**Paracoccidioides brasiliensis** Interferes on Dendritic Cells Maturation by Inhibiting PGE2 Production

Reginaldo K. Fernandes1☯*, Tatiana F. Bachiega1☯, Daniela R. Rodrigues1☯, Marjorie de A. Golim2☯, Luciane A. Dias-Melicio3☯, Helanderson de A. Balderramas1☯, Ramon Kaneno1☯, Ângela M. V. C. Soares1☯*

1 Department of Microbiology and Immunology, Biosciences Institute, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil, 2 Flow Cytometry Laboratory, Hemocenter, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil, 3 Department of Pathology, Botucatu Medical School, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil

☯ These authors contributed equally to this work.
* regiskeller@msn.com (RKF); acsoares@ibb.unesp.br (AMVCS)

**Abstract**

Paracoccidioidomycosis (PCM) is a systemic mycosis, endemic in most Latin American countries, especially in Brazil, whose etiologic agent is the thermomorphic fungus of the genus **Paracoccidioides**, comprising cryptic species of **Paracoccidioides brasiliensis**, S1, PS2, PS3 and **Paracoccidioides lutzii**. The mechanisms involved in the initial interaction of the fungus with cells of the innate immune response, as dendritic cells (DCs), deserve to be studied. Prostaglandins (PGs) are eicosanoids that play an important role in modulating functions of immune cells including DCs. Here we found that human immature DCs derived from the differentiation of monocytes cultured with GM-CSF and IL-4 release substantial concentrations of PGE2, which, however, were significantly inhibited after challenge with **P. brasiliensis**. In vitro blocking of pattern recognition receptors (PRRs) by monoclonal antibodies showed the involvement of mannose receptor (MR) in PGE2 inhibition by the fungus. In addition, phenotyping assays showed that after challenge with the fungus, DCs do not change their phenotype of immature cells to mature ones, as well as do not produce IL-12 p70 or adequate concentrations of TNF-α. Assays using exogenous PGE2 confirmed an association between PGE2 inhibition and failure of cells to phenotypically mature in response to **P. brasiliensis**. We conclude that a **P. brasiliensis** evasion mechanism exists associated to a dysregulation on DC maturation. These findings may provide novel information for the understanding of the complex interplay between the host and this fungus.

**Introduction**

Paracoccidioidomycosis (PCM) is a systemic mycosis, endemic in most Latin American countries, especially in Brazil, whose etiologic agent is the thermomorphic fungus of the genus
Paracoccidioides, comprising cryptic species of Paracoccidioides brasiliensis, S1, PS2, PS3 and Paracoccidioides lutzii [1–3]. Hosts are infected through the respiratory tract by mycelium propagules found in soil that reach the alveoli where conidia convert to yeast, the infective form [4, 5]. Thereafter, yeasts can disseminate by lympho-haematogenous route, inducing a disease with a wide spectrum of symptoms in a small number of individuals suggesting that in most of exposed subjects innate and adaptive mechanisms efficiently assure resistance [6, 7]. Studies in human and animals have shown that resistance to P. brasiliensis is determined by a Th1 response [8–15] with TNF-α and IFN-γ playing an essential role [16], while the susceptibility involves a Th2 response with main participation of IL-4, IL-5, IL-10 and TGF-β [17–19]. Recently, an important study showed that individuals with PCM infection (PI) present a predominant Th1 response while those with chronic/adult form (AF) develop a Th17/Th22 pattern. The acute, subacute/juvenile form (JF) is the most severe form of the disease being characterized by Th2/Th9 type response [20]. Although aforementioned studies have shown that resistance/susceptibility in PCM can be explained by the involvement of different subpopulations of CD4+ cells, the mechanisms leading to preferential induction of any subpopulation are still unclear and those involved in the initial interaction of the fungus with cells of the innate immune response, mainly the dendritic cells (DCs), deserve to be studied.

Dendritic cells have the primary function to bind, capture, kill and process microorganisms, and migrate to peripheral lymphoid organs where they mature for efficiently triggering and instructing a T cell response [21–23]. Thus, the nature of the DCs/microorganisms interactions defines the predominant type of effector T cells. However, for most of organisms, including fungi, the receptors signaling pathways and other molecules involved in this modulation are poorly understood. Among them pattern recognition receptors (PRRs) take an important role in the binding of microorganism to DC [24, 25] triggering events that modulate the phagocytosis, antigen processing, induction of oxidative metabolism, and cytokine production [26–29].

P. brasiliensis induces migration of DCs from murine lungs to lymph nodes, however, bone marrow-derived DCs of these mice have low capability to induce a Th1 response [30], confirming previous studies on susceptible animals showing that fungus inhibits the expression of class II MHC molecules as well as IL-12 and TNF-α production by DCs [31], while induces IL-10 production by regulatory DCs [32]. On the other hand, human DCs in response to P. brasiliensis express CD83, CD80, CD86, and CCR7, and produce TNF-α, IL-6 and IL-12p40 [33]. Accordingly, genes encoding the cytokines IL-12 and TNF-α and chemokines CCL22, CCL27 and CXCL10 are positively regulated in DCs infected with the fungus [34]. Calich’s group proposed that an exacerbated inflammatory early responsiveness can hinder the development of a protective specific immune response. In this context, it was observed that DCs from susceptible animals secrete high levels IL-12 and TNF-α and promote an exacerbated proinflammatory response that in turn induces T cell anergy. In opposition, in resistant animals production of proinflammatory cytokines is accompanied by high levels of TGF-β and concomitant induction of regulatory T cells. This regulated response facilitates the development of IFN-γ, IL-4 and IL-17 producing effector T cells [35]. Although these studies have elucidated some aspects of the P. brasiliensis/DCs interaction, the possible role of other potential modulators of this interaction deserves to be considered. Among these modulators we highlight the importance of eicosanoids, such as prostaglandin E2 (PGE2).

PGE2 plays an important role in modulating the immune response [36]. Its effects are preferably suppressor and can already be detected during the development of the innate immune response, since it inhibits granulocyte functions [37] as well as the phagocytosis and killing functions by alveolar macrophages [38, 39] and monocytes [40–42]. This eicosanoid by suppressing NK cells activating cytokines such as IL-12 and IL-15 [43, 44] inhibits the cytolytic effector functions of these cells [45, 46]. PGE2 is also a potent suppressor during adaptive immune...
response. Its effect can be direct on T cell proliferation since it inhibits IL-2 production \[47\] and the expression of its receptor \[48\]–\[49\]. However, the most important effect of PGE₂ on adaptive immune response is the ability to regulate the balance between different CD4 responses. In this context, PGE₂ shifts the balance from Th₁ response toward Th₂, by regulating IFN-γ, a Th₁ cytokine, but not the Th₂ cytokines IL-4 and IL-5 \[50, 51\]. In addition to these direct effects on CD4 T cells, the suppressive effect of PGE₂ on Th₁ response can also result from its action on APC cells. It inhibits IL-12 production by monocytes \[52\] and DCs \[53, 54\] as well as the expression of the receptor for this cytokine \[55\]. These findings were confirmed by more recent studies showing that skewed Th₂ immune response involves expression of cyclooxygenase-2 (COX-2) in DCs \[56\]. Others have shown that PGE₂ produced by DCs may be involved in regulatory T cells (Tregs) expansion \[57\]. Despite all these studies demonstrating the suppressor impact of PGE₂ during the induction of immune responses, some studies have shown the importance of this mediator in the induction of fully mature DCs capable of homing to lymph nodes and to be highly effective in priming naïve T cells \[58, 54, 59, 60\].

According to these studies the greater or lesser ability of microorganisms to induce PGE₂ production by DCs can influence the maturation of these cells in response to these microorganisms. Additionally, this ability can be influenced by the type of PRR to which this organism will bind in DCs. Little is known about the receptors involved in PGs production by DCs, but TLR2, dectin-1, and DC-SIGN appears to be the most involved \[61\].

Here, we aimed to investigate whether human DCs produce PGE₂ in response to \textit{P. brasiliensis} of high and low virulence, the role of PRRs in this production and whether these mediators modulate the DC maturation in response to the fungus.

**Material and Methods**

**Subjects**

Dendritic cells were differentiated from monocytes of healthy blood donors from University Hospital of the School of Medicine Botucatu, São Paulo State University, UNESP, after signature of informed consent form. The study was approved by the Institution Research Ethics Committee (registration number: 375/2011).

**Fungi**

We used yeast cells of high virulent and low virulent strains of \textit{P. brasiliensis} (Pb18 and Pb265, respectively). To ensure virulence, the isolate was used after three serial animal passages. Yeast cells were then maintained by weekly sub-cultivation in agar GPY medium (2% glucose, 1% peptone, 0.5% yeast extract and 2% agar medium GPY at 37°C) and used on the sixth day of culture. To obtain individual cells, the fungal suspension was homogenized with glass beads in a \textit{Vortex} homogenizer (three cycles of 10 s), followed by sedimentation of undissolved lumps at 37°C for 5 minutes. Supernatants with most single cells were collected and counted in a \textit{Neubauer} chamber, using a phase contrast microscope, considering bright cells as viable, since dead cells are black. Yeast cells were adjusted to 2 x 10⁵ cells/mL and only suspensions with viability ≥ 90% were used.

**Generation of monocyte-derived DCs (mo-DCs)**

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll- Hypaque density gradient (Sigma-Aldrich, St. Louis, MO, USA) (centrifugation at 405 g for 30 minutes). Cells were collected and erythrocytes were eliminated by treatment with lysis buffer for 5 minutes at room temperature and 2 times washing with RPMI 1640 culture medium (Sigma-Aldrich). Then,
cells were suspended in complete culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/mL gentamicin and 10% inactivated fetal bovine serum) seeded in six-well tissue culture plates (5 x 10^6 cells /mL) and allowed to adhere for 2 h at 37°C in an atmosphere of 5% CO₂. Non-adherent cells were then removed by washing plates with RPMI 1640 culture medium and monocyte rich cultures were incubated with complete culture medium containing 80 ng/mL of rH IL-4 and 80 ng/mL of rH GM-CSF (R&D Systems, Inc, Minneapolis, MN, USA) for 7 days. After this period, loosely adherent cells (considered as immature dendritic cells) were collected, washed with RPMI 1640, seeded in to 24-well tissue culture plates (10^6 cells/mL) and submitted to the different treatments. Flow cytometry assays identified the collected cells as having the CD14low/CD1ahigh/CD83low phenotype, which is characteristic of immature DC. Cells viability was checked during the experiments by using trypan blue exclusion test.

**PGE₂ production by DCs**

Immature DCs (10^6/mL) were challenged with Pb18 or Pb265 (2 x 10^5 yeasts/mL) using a DCs/yeasts ratio of 5:1, or treated with LPS (5 μg/mL) for 1, 2, 4, 8, 12, 18, 24 or 48 h. Supernatants were harvested and assayed for PGE₂ levels using a competitive enzyme-linked immunosorbent assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). In some experiments, DCs were incubated for 2h with monoclonal antibodies: anti-TLR2 (2 μg/10^6 cells), and/or anti-MR (2 μg/10^6 cells) (monoclonal antibodies purchased from Biolegend, San Diego, CA, USA), anti-dectin-1 (3 μg/10^6 cells) and anti-DC-SIGN (4 μg/10^6 cells) before fungus challenge (monoclonal antibodies purchased from R&D Systems, Minneapolis, MN, USA). These concentrations were chosen because they induced the highest percentages of blockage in previous experiments (data not shown). The protocols were designed in order to block three receptors keeping only one available.

**Flow Cytometry analysis**

Monocyte-derived immature DCs were phenotyped by flow cytometry for CD14 (PerCP), CD1a (FITC) and CD83 (PE) expression in a FACSCalibur™ flow cytometer (BD-Becton, Dickinson and Company, San Diego, CA, USA) using the CellQuest software (BD-Becton-Dickinson, Company). In subsequent experiments immature DCs (10^6/mL) challenged with Pb18 or Pb265 or treated with LPS and respective controls were evaluated for expression of HLA-DR (FITC), CD40 (PerCP), CD80 (FITC), CD83 (PE), CD86 (APC), CCR5 (FITC), CCR7 (PerCP) e CXCR4 (PerCP). In some experiments 100 μg/mL of exogenous PGE₂ was added to DCs challenged with the fungus.

**Cytokine production**

Immature DCs (10^6/mL) were challenged with Pb18 or Pb265 or treated with LPS (5 μg/mL) for 48 h and culture supernatants were evaluated for IL-12p70 and TNF-α production using commercial duoset ELISA kits (BD OptEIA-Becton, Dickinson and Company). IL-12p70 production was also determined by using an other commercial duoset ELISA kit (R&D Systems) and a cytometric bead assay (CBA) (BD-Becton,Dickinson and Company). In some experiments 100 μg/mL of exogenous PGE₂ was added to DCs challenged with the fungus.

**Statistical analysis**

Statistical analysis was performed by using the *GraPhpad Prism* Version 5.01 for Windows, GraphPad Software, Inc. (San Diego, CA, USA). Significant differences among groups were
determined by analysis of variance test (ANOVA) for dependent samples, and the averages compared by Multiple Correlations Tukey-Kramer test, assuming as true every case in which the probability of error was less than 5% (p<0.05).

**Results**

**PGE$_2$ production by DCs challenged with *P. brasiliensis***

After confirmation that cells had a phenotype of immature DCs (CD14$^{\text{low}}$/CD1a$^{\text{high}}$/CD83$^{\text{low}}$) we aimed to evaluate whether they produce PGE$_2$ in response to challenge with *P. brasiliensis*. We tested both, Pb18 and Pb265, precisely because differences in the ability to produce PGE$_2$ and the consequences of this process, for example, greater or lesser ability to induce maturation of DCs, could be one of the factors that determine the differences in virulence presented by these two strains.

 Cultures were evaluated at 1, 2, 4, 8, 12, 18, 24 and 48 h. Cell stimulation with LPS was considered as a positive control for PGE$_2$ production. Despite some differences detected among the periods PGE$_2$ production by control DCs was relatively stable, with minimum and maximum production of 80.304 ± 10.94 pg/mL (12 h) and 119.434 ± 6.141 pg/mL (48 h), respectively. Stimulation with LPS promoted a significant and progressive increase in PGE$_2$ production (158.8 ± 26.30 pg/mL at 1 h to 372.8 ± 36.36 pg/mL at 48 h). On the other hand, challenge with Pb18 and Pb265, significantly inhibited PGE$_2$ production emphasizing that in some periods (2, 8 and 18 h) inhibition induced by Pb265 was higher than Pb18 (Fig. 1).

**Involvement of MR, TLR2, Dectin-1 and DC-SIGN in PGE$_2$ inhibition induced by *P. brasiliensis***

Once observed that Pb18 and Pb265 are able to inhibit PGE$_2$ production by DCs, we aimed to evaluate which PRRs are involved in this process. For this purpose, we used a schedule in which three receptors were blocked by specific antibodies and only one remained available each time. After blocking, cells were challenged with Pb18 or Pb265 for 4 or 24 h. We found that PGE$_2$ production was inhibited only when mannose receptor (MR) were kept available, indicating its role in the process. Individual availability of the TLR2, Dectin-1 or DC-SIGN resulted in levels quite similar or even higher than those detected for control DCs (Fig. 2).

**DCs maturation induced by *P. brasiliensis***

Our next objective was to evaluate whether *P. brasiliensis* induces maturation of human DCs featured by phenotypic changes. For this purpose expression of CD40, CD80, CD83, CD86, HLA-DR, CXCR4, CCR5 and CCR7 was assessed 48 h after fungus challenge. This period was chosen as ideal for maturation analysis, because it was when we detected the higher levels of PGE$_2$ after cells stimulation with LPS. We observed that challenge with Pb18 or Pb265 was not able to increase the number of DCs expressing CD40, CD83, CCR5 and CCR7. Conversely, other maturation markers CD80, HLA-DR, and CXCR4 were reduced by both strains while CD83 and CD86 were reduced by Pb265 (Fig. 3). CD80 was the only molecule with increased percentage of cells after challenge with strain Pb18. Nevertheless, when median of fluorescence intensity (MFI) was evaluated, we observed a significant decrease in response to two strains suggesting that expression of this molecule, similarly to the other above cited, was not positively regulated by *P. brasiliensis*. Together, these data demonstrate that the yeast fail to induce DCs maturation. The finding that the fungus does not induce increased expression of molecules involved in cells maturation or even in some cases inhibits this expression is not related to the lack of cell viability. Viability checking, frequently performed, was always above 90%.
Some results with LPS are also a further demonstration of fungal effects reliability. Although expression of some molecules has not been increased probably due to problems with antibodies labeling (cytometry assays), other molecules such as CD83 and CD40 were increased in response to LPS, whereas depressed in response to the fungus.

Cytokines production by DCs challenged with *P. brasiliensis*

In addition to the analysis of surface molecules, we aimed to evaluate IL-12 and TNF-α production, whose increases are also indicative of DCs maturation. We observed that both Pb265 and Pb18 induce DCs to release TNF-α but the levels were always lower than those induced by LPS (Fig. 4). On the contrary, we found that *P. brasiliensis* is not able to induce significant IL-12p70 production by DCs. Negative results were obtained by both ELISA (two different kits) and CBA assay. Overall, the results reinforce the view that the fungus is not able to induce...
maturation of DCs, as evidenced by the lack of changes on expression of molecules and production of some cytokines essential for activating CD4+ lymphocytes.

**Effect of exogenous PGE2 on the phenotypic maturation of DCs challenged or not with *P. brasiliensis***

With exception of CD40 and CXCR4, exogenous PGE2 significantly increased the percentage of control cells (not challenged) expressing all the tested molecules (CD80, CD83, CD86, HLA-DR, CCR5, and CCR7) which confirms the effect of this mediator on positively modulating phenotypic maturation of DCs. Of most importance, it also increased the percentage of cells challenged with Pb18 and Pb265 expressing all the molecules, although the results for CD40, CD86 and CXCR4 (for Pb265) were not significant. In relation to HLA-DR the results detected for control and challenged cells were confirmed by MFI assays (Fig. 5).
In addition to modulation of DCs phenotype, exogenous PGE2 also promote alterations in TNF-α production (Fig. 6), whose levels after treatment were similar to those induced by LPS (Fig. 4). On other hand, DCs did not produce IL-12p70 even after exogenous PGE2 treatment. Taken together, data on DC phenotype and cytokine production suggest that *P. brasiliensis* fails to induce DC maturation, at least in part because PGE2 production is inhibited.
Discussion

Studies on the modulatory role of PGE$_2$ on host immune response to *P. brasiliensis* are scarce, particularly in relation to DCs. In this context, this study aimed to evaluate whether human DCs produce PGE$_2$ in response to challenge with high and low virulent strains of *P. brasiliensis*, the involvement of PRRs in this process, as well as the modulatory role of this eicosanoid on maturation of these cells. These objectives were supported by previous results from our laboratory showing that Pb18 and Pb265 induce PGE$_2$ production by monocytes and that this eicosanoid, in an autocrine way, inhibits antifungal activity of these cells [40–42]. Thus, this fungus would induce PGE$_2$ production by monocytes as an escape mechanism from effector functions of these cells. However, contrary to detected for monocytes, in the present study we observed
that both Pb18 and Pb265 inhibit PGE$_2$ production by DCs. In addition, PGE$_2$ inhibition was associated with impaired maturation of DCs in response to the fungus, as confirmed by low expression of CD40, CD80, CD83, CD86, HLA-DR, CXCR4, CCR5, and CCR7.

This association between inhibition of PGE$_2$ and no DC maturation was strongly indicative that lack of adequate levels of this mediator is responsible for maturation failure. Experiments adding exogenous PGE$_2$ to cultures challenged with the fungus confirmed this mechanism since the treatment induced an increase in the percentage of cells expressing CD80, CCR7, CCR5 and HLA-DR in response to both strains, and of CD83 and CXCR4 in response to Pb265. Our findings are corroborated by previous reports that addition of PGE$_2$ to a mixture of cytokines such as IL-$\beta$ and TNF-$\alpha$ is essential for DCs maturation, since it considerably increases the expression of costimulatory molecules by these cells [59], and is fundamental for the expression of CCR7 [60].

CD40 is a critical coestimulatory molecule in the activation of T [62] lymphocytes, together with CD80, CD86 and mainly HLA-DR [63, 64]. CD83 molecule, besides participating in T cell activation is the main marker of DC maturation [65]. CCR7 binds the chemokines CCL19 and CCL21 derived from lymph nodes and its expression on DCs increases their ability to migrate to these organs [59, 60]. Therefore, our results showing that $P$. brasiliensis, by inhibiting PGE$_2$ production, does not effectively induce an increase in the expression of these molecules,
strongly suggest that, in vivo, the contact of fungus with DCs hinders the migration of these cells to secondary lymphoid organs, as well as their ability to activate T cells and hence initiate an adaptive immune response. Our results allow us to suggest that *P. brasiliensis* uses opposite mechanisms to escape monocytes and DCs responses, since increased production of PGE2 by monocytes inhibits their fungicidal mechanism, while impaired production by DCs avoid their maturation.

We also demonstrated that mannose receptor (MR) is the PRR involved in PGE2 inhibition by *P. brasiliensis* which emphasizes the role of this receptor in the escape mechanisms of some fungi. Accordingly, endocytosis of *Candida albicans* by MR pathway results in inhibition of
NADPH oxidase pathway that is essential for fungus elimination by phagocytes [66]. Specifically in relation to *P. brasiliensis* previous studies suggest that it can use MR as an evasion mechanism. A particular study has shown that gp43 fraction, the immunodominant antigen of *P. brasiliensis*, binds to MR to inhibit phagocytic and fungicidal capacity of murine peritoneal macrophages [67]. In a recent study, we observed that *P. brasiliensis* phagocytized by binding to MR is able to grow inside human monocytes. In addition, IL-18 positively modulates this process by increasing fungus binding to MR receptors [68].

We also evaluated whether DCs challenged with the fungus increase their production of IL-12 and TNF-α, two important cytokines for DCs maturation. We observed that exposition of DCs to *P. brasiliensis* does not induce production of IL-12p70. DCs and macrophages are the main source of this cytokine in response to intracellular microorganisms [69, 70] which has a key role in the modulation of Th1 response. Therefore, fail of DCs to produce IL-12p70 in response to *P. brasiliensis* avoid their role in instructing CD4+ to a Th1 response essential for host resistance to this microorganism. This result is in agreement with previous studies showing that *P. brasiliensis* or its main antigen gp43 inhibit IL-12 production by murine BM-DCs [71]. However, fail of DCs to produce IL-12 in response to the fungus is not associated to inhibition on PGE2 levels, as exogenous treatment did not result in cytokine increase. Indeed, previous studies reported that DCs activated in the presence of PGE2 lose their ability to secrete IL-12 [72, 67, 73].

TNF-α is a pleiotropic cytokine that regulates a broad range of biological events, including cell differentiation, proliferation, tissue development and death, as well as inflammation, innate and adaptive immune responses [74–77]. DCs maturation is highly dependent on TNF production [78–80] and we observed that fungus induce TNF-α production by DCs, but it levels were significantly increase after PGE2 treatment. This finding lead us to consider that inhibition of PGE2 results in the production of lower levels of TNF-α, that are insufficient to ensure DCs maturation.

Our findings support studies with PCM patients showing that only DCs from treated patients are effectively activated and show high expression of HLA-DR, CD86 and DC-SIGN, as well as IL-12 production. Thus, during active disease a dysregulation in DCs maturation can be detected and consequent fail to provide optimal costimulation for T cell proliferation may occur [81].

It has to be emphasized that no significant differences between responses induced by Pb18 and Pb265 were observed. The capacity of modulating host immune response by fungus strains is dependent on variations in their cell wall components [82, 16, 83] since they can account for fungus binding to different receptors of the innate immune response. More virulent strains of *P. brasiliensis* (as Pb18) show a smaller amount of β-glucan in their wall composition, while strains with low virulence (as Pb265) have a large amount of this carbohydrate [84] which indicates that these two strains present different capacities to bind to dectin-1, the receptor that recognizes β-glucan. In this context, as PG inhibition involves MR and not dectin-1, variations in β-glucan wall presented by two strains possibly do not interfere with this process, which can explain the similar responses detected between the two strains.

Overall, our results allow include *P. brasiliensis* in the growing list of microorganisms that impair DCs maturation and functions to evade a protective adaptive response. This mechanism has been detected in diseases caused by varicella-zoster [85] herpes simplex [78] vaccinia [86], measles [87], *Trypanosoma cruzi* [88], *Salmonella* [89], *Mycobacterium tuberculosis* [90, 91], *Mycobacterium leprae* [92] and *Cryptococcus gattii* [80].

In summary, we have found that *P. brasiliensis*, by binding to MR, inhibits PGE2 production by DCs which results in the production of lower TNF-α levels and consequent deregulation on DCs maturation in response to this fungus. However, the consequences of this process for
delivering signals required for the induction of an efficient T cell response against the fungus, need to be determined. To answer this question, in a current study in our lab, we aimed to analyzing the global transcriptional profile of DCs in response to the fungus, as well as of CD4+ cells in response to DCs. Together, the results will provide novel information for understanding the complex interplay between the host and *P. brasiliensis* and may support further therapeutic approaches.

**Acknowledgments**

We thank the healthy volunteers for their willingness to participate in this study.

**Author Contributions**

Conceived and designed the experiments: RKF TFB DRR HAB. Performed the experiments: RKF MAG LAD-M RK AMVCS. Analyzed the data: RKF MAG RK. Contributed reagents/materials/analysis tools: RK AMVCS. Wrote the paper: RKF AMVCS RK. Software used in analysis: MAG.

**references**

1. Brummer E, Castaneda E, Restrepo A (1993) Paracoccidioidomycosis: an update. Clin Microbiol Rev 6:89–117. PMID: 8472249
2. Matute DR, McEwen JG, Puccia R, Montes BA, San-Blas G, et al. (2006) Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol Biol Evol 23: 65–73. PMID: 16151188
3. Teixeira MM, Theodoro RC, de Carvalho MJ, Fernandes L, Paes HC, Hahn RC, Mendoza L, et al. (2009) Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides genus*. Mol Phylogenet Evol 52: 273–283 doi: 10.1016/j.ympev.2009.04.005 PMID: 19376249
4. Terçarioli GR, Bagagli E, Reis GM, Theodoro RC, Bosco Sde M, Macoris SA, et al. (2007) Ecological study of *Paracoccidioides brasiliensis* in soil: growth ability, conidia production and molecular detection. BMC Microbiol 7:92. PMID: 17953742
5. McEwen JG, Bedoya V, Patiño MM, Salazar ME, Restrepo A (1987) Experimental murine paracoccidiomycosis induced by the inhalation of conidia. J Med Vet Mycol 25:165–75. PMID: 3612432
6. Calich VL, Da Costa TA, Felonato M, Arruda C, Bernardino S, Loures FV, et al. (2008) Innate immunity to *Paracoccidioides brasiliensis* infection. Mycopathologia 165:223–36. PMID: 18777631
7. Benard G (2008) An overview of the immunopathology of human paracoccidiomycosis. Mycopathologia 16:209–21.
8. Shikanai-Yasuda MA, Telles Filho Fde Q, Mendes RP, Colombo AL, Moretti ML (2006) Guidelines in paracoccidiomycosis. Rev Soc Bras Med Trop 39:297–310. PMID: 16906260
9. Calich VLG, Russo M, Vaz CAC, Burger E, Singer-Vermes LM (1994) Resistance mechanism to experimental *Paracoccidioides brasiliensis* infection. Cienc Cult 46:455–61.
10. de Almeida SR, de Moraes JZ, de Camargo ZP, Gesztesi JL, Mariano M, Lopes JD (1998) Pattern of immune response to GP43 from *Paracoccidioides brasiliensis* in susceptible and resistant mice is influenced by antigen-presenting cells. Cell Immunol 190:68–76. PMID: 9926448
11. Calich VLG, Kashino SS (1998) Cytokines produced by susceptible and resistant mice in the course of *Paracoccidioides brasiliensis* infection. Braz J Med Biol Res 31:615–23. PMID: 998765
12. Kashino SS, Fazioli RA, Cafalli-Favati C, Meloni-Bruneri LH, Vaz CA, Burger E, et al. (2000) Resistance to *Paracoccidioides brasiliensis* infection is linked to a preferential Th1 immune response, whereas susceptibility is associated with absence of IFN-gamma production. J Interferon Cytokine Res 20:89–97. PMID: 10670655
13. Oliveira SJ, Mamoni RL, Musatti CC, Papaioordanou PMO, Blotta MHSL. (2002) Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidiomycosis: comparisons with infected and non-infected controls. Microbes Infected 4:139–44. PMID: 11880044
14. Mamoni RL, Blotta MHSL (2005) Kinetics of cytokines and chemokines gene expression distinguishes *Paracoccidioides brasiliensis* infection from disease. Cytokine 32:20–29. PMID: 16174562
15. Livonesi MC, Souto JT, Campanelli AP, Maffei CM, Martinez R, Rossi MA, et al. (2008) Deficiency of IL-12p40 subunit determines severe paracoccidioidomycosis in mice. Med Mycol 46:637–46. doi: 10.1080/13693780801982762 PMID: 18608917
16. Calvi SA, Peracozi MT, Mendes RP, Marcondes-Machado J, Fecchio D, Marques SA, et al. (2003) Effect of cytokines on the in vitro fungicidal activity of monocytes from paracoccidioidomycosis patients. Microbes Infect 5:107–13. PMID: 12650768
17. Benard G, Romano CC, Cacere CR, Juvenale M, Mendes-Giannini MJ, Duarte AJ (2001) Imbalance of cytokines and chemokines gene expression distinguishes Paracoccidioides brasiliensis infection from disease. Cytokine 32:20–29. PMID: 16174652
18. Mamoni RL, Nouér SA, Oliveira SJ, Musatti CC, Rossi CL, Camargo ZP, et al. (2002) Enhanced production of specific IgG4, IgE, IgA and TGF-beta in sera from patients with the juvenile form of paracoccidioidomycosis. Med Mycol 40:153–9. PMID: 12058728
19. Mamoni RL, Blotta MHSL (2008) Kinetics of cytokines and chemokines gene expression distinguishes Paracoccidioides brasiliensis infection from disease. Cytokine 32:20–29. PMID: 16174652
20. de Castro LF, Ferreira MC, da Silva RM, Blotta MH, Longhi LN, Mamoni RL (2003) Characterization of the immune response in human paracoccidioidomycosis. J Infect 67:470–85. doi: 10.1016/j.jinf.2003.07.019 PMID: 1237434
21. Cella M, Sallusto F, Lanzavecchia A (1997) Origen, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol 9:10–6. PMID: 9039784
22. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271–96. PMID: 1910679
23. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–52 Review. PMID: 9521319
24. Van Vliet SJ, Den Dunnen J, Gringhuis SI, Geijtenbeek TB, Van Kooyk Y (2007) Innate signaling and regulation of dendritic cell immunity. Curr Opin Immunol 19:435–40. PMID: 17629469
25. Van Vliet SJ, Garcia-Vallejo JJ, Van Kooyk Y (2008) Dendritic cells and C-type lectin receptors: coupling innate to adaptive immune responses. Immunol Cell Biol 86:580–7. doi: 10.1038/icb.2008.55 PMID: 18679407
26. Van Kooyk Y (2008) C-type lectins on dendritic cells: key modulators for the induction of immune responses. Biochem Soc Trans 36:1478–81. doi: 10.1042/BST0361478 PMID: 19021579
27. Geijtenbeek TB, Gringhuis SI (2009) Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol 9:465–79. doi: 10.1038/nri2596 PMID: 19521399
28. Watts C, West MA, Zaru R (2010) TLR signalling regulated antigen presentation in dendritic cells. Curr Opin Immunol 22:124–30. doi: 10.1016/j.coi.2009.12.005 PMID: 20833398
29. Zanoni I, Granucci F (2010) Regulation of antigen uptake, migration, and lifespan of dendritic cells by Interferes on Dendritic Cells Function. Immunol Cell Biol 88:637–47. doi: 10.1038/icb.2010.52 PMID: 20556351
30. Almeida SR, Lopes JD (2001) The low efficiency of dendritic cells and macrophages from mice susceptible to Paracoccidioides brasiliensis in inducing a Th1 response. Braz J Med Biol Res 34:529–37. PMID: 11285466
31. Ferreira KS, Lopes JD, Almeida SR (2004) Down-regulation of dendritic cell activation induced by Paracoccidioides brasiliensis. Immunol Lett 94:107–14. PMID: 15234542
32. Ferreira KS, Bastos KR, Russo M, Almeida SR (2007) Interaction between Paracoccidioides brasiliensis and pulmonary dendritic cells induces interleukin-10 production and toll-like receptor-2 expression: possible mechanisms of susceptibility. J Infect Dis 196:1108–15. PMID: 17763336
33. Fornazim MC, Mamoni RL, Blotta MHSL (2008) Human dendritic cells pulsed with low virulence strain of Paracoccidioides brasiliensis (Pb265) induce the proliferation of IFN-y and IL-17 producing cells. XXXIII Congress of the Brazilian Society for Immunology 1:1–3.
34. Tavares AH, Derengowski LS, Ferreira KS, Silva SS, Macedo C, Bocca AL, et al. (2012) Murine dendritic cells transcriptional modulation upon Paracoccidioides brasiliensis infection. PLoS negl Trop Dis 6:1459.
35. Pina A, de Araujo EF, Felonato M, Loures FV, Feriotti C, Bernardino S, et al. (2013) Myeloid dendritic cells (DCs) of mice susceptible to paracoccidioidomycosis suppress T cell responses whereas myeloid and plasmacytoid DCs from resistant mice induce effector and regulatory T cells. Infect Immun 81:1064–77. doi: 10.1128/IAI.00736-12 PMID: 23340311
36. Kalinski P (2012) Regulation of immune responses by prostaglandin E2. J Immunol 188:21–8. doi: 10.4049/jimmunol.1101029 PMID: 22187483
37. Smith RJ (1977) Modulation of phagocytosis by and lysosomal enzyme secretion from guinea-pig neutrophils: effect of nonsteroid anti-inflammatory agents and prostaglandins. J Pharmacol Exp Ther 200:647–657. PMID: 850132
38. Aronoff DM, Canetti C, Peters-Golden M (2004). Prostaglandin E₂ inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. J Immunol 173: 559–565. PMID: 1520817

39. Serezani CH, Chung J, Ballinger MN, Moore BB, Aronoff DM, Peters-Golden M (2007). Prostaglandin E₂ suppresses bacterial killing in alveolar macrophages by inhibiting NADPH oxidase. Am J Respir Cell Mol Biol 37:562–570. PMID: 17585108

40. Soares AM, Calvi SA, Peraçoli MT, Fernandez AC, Dias LA, Dos Anjos AR (2001) Modulatory effect of Prostaglandin E₂ on human monocyte activation for killing of high and low-virulence strains of Paracoccidioides brasiliensis. Immunology 102:480–5. PMID: 11326382

41. Bordon AP, Dias-Melicio LA, Acorí MJ, Calvi SA, Serrão Peraçoli MT, Victoriano De Campos Soares AM (2007) Prostaglandin E₂ inhibits Paracoccidioides brasiliensis killing by human monocytes. Microbes Infect 9:744–7. PMID: 17400014

42. Bordon-Graciani AP, Dias-Melicio LA, Acorí-Valério MJ, Araujo JP Jr, De Campos Soares AM (2012) Inhibitory effect of PGE₂ on the killing of Paracoccidioides brasiliensis by human monocytes can be reversed by cellular activation with cytokines. Med Mycol 50:726–34. doi: 10.3109/13693786.2012.676740 PMID: 22548241

43. Walker W, Rotondo D (2004) Prostaglandin E₂ is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-γ synthesis. Immunology 111: 298–305. PMID: 15009430

44. Joshi PC, Zhou X, Cuchens M, Jones Q (2001) Prostaglandin E₂ suppressed IL-15-mediated human NK cell function through down-regulation of common γ-chain. J Immunol 166: 885–891. PMID: 11145664

45. Bankhurst AD (1982) The modulation of human natural killer cell activity by prostaglandins. J Clin Lab Immunol 7: 85–91. PMID: 6951051

46. Goto T, Herberman RB, Maluish A, Strong DM (1983) Cyclic AMP as a mediator of prostaglandin E induces suppression of human natural killer cell activity. J Clin Immunol 130:1350–1355. PMID: 6185577

47. Walker C, Kristensen F, Bettens F, deWeck AL (1983) Lymphokine regulation of activated (G1) lymphocytes. J Immunol 146:1791–1796. PMID: 2849551

48. Rincón M, Tugores A, López-Rivas A, Silva A, Alonso M, De Landázuri MO, et al. (1988) Prostaglandin E₂ and the increase of intracellular cAMP inhibit the expression of interleukin 2 receptors in human T cells. Eur J Immunol 18:1791–1796. PMID: 2849551

49. Kolenko V, Rayman P, Roy B, Cathcart MK, O’Shea J, Tubbs R, et al. (1999) Downregulation of JAK3 protein levels in T lymphocytes by prostaglandin E₂ and other cyclic adenosine monophosphate-elevating agents: impact on interleukin-2 receptor signaling pathway. Blood 93:2308–2318. PMID: 10009941

50. Betz M, Fox BS (1991) Prostaglandin E₂ inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol 146:108–113. PMID: 1845802

51. Snijdewint FG, Kaliński P, Wierenga EA, Bos JD, Kapsenberg ML (1993) Prostaglandin E₂ differentially modulates cytokine secretion profiles of human T helper lymphocytes. J Immunol 150:5321–5329. PMID: 8390534

52. van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA (1995) Prostaglandin-E₂ differentially modulates cytokine secretion profiles of human T helper lymphocytes. J Immunol 150:5321–5329. PMID: 8390534

53. van der Pouw Kraan TC, Boeije LC, Smeenk Rj, Wijdenes J, Aarden LA (1995) Prostaglandin-E₂ is a potent inhibitor of human interleukin 12 production. J Exp Med 181:775–779. PMID: 7836930

54. Kaliński P, Hilken CM, Snijders A, Snijdewint FG, Kapsenberg ML (1997) IL-12-deficient dendritic cells, generated in the presence of prostaglandin E₂, promote type 2 cytokine production in maturing human naive T helper cells. J Immunol 159:28–35. PMID: 9200435

55. Kaliński P, Schultemaker JH, Hilken CM, Kapsenberg ML (1998) Prostaglandin E₂ induces the final maturation of IL-12-deficient CD1a⁻CD83⁻ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J Immunol 161:2804–2809. PMID: 9743339

56. Bansal K, Sinha AY, Ghorpade DS, Togarsimalmath SK, Patil SA, Kaveri SV, et al. (2010) Src homology 3-interacting domain of Rv1917c of Mycobacterium tuberculosis induces selective maturation of human dendritic cells by regulating PI3K-MAPK-NF-kappaB signaling and drives Th2 immune responses. J Bio Chem 285:36511–22. doi: 10.1074/jbc.M110.158055 PMID: 20837474

57. Rieser C, Böck G, Kloker H, Bartsch G, Thurnher M (1997) Prostaglandin E₂ and Tumor Necrosis Factor α Cooperate to Activate Human Dendritic Cells: Synergistic Activation of Interleukin 12 Production. J Experimental Medicine 186:1603–1608. PMID: 9348319
59. Luft T, Jefford M, Luetjens P, Toy T, Hochrein H, Masterman K-A, et al. (2002) Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E2 regulates the migratory capacity of specific DC subsets. Blood 100:1362–1372. PMID: 12149219

60. Scandella E, Men Y, Gillessen S, Förster R, Groettrup M (2002) Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells Blood 100:1354–1361. PMID: 12149218

61. Valera I, Fernández N, Trinidad AG, Alonso S, Brown GD, Alonso A, Crespo MS (2008) Costimulation of dectin-1 and DC-SIGN triggers the arachidonic acid cascade in human monocyte-derived dendritic cells. J Immunol 180:5727–36. PMID: 18390758

62. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ (2009) Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol 229:152–72 Review. doi: 10.1111/j.1600-065X.2009.00782.x PMID: 19426221

63. Hart DN (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90:3245–3287. PMID: 9345009

64. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252. PMID: 9521319

65. Zhou L, Tedder TF (1996) CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. Proc Natl Acad Sci U S A 93:2588–92. PMID: 8637918

66. Donini M, Zenaro E, Tamassia N, Dusi S (2007) NADPH oxidase of human dendritic cells: role in Candida albicans killing and regulation by interferons, dectin-1 and CD206. Eur J Immunol 37:1194–203. PMID: 17407098

67. Popi AF, Lopes JD, Mariano M (2002) Gp43 from Paracoccidioides brasiliensis inhibits macrophage functions. An evasion mechanism of the fungus. Cell Immunol 218:87–94. PMID: 12470616

68. Dias-Melicio LA, Fernandes RK, Golim MA, Rodrigues DN, Soares AMVC (2011) Interleukin-18 promotes growth of Paracoccidioides brasiliensis within human monocytes via mannose receptor modulation. Inflammation Research 60:S167.

69. Ma X, Trinchieri G (2001) Regulation of interleukin-12 production in antigen-presenting cells. Adv Immunol 79:55–92. PMID: 11680011

70. O’Shea JJ, Paul WE (2002) Regulation of Th1, differentiation–controlling the controllers. Nat Immunol. 3:506–508. PMID: 12032561

71. Ferreira KS, Lopes JD, Almeida SR (2004) Down-regulation of dendritic cell activation induced by Paracoccidioides brasiliensis. Immunology Letters 94:107–114. PMID: 15234542

72. Karliški P, Schuitemaker JH, Hilkenks CM, Kapsenberg ML (1998) Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J Immunol 161:2804–9. PMID: 9743339

73. Harizi H, Gualde N (2004) Inhibition of IL-6, TNF-a, and cyclooxygenase-2 protein expression by prostaglandin E2. Cell Immunol 223:120–26. PMID: 15267510

74. Harizi H, Gualde N (2005) The impact of eicosanoids on the crosstalk between innate and adaptive immunity: the key roles of dendritic cells. Tissue Antigens 65:507–514. PMID: 15896197

75. Vassiliou E, Jing H, Ganea D (2003) Prostaglandin E2 inhibits TNF production in murine bone marrow-derived dendritic cells. Cell Immunol 223:120–26. PMID: 15267510

76. Ritter U, Meissner A, Ott J, Körner H (2003) Analysis of the maturation process of dendritic cells deficient for TNF and lymphotoxin-alpha reveals an essential role for TNF. J Leukoc Biol 74:216–22. PMID: 12885938

77. Chu WM (2012). Tumor necrosis factor. Cancer Letters 328:222–225. doi: 10.1016/j.canlet.2012.10.014 PMID: 23085193

78. Salio M, Cella M, Suter M, Lanzavecchia A (1999) Inhibition of dendritic cell maturation by herpes simplex virus. Eur J Immunol 29:3245–53. PMID: 10540336

79. Ritter U, Meissner A, Ott J, Körner H (2003) Analysis of the maturation process of dendritic cells deficient for TNF and lymphotoxin-alpha reveals an essential role for TNF. J Leukoc Biol 74:216–22. PMID: 12885938

80. Huston SM, Li SS, Stack D, Timm-McCann M, Jones GJ, Islam A, et al. (2013) Cryptococcus gattii is killed by dendritic cells, but evades adaptive immunity by failing to induce dendritic cell maturation. J Immunol 191:249–61. doi: 10.4049/jimmunol.1202707 PMID: 23740956

81. Sato PK, Oshiro TM, Diogo CL, Passos EC, Shikanai-Yasuda MA (2011) Characterization of monocyte-derived dendritic cells from patients with active and treated paracoccidioidomycosis. Scand J Immunol 74:609–18. doi: 10.1111/j.1365-3083.2011.02614.x PMID: 21854409
82. Silva CL, Alves LM, Figueiredo F (1994) Involvement of cell wall glucans in the genesis and persistence of the inflammatory reaction caused by the fungus Paracoccidioides brasiliensis. Microbiology 140:1189–94. PMID:8025684

83. Anjos AR, Calvi SA, Ferracini R, Peracoli MT, Silva CL, Soares AM (2002) Role of Paracoccidioides brasiliensis cell wall fraction containing beta-glucan in tumor necrosis factor-alpha production by human monocytes: correlation with fungicidal activity. Med Mycol 40:377–82. PMID:12230216

84. San-Blas G (1993) Biochemical and physiological aspects in the dimorphism of Paracoccidioides brasiliensis. Arch Med Res Autumn 24:267–8 Review. PMID:8298276

85. Abendroth A, Morrow G, Cunningham AL, Slobedman B (2001) Varicella-zoster virus infection of human dendritic cells and transmission to T cells: implications for virus dissemination in the host. J Virol 75:6183–92. PMID:11390620

86. Engelmayr J, Larsson M, Subklewe M, Chahroudi A, Cox WI, Steinman RM, et al. (1999) Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. J Immunol 163:6762–8. PMID:10586075

87. Servet-Delprat C, Vidalain PO, Bausinger H, Manié S, Le Deist F, Azocar O, et al. (2000) Measles virus induces abnormal differentiation of CD40 ligand-activated human dendritic cells. J Immunol 164:1753–60. PMID:10657621

88. Van Overtvelt L, Vanderheyde N, Verhasselt V, Ismaili J, De Vos L, Goldman M, et al. (1999) Trypanosoma cruzi infects human dendritic cells and prevents their maturation: inhibition of cytokines, HLA-DR, and costimulatory molecules. Infect Immun 67:4033–40. PMID:10417171

89. Bueno SM, Riquelme S, Riedel CA, Kalergis AM (2012) Mechanisms used by virulent Salmonella to impair dendritic cell function and evade adaptive immunity. Immunology 137:28–36. doi: 10.1111/j.1365-2567.2012.03614.x PMID: 22703384

90. Hanekom WA, Mendillo M, Manca C, Haslett PA, Siddiqui MR, Barry C 3rd, et al. (2003) Mycobacterium tuberculosis inhibits maturation of human monocyte-derived dendritic cells in vitro. J Infect Dis 188:257–66. PMID:12854081

91. Madan-Lala R, Sia JK, King R, Adekambi T, Monin L, Khader SA, et al. (2014) Mycobacterium tuberculosis impairs dendritic cell functions through the serine hydrolase Hip1. J Immunol 192:4263–72. doi: 10.4049/jimmunol.1303185 PMID:24659689

92. Hashimoto K, Maeda Y, Kimura H, Suzuki K, Masuda A, Matsuoka M, Makino M (2002) Mycobacterium leprae infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. Infect Immun 70:5167–76. PMID: 12183567