. integrifolia* L.f.) was purified as previously described [17]. The lectin

recombinant form (rArtinM) was expressed in *Escherichia coli* BL21 transformed with pExp-ArtinM and purified as previously reported [18]. ArtinM preparations (from plant or recombinant) contained less than 0.05 ng/ml of bacterial endotoxin, as determined by the *Limulus amoebocyte* lysate assay (Sigma Chemical Co., St. Louis, USA).

ArtinM administration and intravenous infection of mice

Mice divided into 4 groups of five mice each were injected subcutaneously (s.c.) with 0.5 μ g ArtinM in 50 μ L PBS. The treatments varied according to the number of ArtinM injections (1–3 doses) and time intervals between each ArtinM injection (Table 1). To determine the most effective regimen of ArtinM administration, all groups were challenged by intravenous (i.v.) injection into the lateral tail vein of 0.1 ml of the inoculum (1×10^6 *P. brasiliensis* yeast cells). The uninfected control mice were inoculated with 0.1 mL of sterile pyrogen-free PBS. On the basis of the results provided by these screening procedures, the protocol in which the mice were injected with two s.c. doses of ArtinM or rArtinM on days 10 and 3 before infection was employed in the subsequent experiments. The control mice, which were submitted to a similar regimen of injections, received PBS (50 μ g) instead of ArtinM. The course of infection was evaluated on days 14 and 30 after exposure to the fungus.

Quantification of colony-forming units (CFU)

The mice were killed by exsanguination on days 14 and 30 postinfection and examined for fungal burdens in the lungs, liver and spleen, as previously described [19]. Briefly, one lung of each animal was aseptically removed and disrupted in 1 ml PBS containing 1.6 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co.), using a tissue homogenizer (Ultra-Turrax T25 Basic, IKA Works, Inc., Wilmington, USA). The number of viable *P. brasiliensis* organisms was determined by plating crude and serially diluted homogenates onto brain heart infusion agar (BHI; Oxoid Ltd., Hampshire, UK), supplemented with 4% (v/v) heat-inactivated fetal calf serum (FCS; Invitrogen,

Table 1 Prophylaxis groups.

Groups	Treatment regimens ^a (days before infection)
C	No Artin M ^b
1	17, 10 and 3
2	10 and 3
3	10
4	3

^aMice received 0.5 μ g Artin M subcutaneously.

^bMice received PBS in a regimen similar to that of group 1.

Camarillo, CA, USA) and incubated at 36°C for 15 days. The lower limit of detection was 10 CFU. The number of *P. brasiliensis* colonies per gram of organ was given as the mean and standard deviation (SD). The lung homogenate supernatants were also separated from cell debris by centrifugation at 2,000 g for 15 min, and stored at -20°C until ELISA-based cytokine and nitric oxide (NO) measurements were performed.

Histopathology

On day 30 postinfection, the lungs were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) and examined by light microscopy with a Axiophot photomicroscope (Carl Zeiss, Jena, Germany) coupled with a JVC TK-1270 camera (Victor Company of Japan Ltd, Tokyo, Japan). The area of individual granulomas, as well as the total area of the lung sections and the area taken by granulomas per slide, was measured by computer-aided image analysis (ImageJ 1.37v, National Institutes of Health, Bethesda, USA).

ELISA-based cytokine detection assay

The levels of IL-12p70, IL-4, IL-10, IFN-γ, and TNF-α in the supernatants of the lung homogenate were measured by capture enzyme-linked immunosorbent assay (ELISA) with antibody pairs purchased from Pharmingen (San Diego, USA). The ELISA procedure was performed according to the manufacturer's protocol. The cytokine concentrations were determined with reference to a standard curve for serial two fold dilutions of the murine recombinant cytokines.

NO production

NO production was quantified by the standard Griess reaction. Briefly, 50 µl of supernatants from the lung homogenates were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, and 2.5% H₃PO₄) at room temperature for 10 min. The absorbance was measured at 550 nm in the Microplate Scanning Spectrophotometer (Power Wavex – BIO-TEK Instruments, Inc., Winooski, USA). The conversion of absorbance into micromolar concentrations of NO was deduced from a standard curve using a known concentration of NaNO₂.

Statistical analysis

Statistical determinations of the difference between means of experimental groups were performed using one-way

analysis of variance (ANOVA) followed by Bonferroni post test. Differences that provided $P < 0.05$ were considered to be statistically significant. All experiments were performed at least three times.

Results

Screening the regimen of ArtinM administration

In order to evaluate whether the prophylactic administration of ArtinM is able to interfere in PCM in the murine model and to determine the most effective regimens of ArtinM administration in conferring protection against the disease, groups of BALB/c mice were submitted to treatments that varied according to the number and time intervals of injections (see Table 1) and then i.v. inoculated with *P. brasiliensis* yeasts. Histopathology examination of the lungs showed (Fig. 1) that mice from group 2, i.e., those injected with ArtinM on days 10 and 3 before infection, presented the most preserved organ architecture, a mild leukocyte infiltration, and absence of yeast cells (Fig. 1D). In contrast, severe lesions were observed in the lungs of the untreated mice (control group, Fig. 1B). Intermediate severity of lesions was observed in the lungs of mice from the screened groups 1 (Fig. 1C), 3 (Fig. 1), and 4 (Fig. 1F). Morphometric analysis revealed that granuloma density was significantly lower in the lungs of mice from groups 2 (0.66 granuloma/mm²) and 4 (1.5 granuloma/mm²) (Fig. 2A), that received ArtinM on days 10 and 3 before infection, and only on day 3 before infection, respectively. On the other hand, the lung of mice treated according to protocols 1 and 3 had less than 3 granuloma/mm², which was quite different from the almost 4 granuloma/mm² counted in the pulmonary tissue of mice from the untreated control group. The outstanding results of group 2, as judged from histopathology criteria, were reinforced by the results of the fungal burden analysis, performed by CFU recovery from tissue. The pulmonary fungal burden was 95% reduced in group 2, compared with the control group (Fig. 2B). Intriguingly, group 3, comprised of mice treated only on day 10 before infection, had a larger recovery of CFU than the control group. Since ArtinM administration protocol 2 was unequivocally the most efficient, it was used in all subsequent experiments.

Prophylaxis with ArtinM protects mice against *P. brasiliensis* infection

The effects of ArtinM and rArtinM administration using protocol 2 were investigated in detail. Pulmonary histopathology in the untreated animals on day 30 after i.v. infection showed multiple sites of focal and confluent epithelioid granulomas with lymphomonocytic halos circumscribing

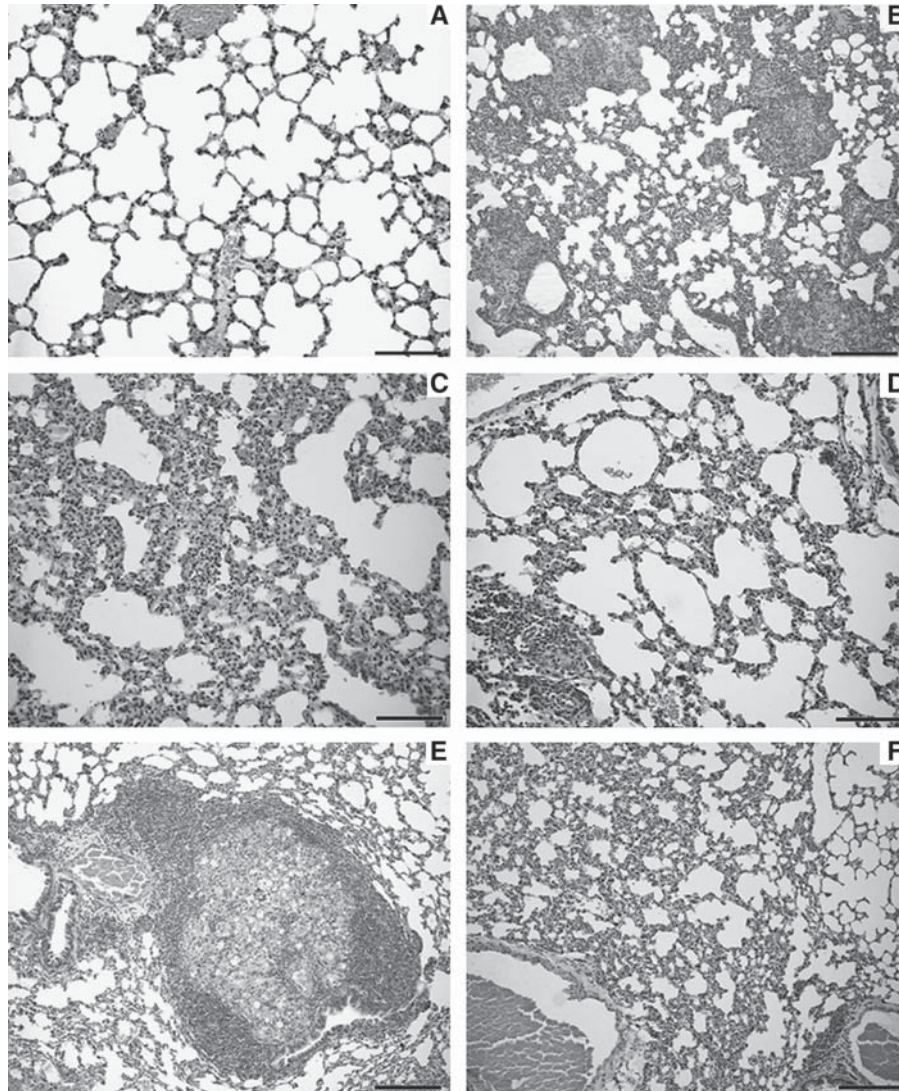


Fig.1 Lung histopathology of infected mice submitted to the screening protocols of ArtinM prophylactic administration. Mice were i.v. infected with 10^6 *P. brasiliensis* yeast cells and analysis was performed at day 30 after infection. The panels show representative lung sections from mice that were not infected (A); that were infected and not treated (B); that were infected and prophylactically treated according to protocol 1 (C), protocol 2 (D), protocol 3 (E), or protocol 4 (F). The most preserved lung structure is observed in the mice treated according to protocol 2 (D), whereas the other groups showed variable degrees of parenchyma lesions. The lung sections were H&E stained. Scale bars indicate 200 μ m.

yeast cells (Fig. 3A). In addition, multiple and confluent typical epithelioid granulomas were observed in the liver and spleen from all mice of this control group (data not shown). In contrast, in the lungs of mice prophylactically injected with ArtinM or rArtinM (Fig. 3B & C), no granulomas or yeast cells were seen, and the bronchoalveolar architecture was well preserved. There were no granulomas in the liver or spleen at any of the analyzed times (data not shown). The CFUs of *P. brasiliensis* yeast cells recovered from the lungs of at 30 days postinfection were examined. The CFU recovered from the lungs of the mice

prophylactically treated (Fig. 3D) with either ArtinM or rArtinM was 91% or 81% lower than the number recovered from the non-treated control mice, respectively. These observations were reinforced by the fact that on day 30 postinfection no yeast cells were recovered from the liver or spleen of any ArtinM-treated mice, while the control mice still presented infectious burdens at this time (data not shown). The CFU data concur with the histopathology results and provide strong evidence that administration of both ArtinM and rArtinM according to protocol 2 exerts a protective effect against *P. brasiliensis* infection.

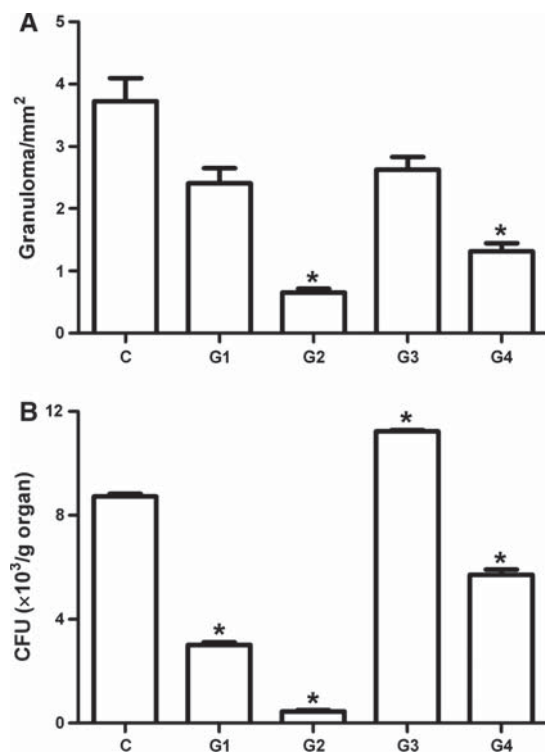


Fig. 2 Granuloma incidence and fungal burden in infected mice submitted to the screening protocols of ArtinM prophylactic administration. Mice were i.v. infected with 10^6 *P. brasiliensis* yeast cells and analysis was performed at day 30 after infection. (A) Morphometric analysis of lung sections in terms of granulomas/mm² of tissue. (B) Pulmonary CFU recovery. Each group of five mice was not treated (control, C) or prophylactically treated according to protocols 1, 2, 3, or 4 (G1-G4). The bars depict means and SD. * $P < 0.05$ versus control group.

Production of pro- and anti-inflammatory cytokines and NO in *P. brasiliensis* infected-mice

Since the native and recombinant ArtinM treatment was effective in protecting mice against *P. brasiliensis* infection, the question of whether an adequate milieu of pulmonary cytokines correlated with the advantageous effect of lectin administration was addressed. Analysis of the production of pro- and anti-inflammatory cytokines in the supernatants of lung homogenates from the *P. brasiliensis*-infected mice treated with ArtinM or rArtinM showed that on day 14 postinfection, the levels of IL-12p70, IFN- γ , IL-10, and IL-4 were not significantly different from those of the control group (Fig. 4, A, C–E). Only the TNF- α levels were found to be higher in the lung of ArtinM treated groups (Fig. 4B). On day 30 postinfection, higher concentrations of TNF- α (Fig. 4B) and IL-12 (Fig. 4A) were detected among the ArtinM-treated mice as compared with the controls. The differences between treated and untreated mice were still more evident when the time course of cytokines production was analyzed. A significantly lower

($P < 0.05$) amount of IL-12p70 was produced by the control mice on day 30 postinfection than on day 14 postinfection, whereas in ArtinM-treated mice the amount of IL-12p70 was stable or augmented during the course of infection (Fig. 4A). The levels of IFN- γ (Fig. 4C) and IL-4 (Fig. 4D) were stable in the treated groups, and variable in the untreated group. Lower IFN- γ and higher of IL-4 levels were detected on day 30 compared with these same levels on day 14 postinfection. The IL-10 levels increased in all groups from day 14 to 30 postinfection (Fig. 4E). Assays to evaluate nitrite production were performed in parallel with cytokine detection. Low levels of nitrite were detected on day 14 postinfection in the lungs of mice from all groups (Fig. 4F). On the other hand, on day 30 postinfection the prophylaxis group had much higher nitrite levels than the control ($P < 0.05$).

Discussion

Following the demonstration that ArtinM administration confers protection against subsequent challenge with *Leishmania* through induction of Th1-biased immunity, which in turn does not depend on the immunization with specific antigen(s) [14], new studies concerning the applicability of this lectin as an immunomodulatory agent have been performed. ArtinM has been shown to be useful with other intracellular pathogens [13,15] and to be effective in *P. brasiliensis* infection when it is therapeutically administered [13]. The current study was undertaken to investigate whether the prophylactic administration of ArtinM, in either its native or recombinant form, could also modify the course of *P. brasiliensis* infection. We found that protection was conferred by prophylactic administration of either form of the lectin. The Th1-prone immune response that developed exerted a direct beneficial effect on the severity of the disease, as determined by lung histopathology and CFU counts.

Whereas mice given no lectin exhibited extensive granulomatous lesions that characterize severe PCM, the animals that received prophylaxis with ArtinM or rArtinM showed significantly lower number of granulomas and decreased pulmonary fungal burden. Moreover, the lungs of only a few ArtinM-treated mice presented abnormalities, such as mild granulomatous inflammation, which were never accompanied by the dissemination of the fungus to the liver and spleen (data not shown) as found with untreated mice. As an exception, higher numbers of CFU were recovered from the lungs of mice treated as per protocol 3 compared with untreated control mice. We cannot explain these results, especially considering that the tissue injury was not more severe in group 3 mice and a low number of granuloma/mm² was found in their lungs.

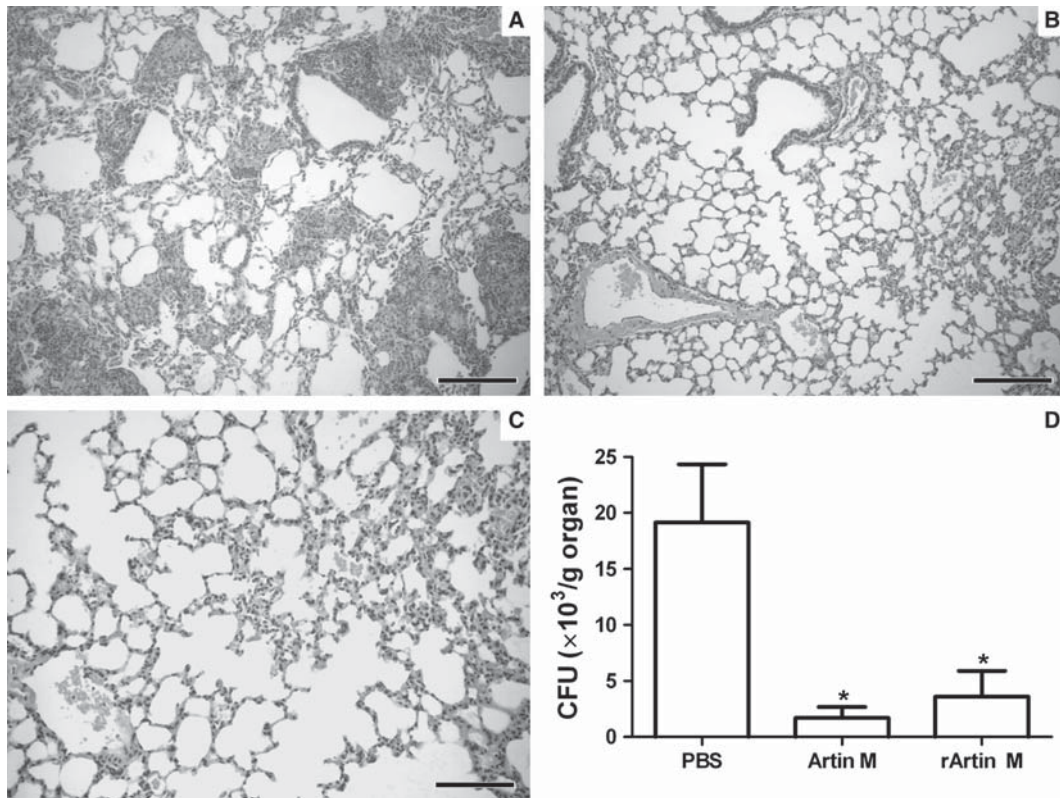


Fig. 3 Prophylaxis with natural or recombinant forms of Artin M protects mice against *Paracoccidioides brasiliensis* infection. Mice were i.v. infected with 10^6 *P. brasiliensis* yeast cells and analysis was performed at day 30 after infection. Lung histopathology of *P. brasiliensis*-infected mice treated with Artin M (B), rArtin M (C) or PBS (A); sections were H&E stained. The mice injected with PBS presented focal and confluent epithelioid granulomas circumscribing a large number of yeast cells. No granulomas or yeast cells are visualized in the mice pre-treated with Artin M (B) or rArtin M (C). (D) Pulmonary CFU recovery from mice treated with Artin M or rArtin M; control mice received PBS only. Scale bars on panels A, B, and C indicate 200 μ m. The bars on panel D depict means and SD of CFU obtained from groups of five mice at day 30 after infection. * $P < 0.05$ versus control group.

Considering the key role exerted by cytokines [20–24] in the course of paracoccidioidomycosis, their concentrations in the lung homogenates obtained from the control and ArtinM-treated mice were compared. The treated group exhibited an increase in the pulmonary proinflammatory cytokine levels, as well as in the nitrite levels, as compared to the control group. These observations are similar to the Th1 profile required for protection against PCM [11] and agree with our previous data concerning ArtinM therapy against PCM [13]. We have already demonstrated that the protection induced by ArtinM is attributable to the lectin interaction with TLR2 [13]. On the other hand, Loures and colleagues [25] established that TLR2 expression determines a beneficial effect on *P. brasiliensis* pulmonary infection, which is due to a negative control on Th17 immunity and tissue pathology. This TLR2 activity can be related with the absence of inflammatory injury following ArtinM administration in non-infected mice (not shown), in addition to the non-exacerbated inflammatory response in *P. brasiliensis* infected mice following therapy [13] or

prophylaxis with ArtinM. Such an effect of ArtinM is advantageous in comparison to the administration of exogenous IL-12 since it diminished the severity of the infection, but also induced a high inflammatory response [20]. A complementary explanation for the ArtinM advantageous effect may be the fact that the induced high IL-12 production was balanced by the deactivating cytokine IL-10, detected in substantial concentrations. Nevertheless, IL-10 levels in the ArtinM-treated mice were significantly lower than those seen in non-treated animals. This result constitutes a distinction between the prophylactic and therapeutic effects of ArtinM on PCM, i.e., the animals undergoing therapy presented IL-10 levels as high as the ones detected in non-treated mice [13]. In spite of this distinction, the effects of ArtinM were equally beneficial whether administered in a prophylactic or a therapeutic regimen. Our results indicate that both regimens of ArtinM administration generate a lung environment that efficiently controls fungal growth and avoids systemic dissemination of the etiologic agent. In addition, as in the

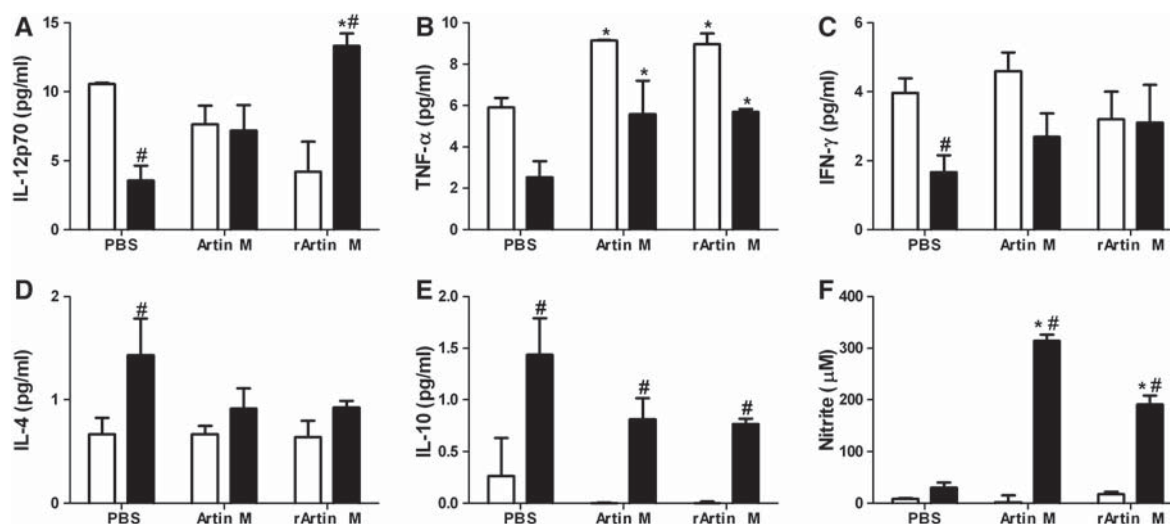


Fig. 4 Prophylaxis with ArtinM or rArtinM increases proinflammatory cytokines and NO production. Mice were i.v. infected with 10^6 *P. brasiliensis* yeast cells and analysis was performed at days 14 (white bars) and 30 (black bars) after infection. The mice were treated with Artin M, rArtin M, or PBS. The lung homogenates were analyzed for IL-12 (A), TNF- α (B), IFN- γ (C), IL-4 (D), IL-10 (E), and NO (F). Data are reported as the mean and SD for five mice per group performed in duplicate. * $P < 0.05$ versus control group. # $P < 0.05$ versus day 14.

case of the therapeutic protocol, the prophylactic administration of ArtinM did not elicit an anti-ArtinM humoral response (data not shown).

PCM is usually fatal in the absence of specific treatment. Most often, treatment lasts for up to 2 years, patients experience toxic drug side effects and relapses. In addition, host immune imbalance limits the efficacy of anti-fungal chemotherapy [26]. For these reasons, one important strategy to fight fungal disease is to improve the host immune reactions. Although various cytokines have proven to be beneficial in fungal infections, the Th1/Th2-cell balance itself seems to be the ultimate target of immunotherapy [27]. The demonstration that ArtinM administration promotes high IL-12 production supports the idea of the pharmaceutical applicability of ArtinM. This is because ArtinM favors Th1 immunity and NO production, whose importance in the protection against intracellular pathogens is well established. Considering the observation that protection against *P. brasiliensis* was maintained for more than 4 weeks in ArtinM-treated mice, we hypothesize that the lectin effects on the innate immunity have repercussions on the adaptive response, a supposition supported by a vast literature data regarding the bridge between innate and adaptive immunity (reviewed by [28,29]). Consequently, the occurrence of a robust cell-mediated immunity, characteristically required to confer protection against *P. brasiliensis* infection, might be favored. Besides, since T cells also express TLRs, a direct interaction of ArtinM with cells of the adaptive immunity is plausible and, in this case, it could also contribute to maintaining the protection observed

in the treated mice. On the basis of recent demonstrations that TLR2 controls both the expansion and function of T regulatory cells [30] and the observation that ArtinM-treated mice show high pulmonary levels of IL-10, we can also suppose that ArtinM interaction with TLR2 on cells of the innate immunity reverberates in Tregs, thus minimizing a deleterious effect eventually caused by strong and uncontrolled inflammatory responses. This hypothesis is consistent with our findings of a mild inflammatory response in *P. brasiliensis* infected mice after therapy [13] or prophylaxis with ArtinM, in spite of the detection of high IL-12 and NO levels. Nonetheless, a further detailed investigation is obligatory to clarify the apparent deleterious effect exerted by ArtinM treatment. This was observed when in our initial screening of protocols for the administration of this lectin, a single dose was given to mice on day 10 before infection.

It is probable that the valuable immunomodulation provided by ArtinM is related to its pleiotropic effects. An overall study to identify the cells of the immune system that correspond to targets for ArtinM recognition and signaling is being performed in our laboratory.

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