RESEARCH ARTICLE

In vitro challenge of human dendritic cells with *Paracoccidioides brasiliensis* induces preferential generation of T_{reg} cells

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Abstract

Paracoccidioidomycosis (PCM) is a systemic mycosis, endemic to most Latin American countries (especially Brazil) whose etiologic agent is the thermodimorphic fungus *Paracoccidioides brasiliensis* (Pb). Host resistance/susceptibility to PCM has been explained by the involvement of different subpopulations of $CD4^+$ cells. However, the mechanisms leading to preferential induction of any subpopulation are still unclear, and the participation of dendritic cells (DCs) must be highlighted. These cells bind, capture, kill, process microorganisms and migrate to peripheral lymphoid tissue where they maturate, efficiently trigger, and drive $CD4^+$ T cell-mediated immune responses. The nature of the interaction of these cells with each microorganism defines $CD4^+$ cell differentiation. Few studies have evaluated which subsets of CD4+ cells are preferentially induced after the interaction of human DCs/Pb. Here, we show that *in vitro* challenge of DCs with Pb results in the preferential induction of T_{reg} cells.

Keywords: *Paracoccidioides brasiliensis*; Human Dendritic Cells; T_{reg} Cells

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis, endemic to most Latin American countries especially Brazil. The etiological agent of PCM is the thermodimorphic fungus of the genus *Paracoccidioides, which includes* the three cryptic species of *Paracoccidioides brasiliensis (Pb),* denominated S1, PS2, PS3 and the specie *Paracoccidioides lutzii* [1-3]. Hosts are usually infected through the respiratory tract by mycelium propagules found in soil. These structures, reach the lung alveoli, where they develop into yeasts that can disseminate via the lymphohematogenous route to several organs, which may result in a broad spectrum of clinical manifestations. However, most infected people are asymptomatic, present a positive cutaneous test for paracoccidioidin, and are referred to as the PCM-infection (PI) group. In contrast, a small number of individuals show one of two symptomatic forms of the disease: the chronic/adult form (AF) or acute/juvenile form (JF). The AF form predominantly presents in adult male individuals and can be unifocal, when a single organ is affected (usually the lungs) or multifocal, when two or more organs are involved (mainly the lungs and the oral mucosa). In contrast, JF is predominantly observed in young patients of both genders and is characterized by systemic lymph node involvement, hepatosplenomegaly, and bone marrow lesions [4]. Pioneering studies in human and animals have shown that resistance to *Pb* involves the host Th_1 response [5-12], in which TNF-α and IFN-γ play an essential role [13]. Susceptibility, however, is associated with the Th_2 response, which mainly involves IL-4, IL-5, IL-10 and TGF-β [14, 15]. More recently, the roles of Th_{17}/Th_{22} and T_{reg} cells have been investigated in patients. PI individuals present a predominantly Th_1 response while those with AF display a mixed Th_{17}/Th_{22} profile. Conversely, JF patients are characterized by Th_2/Th_9 responsiveness. Of note, a high T_{reg} cells were detected with high frequency in AF patients. These cells may have a dual role in this group. In addition to dampening the exacerbated inflammatory response, T_{reg} cells can inhibit macrophage activation. The inhibition of macrophages is an important effector mechanism against the fungus, which is activated by Th_1 cells [16].

Although studies have shown that resistance/susceptibility in PCM can be explained by the involvement of different $CD4^+$ subsets, the mechanisms determining the preferential differentiation of individual subsets remain unclear. Dendritic cells primarily bind, capture, kill, and process microorganisms. These cells migrate to peripheral lymphoid tissues where they trigger and instruct the T cell immune response [17-19]. The nature of the interaction of these cells with each microorganism will define the $CD4^+$ T cell response. Some studies have focused on the DC/Pb interaction and showed that DCs and macrophages induce the Th_1 response with low efficiency in susceptible mice and humans [20-24]. However, studies assessing $CD4^+$ cell subsets profiles after interactions with human DCs are scarce.

In this study, we observed that DC/Pb interaction does not result in the generation of Th_1, Th_2 or Th_{17} cells. However, it did induce the differentiation of T_{reg} cells.

2 Materials and Methods

2.1 Participants

Blood samples were collected from healthy adult donors who signed the written informed consent approved by the Research Ethics Committee School of Medicine of Botucatu, São Paulo State University (Unesp) (registration number: 32809914.2.0000.5411).

2.2 Fungus

We used yeast cell suspensions of high- and low-virulence strains of *Paracoccidioides brasiliensis* (Pb18 and Pb265, respectively). Cultivation, obtainment of individual cells, viability determination, and concentrations adjustment were performed as previously described [25].

2.3 Monocyte Purification and *In Vitro* Differentiation

Human monocyte-derived DCs were generated as follows: mononuclear cells were obtained by centrifugation of peripheral blood at 405 × *g* for 30 min on a Ficoll-Paque density gradient (GE Healthcare, Uppsala, Sweden). Mononuclear cells were harvested, and any remaining erythrocytes were lysed by treatment with hypotonic lysis solution for 5 min. Cell suspensions were washed twice with RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) and the cell pellet was resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/mL gentamicin and 10% inactivated fetal bovine serum (complete culture medium), and adjusted to 10^7 mononuclear cells/mL. The mononuclear cells were then centrifuged at 800 × *g* for 20 min on a Percoll density gradient (GE Healthcare, Uppsala, Sweden) to isolate monocytes from lymphocytes. Cells at the interface were collected, and monocytes were purified by negative magnetic selection of $CD14^+/CD3^-$ cells. Lymphocytes in the pellet were collected, washed, and frozen at -80°C. The isolated monocytes were suspended in complete culture medium and cultured with human recombinant IL-4 (80 ng/mL; R&D Systems Minneapolis, MN, USA) and GM-CSF (80 ng/mL; R&D Systems) in 6-well culture plates (5.10^5 cells/mL) for seven days. Subsequently, the loosely adhered cells were collected with a pipette, washed with complete medium, and the cell concentration was adjusted to 10^5 DCs/mL and plated in 96 flat-bottomed well plates (100 μL/well).

2.4 DC Immunophenotyping and Analysis of Viability

Generated DCs were transferred to flow cytometry tubes (BD Bioscience, San Diego, CA, USA) and centrifuged at 520 × *g* for 10 min at 4°C. After centrifugation, cells were resuspended in 1 mL of electrolyte solution (ISOTON II) followed by incubation with anti-CD14-PerCP-Cy 5.5 (peridinin chlorophyll protein complex), anti-CD1a-FITC (fluorescein isothyocyanate), anti-CD83-PE (phycoerythrin), and anti-CD11c-APC (allophycocyanin) (BD Bioscience) for 30 min. Next, the cells were washed at 520 × *g* for 10 min, suspended in 450 μL of ISOTON II, and fixed with 50 μL of fixing solution containing 5% formaldehyde (BD Bioscience). DC samples were also labeled with 1 μL/mL Live/Dead Kit (Life Technologies, Eugene, OR, USA). Twenty thousand events were acquired on a FACSCanto flow cytometer (BD Bioscience) and the data analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Unlabeled cells were used as autofluorescence controls for each sample. Results are expressed as the percentage of positive cells.

2.5 Challenge of DCs with *Paracoccidioides brasiliensis*

About 10^5 DCs were challenged with 2.10^4 Pb cells of high (Pb18) or low virulence (Pb265) for 48h (DC:Pb ratio 5:1). This ratio was based on a previous publication [26].

2.6 Isolation of $CD4^+$ Cells

Total lymphocytes obtained as described earlier (section 2.3) were defrosted, washed, and $CD4^+$ cells were purified by negative magnetic selection using MACS® magnetic $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec Inc., Auburn, CA). Isolated cells were

analyzed by flow cytometry to check their phenotype and viability using anti-CD4-PE-Cy7 (phycoerythrin), anti-CD3-FITC (Fluorescein), anti-CD8-PerCP-CY5.5 (Peridinin Chlorophyll Protein Complex) (BD - Becton, Dickinson and Company) monoclonal antibodies, and the Live/Dead Kit (Life Technologies, Eugene, OR, USA).

2.7 Lymphoproliferation Assay

$CD4^+$ cells were labeled with 1 µM/mL Cell Trace™ Far Red Cell Proliferation Kit (Life Technologies, Eugene, OR, USA), adjusted to 10^6/mL, and co-cultured for 96 or 120 h with 10^5 autologous DCs (CD4:DC ratio10:1) previously challenged with Pb18 or Pb265. Phytohemagglutinin (PHA) (1 µg/mL) was used as a polyclonal-positive stimulus. Proliferating cells were phenotyped with monoclonal anti-CD4-PE-Cy7 and anti-CD3-FITC (BD Bioscience) antibodies and analyzed by flow cytometry as described previously.

2.8 Intracellular Expression of IFN-γ, IL-4, and IL-17

$DC/CD4^+$ T cell co-cultures were maintained for 96,120 h and 6 h before each period a Brefeldin solution (1000X, 5 mg/mL) (BioLegend) was added to the cultures to prevent cytokine release from the cytoplasm. After these periods, cultured cells were transferred to microfuge tubes and centrifuged at 10,000 rpm for 60 s. The supernatant was removed and cells washed with 200 µL of 1% PBS-BSA. Subsequently, cells were labeled with anti-CD4-PE-Cy7, and anti-CD3-FITC (BD-Becton, Dickinson and Company) and incubated for 20 min. Cells were then fixed with 100 µL of reagent A of the Fix & Perm kit (Nordic MUbio) for 20 min at room temperature, washed with 0.5% BSA solution and permeabilized with of 100 µL of reagent B of the Fix & Perm kit (Nordic MUbio), followed by incubation with anti-IFN-γ-PE, anti-IL-4-PE, and anti-IL17-Alexa 488 antibodies for 20 min at room temperature. Finally, cells were analyzed for intracellular expression of IFN-γ, IL-4, and IL-17 by flow cytometry.

2.9 Intracellular Expression of Foxp3

$CD4^+$ cells treated as described above were incubated with anti-CD25-APC-H7, anti-CD127-PerCP-Cy5.5, anti-CD3- FITC, and anti-CD4-PE-Cy7 antibodies (BD - Becton, Dickinson and Company) for 20 min at 4°C. Cells were washed with 300 µL of wash buffer (eBiosciense - San Diego, CA) and then suspended in 300 µL of reagent A of the Fix & Perm kit. Cells were then permeabilized with 300 µL of reagent B of the Fix & Perm kit for 30 min at 4°C, washed with 500 µL of wash buffer and centrifuged twice for 5 min at 405 × *g* and once for 60 s at 9,500 × *g*. The supernatant was removed and the cells incubated with anti-Foxp3-PE (phycoerythrin) for 30 min at room temperature. Finally, cells were rinsed with 1 mL of wash buffer and resuspended in 300 µL of isotonic solution + 1% BSA for analysis by flow cytometry (as described above: section 2.4).

2.10 Statistical Analysis

Data analysis was performed using *GraphPad Prism* Version 5.01 for Windows, (GraphPad Software, Inc. San Diego, CA, USA). Differences between results were calculated using the Repeated Measures ANOVA test followed by the Tukey-Kramer test for multiple comparisons. Differences with less than 5% probability of error ($p \leq 0.05$) were considered significant.

3 Results

3.1 CD4$^+$ Lymphocytes Proliferate in Response to Autologous Dendritic Cells Challenged with Pb

Challenging DCs with Pb increased their ability to induce proliferation of autologous CD4$^+$ cells. The proliferative response of CD4$^+$ cells to DCs previously challenged with Pb was higher than the control co-culture (unchallenged DCs) (Figure 1). This difference was observed at both 96 and 120 h of culture but not during the earlier periods (48 and 72 h). Pb18 and Pb265 strains were equally able to prime DCs to induce this activation.

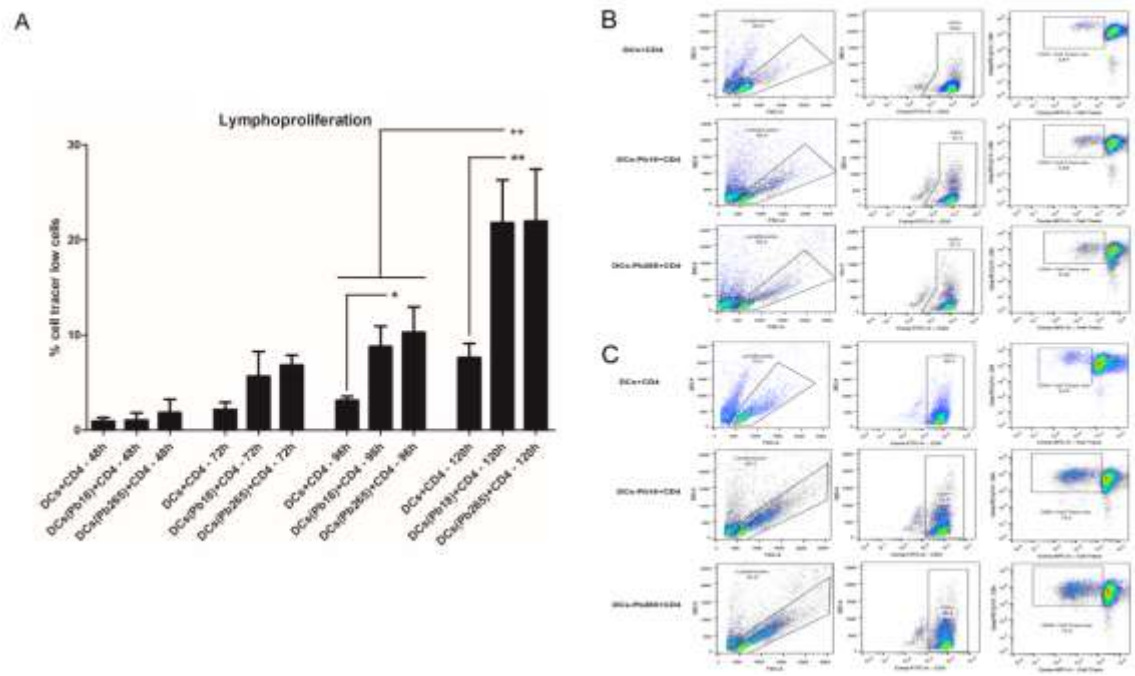

Figure 1. A) Percentage CD4$^+$ T cell proliferation after 48h-120 h co-culture with DCs with or without Pb18 or Pb265 challenge for 48 h. Results are expressed as mean ± SD of cultures performed with cells from 6 participants; *$p < 0.05$; **$p < 0.001$. B) Representative dot plots showing frequency of CD4$^+$ T cell lymphoproliferation at 96 h. C) Representative dot plots showing frequency of CD4$^+$ T cell lymphoproliferation at 120 h.

3.2 Pb-Challenged DCs Challenged Induce a T_{reg} Profile

As CD4$^+$ cells proliferated in response to DCs stimulated with Pb (96 and 120 h), we next investigated the proliferating cells by detection of intracellular cytokines specific to each CD4$^+$ subset. Only a low percentage of CD4$^+$ cells expressed IFN-γ, IL-4, and IL-17 cytokines, indicating a lack of differentiation into Th_1, Th_2 or Th_{17} subsets (Figure 2A, C).

Next, we decided to investigate the presence of T_{reg} cells in these co-cultures. We found a significant percentage of cells expressing CD25 and the intracellular transcription factor Foxp3. In addition, these

cells were negative for CD127, which confirms the T_{reg} phenotype (Figure 3A, B, and C). Both Pb18 and Pb265 strains stimulated DCs to induce proliferation of autologous $CD4^+$ cells.

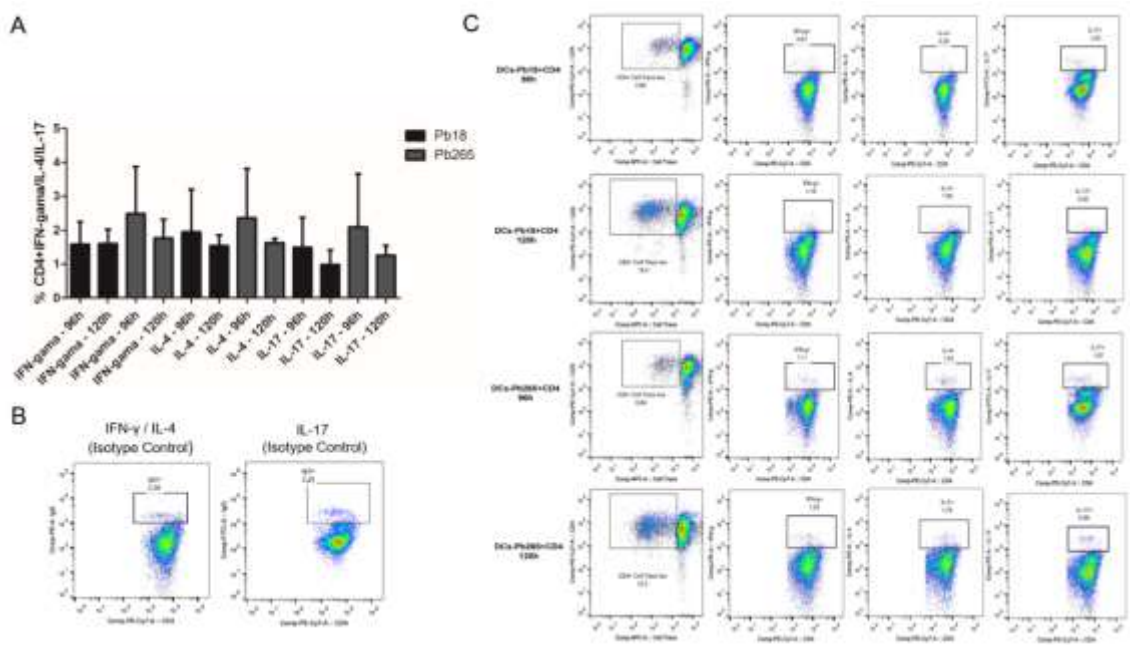

Figure 2. A) Percentage of $CD4^+$ cells expressing IFNγ, IL-4, and IL-17 after 96 h-120 h co-culture with DCs challenged with Pb18 or Pb265 for 48 h. Results are expressed as mean ± SD of cultures performed with cells from 6 participants. B) Isotype control for intracytoplasmic IFN-γ, IL-4, and IL-17. C) Representative dot plots of cytokine expression.

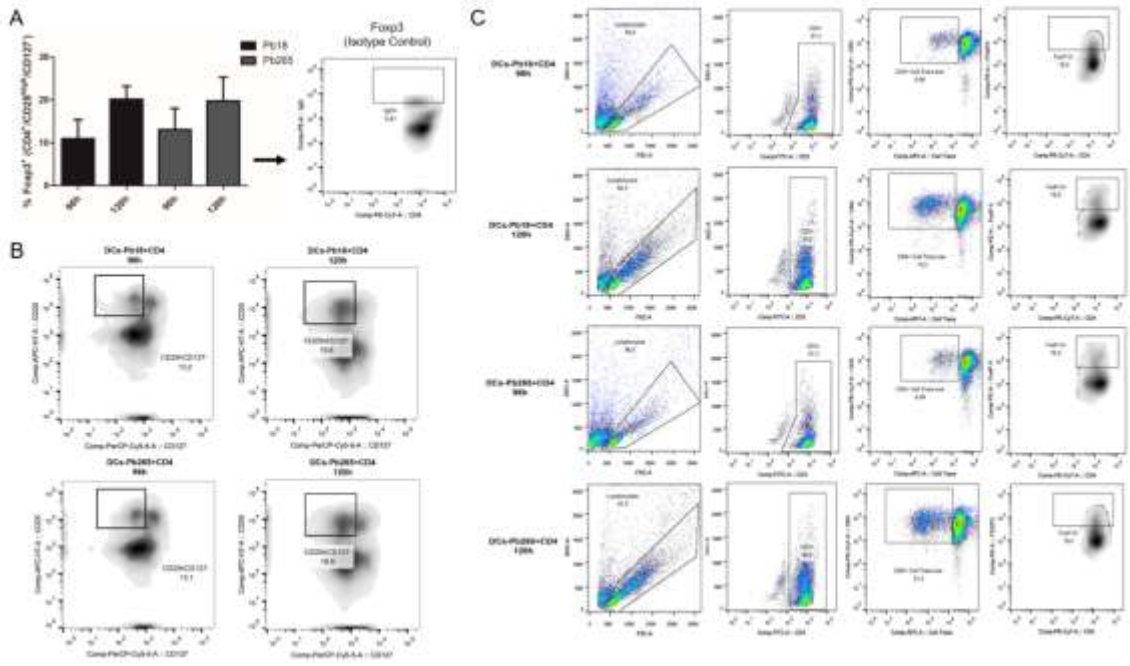

Figure 3. A) Percentage of $CD4^+$ cells expressing CD25 and Foxp3, but not CD127, after 96-120 h co-culture with DCs challenged with Pb18 or Pb265.Dot plot at the right side, shows the isotype control of cytoplasmic Foxp3 labeling. Results are expressed as mean ± SD of cultures performed with cells from 6 participants. B) Representative CD127 and CD25 FACS plots. C) Representative dot plots of Foxp3 expression in $CD4^+$ cells.

4 Discussion

Here we found that after interacting with DCs challenged with either Pb18 or Pb265, $CD4^+$ cells do not differentiate into Th_1, Th_2 or Th_{17} subsets. Instead, these $CD4^+$ cells are induced to a T_{reg} lineage as shown by the expression of Foxp3 and CD25 and the absence of CD127. To the best of our knowledge, this is the first study to demonstrate that human DCs challenged with Pb induce T_{reg} differentiation.

Our results are consistent with others showing that DCs have regulatory functions via the induction of T cell tolerance [27]. Tolerogenic DCs exhibit low expression levels of costimulatory molecules, release low levels of IL-12, and high levels of IL-10, driving $CD4^+$ T cells to differentiate into T_{reg} cells, rather than effector $CD4^+$ cells [28-30].

The mechanisms that lead microorganism-derived antigens to induce immunogenic or tolerogenic DCs are not fully understood, but several studies have shown that these cells can induce immunogenicity or tolerance depending on their state of maturation and localization [28]. It is generally believed that mature DCs trigger immunogenic mechanisms, while the remaining immature DCs tend to induce tolerance. Previously, we determined that Pb inhibits the production of prostaglandin E2 (PGE2) by binding to the mannose receptor of immature human DCs. Here, we have expanded this mechanism by showing that these immature DCs induce T_{reg} differentiation.

These data have contributed to the understanding of immunopathological mechanisms in disease. T_{reg} cells play a key role in immune modulation, as they secrete

the regulatory cytokines IL-10 and TGF-β, which can inhibit the activity of $CD4^+$ effector cells [31, 32]. Pioneering studies showed that a high number of T_{reg} cells are found in lesions of patients with chronic PCM, strongly suggesting that this $CD4^+$ subset contributes to the maintenance of fungi in chronic lesions [33, 34]. Indeed, other studies have reported that T_{reg} cells are strongly associated with immunosuppression during PCM by a cell-cell contact mechanism and IL-10 and TGF-β production [35].

A similar association was detected in studies with experimental models of the infection. Dectin-1 receptor knockout mice show increased T_{reg} expansion that enables uncontrolled fungal growth and dissemination [36]. Mice lacking CCR5, a key receptor for T_{reg} migration, are more efficient in controlling fungal burden and dissemination of the infection [37]. Similar results were found when T_{reg} cells were depleted in susceptible and resistant mice infected with the fungus [38].

Correspondingly, T_{reg} cells facilitate the burden in other fungal infections such as by *Candida albicans*, *Pneumocystis carinii*, and *Aspergillus fumigatus* [39-41]. For instance, it was observed that in mice infected with *A. fumigatus*, T_{reg} cells play a regulatory role due to its ability to inhibit the Th_1 cell, hindering a protective immune response [42].

These studies clearly show an association between Treg-mediated immunosuppression and increased susceptibility to infection. In contrast, other authors have suggested that host resistance depends on the presence of effector mechanisms sufficient to destroy the infectious while being able to regulate and avoid damage to the infected tissue through regulatory responsiveness [43, 44]. Therefore, T_{reg} cells may have a dual role: a deleterious effect by impairing $CD4^+$ effector mechanisms and fungus eradication or a protective role avoiding exacerbated tissue inflammation. In an elegant study, Bazan *et al*. [45], provided evidence for these two T_{reg} roles in PCM. The authors showed that mice depleted of T_{reg} cells with anti-CD25 antibody and infected with Pb had a reduced fungal load. Furthermore, they observed that transference of $CD4^+$ $Foxp3^-$ cells to immunodeficient $Rag1^{-/-}$ mice generated harmful inflammatory reactions in the lungs, while $CD4^+$ $Foxp3^+$ cells induced a protective immune response. However, these authors did not explore the mechanisms involved in T_{reg} generation. Correspondingly, susceptible mice display preferential expansion of myeloid regulatory DCs involved in the impaired proliferation of effector $CD4^+$ cells. In contrast, resistant mice expand both myeloid and plasmacytoid DCs to promote the expansion of Th_1, Th_2, Th_{17}, and T_{regs}, ensuring a controlled effector response that avoids tissue damage [43, 46].

Taken together, our findings strongly suggest that by binding to the mannose receptor, Pb inhibits DC maturation, inducing a regulatory phenotype and consequent $CD4^+$ differentiation to T_{reg} cells.

Conflict of interest disclosure

Authors declare no conflict of interest.

Acknowledgements

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