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Original Article

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Bone marrow–derived mesenchymal stem cells transplantation alters the course of experimental paracoccidioidomycosis by exacerbating the chronic pulmonary inflammatory response

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Abstract

Several studies have shown the potential use of bone marrow mesenchymal stem cells (BM-MSCs) as a therapeutic approach to infectious diseases. Since BM-MSCs can exert antimicrobial properties and influence the immune response against pathogens, our aim was to study the antimicrobial therapeutic potential of BM-MSCs in an experimental model of paracoccidioidomycosis (PCM). BM-MSCs were isolated from BALB/c donor mice. *Paracoccidioides brasiliensis*–infected male BALB/c mice were injected with purified BM-MSCs at 8th week post-infection. Mice were sacrificed at 12th week post-infection. Homing of BM-MSCs was confirmed by cellular labeling with fluorescent lipophilic dye and detected by flow cytometry. We found that, in comparison with nontransplanted infected animals, BM-MSCs-treated and *P. brasiliensis*-infected mice showed a significant increase in (i) fungal burdens, (ii) neutrophils, eosinophils and M2 macrophages counts, and (iii) interleukin (IL)-6, IL-9, GM-CSF, CXCL1, CXCL9, and CCL5 levels, while presenting a decrease in M1 macrophages and Treg cells in lungs. In addition, the histopathological analysis of the lungs showed an increased inflammatory process. This is the first study to our knowledge that evaluates the effects of BM-MSCs treatment in PCM. Our results indicate that the immunoregulatory function of BM-MSCs

may be triggered by the interaction with *P. brasiliensis*, which exacerbates chronic pulmonary inflammatory response.

Key words: bone marrow mesenchymal stem cells, inflammatory response, paracoccidioidomycosis, regenerative therapies.

Introduction

Bone marrow–derived mesenchymal stem/stromal cells (BM-MSCs) are a multipotent nonhematopoietic population of cells that are able to renew themselves, differentiate into multiple cell lineages. In addition, they are and characterized by their tropism to the site of the wound or chronic inflammation.[1] In humans, these cells have a plastic-adherent capability, are positive for CD73, CD90, and CD105, lack the expression of CD45, CD34, CD14, CD11b, CD79a, and HLA class II surface markers, and differentiate into adipogenic, osteogenic, and chondrogenic lineages *in vitro*.[2] In addition to their regenerative capabilities, BM-MSCs tend to have chemotactic properties similar to those immune cells that allow them to respond to injury or inflammation process, thus modulating or regulating the immune response.[3] Several studies have shown that BM-MSCs release many different cytokines, chemokines, extracellular matrix proteins and growth factors that can influence immunity, reduce the inflammatory response and induce tissue repair.[4–8] Cell-based regenerative therapies using autologous BM-MSCs are well accepted, maintain tissue homeostasis and show low tissue rejection, compared to classical pharmacological strategies.[9]

Moreover, it has been proposed that BM-MSCs play a role in host defense.[10] Through a mechanism that involves the recognition of the pathogen via Toll-like receptors (TLRs) present on the cell surface, BM-MSCs may boost antimicrobial response and mitigate damages caused by the host response to the pathogen; such interactions may induce BM-MSCs to function as either pro-inflammatory (MSC1) or immunosuppressive (MSC2) cells.[10,11] At the same time, BM-MSCs may stimulate the production or expression of antimicrobial peptides.[12] *In vivo* studies using animal models of infection (including *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Staphylococcus epidermidis*, *Toxoplasma gondii*, or *Cytomegalovirus*) have shown that BM-MSCs decrease microbial burdens and extend host survival.[11]

Endemic fungal infections are one of the major causes of morbidity and mortality worldwide, and represent a serious public health problem.[13] Among these infections, paracoccidioidomycosis (PCM), which is caused by the dimorphic fungal pathogen *Paracoccidioides* spp., is a mycosis of increasing importance in Latin America.[14,15] Usually, this mycosis is a chronic and progressive illness that involves several organs and systems, mainly the lungs (the primary site of infection) with the appearance of fibrosis, a complication that is associated with loss of respiratory function in 50% of patients.[16] Recently, using a murine model of chronic pulmonary PCM, our group showed that neutrophils are detrimental during the chronic course of the disease development; thus, depletion of neutrophils was associated with better control of infection and reduction of inflammatory response.[17]

Regarding antimicrobial and immunoregulatory roles of mesenchymal stem cells (MSCs), it has been reported that IL-17–positive MSCs were able to inhibit the growth of *Candida albicans* during *in vitro* and *in vivo* studies.[18] However, in another study using a mouse model of asthma triggered by *Aspergillus fumigatus* hyphal extract, MSCs efficiently reduced the neutrophil-mediated allergic airway inflammation through inhibition of the Th17 signaling pathway.[19] Another *in vitro* study described an immunomodulatory effect of BM-MSC on macrophages stimulated by *A. fumigatus* conidia, with a decrease of tumor necrosis factor α (TNFα) and an increase of IL-10.[20] However, the effect of BM-MSC on chronic inflammatory response due to fungal pathogens has not yet been shown. Therefore, the objective of the present work was to study the immunoregulatory and antifungal potential of BM-MSCs in an experimental model of chronic pulmonary PCM.

Methods

BM-MSC isolation, purification and culture

BM-MSCs were obtained from 4-week-old female BALB/c mice from the breeding colony maintained at the Corporación para Investigaciones Biológicas (CIB). Protocols for isolation and purification of BM-MSCs were adapted from a previous report by Rojas et al.[21] Briefly, mice were anesthetized with a solution of ketamine (80 mg/kg) and Xylazine (8 mg/kg) via intramuscular injection. Their femurs and tibias were removed, and bone marrow cells were collected by flushing with Dulbecco's modified eagle medium (DMEM) low glucose (GIBCO, Invitrogen Corporation, Carlsbad, CA, USA) containing penicillin/streptomycin 1% (GIBCO). Approximately 20×10^6 bone marrow cells were seeded in culture flasks (Eppendorf,

Hamburg, Germany) containing DMEM-low glucose supplemented with 1% penicillin – streptomycin, 10% heat-inactivated (37°C for 15 min and 56°C for 20 min, according to manufacturer's recommendation) fetal bovine serum (FBS) (GIBCO) and nonessential amino acids 1% (GIBCO) and were later incubated at 37°C in 5% CO_2. Nonadherent cells were removed by inversion after 48 hours and then washed with PBS twice and maintained for 7 days in supplemented-D-MEM low glucose. In order to exclude hematopoietic stem cells and leucocytes, a magnetic bead-based mouse cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing anti-CD45, anti-CD11b, anti-CD5, anti-Gr1 (Ly-6/C), and anti-Ter 119 monoclonal antibodies was employed. Finally, a total of 1×10^6 purified BM-MSCs was used to seed and amplify in culture. After 4 weeks, the cell population had increased approximately threefold, and the cells were then labeled and used to transplant to the animals.

Ethics statement

This study was carried out in accordance with regulations outlined in Colombian Law 84/1989 and Resolution no. 8430/1993, the European Union, and the Canadian Council on Animal Care. The protocol was approved by the Institutional Ethics Committee of the CIB (Act no.95).

Characterization of BM-MSCs

After isolation and purification of BM-MSCs, flow cytometry was performed to determine expression patterns of murine surface proteins. Fluorescein isothiocyanate (FITC) anti-CD45 (BD Pharmingen, San Diego, CA, USA), phycoerythrin (PE)-Cy5-anti-CD44, allophycocyanin (APC) anti-CD105, PE-Cy7-anti-CD106, APC-anti-TER-119, Pacific blue-anti-SCA-1, and PE-anti-CD73 antibodies (Biolegend, San Diego, CA, USA) were employed. FITC-CD45$^-$ cells were gated from R1 forward scatter (FSC-A) versus side scatter (SSC-A) areas. Those cells negative for CD45 were gated and the other markers were analyzed. A total of 50 000 events were counted. Cells were analyzed using a FACS Canto II system (BD Biosciences, San Jose, CA, USA) and FlowJo V10 software (FlowJo, LLC, Data Analysis software, Ashland, OR, USA). Cell viability was determined by trypan blue exclusion (all samples were over 95%). An additional differentiation assay was performed to demonstrate the plasticity of BM-MSCs. Chondrogenic, adipogenic, and osteogenic differentiation were thus induced *in vitro* using a StemPro® (Waltham, MA, USA) differentiation kit, according to manufacturer's instructions. The StemPro® kit contains all reagents required for inducing MSCs to be committed to the chondrogenesis, adipogenesis or osteogenesis pathways and generate chondrocytes, adipocytes, and osteocytes, respectively.

BM-MSCs tracking

BM-MSCs were labeled with a fluorescent lipophilic carbocyanine dye, and chloromethylbenzamide (CM-Dil) dye according to manufacturer´s instructions (CellTracker, Invitrogen, Switzerland). Briefly, CM-Dill was diluted in a concentration of 1 uM in culture medium and added to the culture flask containing the cells in a confluence around 80%–95%. The culture was incubated at 37°C, 5% CO_2 for 30 min. Then, cells were washed twice with phosphate-buffered saline (PBS). Labeled BM-MSCs were maintained in culture for 24 hours and then transplanted intravenously in BALB/c mice infected with *P. brasiliensis* (at 4 weeks post-infection). The homing of BM-MSCs into the different organs was evaluated as the presence of CM-Dil-labeled BM-MSCs was detected in lungs, livers, and spleens at 1st, 7th, 14th, and 28th day post-transplant. Tissues were then homogenized, and CM-Dil fluorescence was determined by flow cytometry.

Mouse model of chronic pulmonary paracoccidioidomycosis

In this study we used a highly virulent strain of *P. brasiliensis* (Pb18) as previously described by Puerta-Arias et al.[17] The model of infection was developed according to previous reports[17,22]; briefly, inbred BALB/c 6 to 8 week-old male mice were intranasally (i.n.) infected with 1.5×10^6 *P. brasiliensis* yeast cells contained in 60 μl of PBS. The total inoculum was split into two equal doses instilled intranasally at 5–10 minute (min) intervals. The control group was inoculated with 60 μl of PBS. A total of 110 mice were used in this study. Forty male mice were separated into two experimental groups consisting of infected or noninfected control mice. Mice in both groups were split into subgroups in order to undergo the following treatment regimens: nontransplanted and transplanted. Each sub-group consisted of 10 animals (5 for histopathological analysis and 5 for colony-forming units (cfu), cellular and cytokine and chemokine levels). In addition, 40 mice were used for BM-MSCs tracking experiments, and 30 female mice were used as donors.

Transplant of BM-MSCs

Infected and noninfected mice were intravenously injected with a single dose of 1×10^6 BM-MSCs at 8th week post-challenge. Mice were sacrificed after the 12th week p.i., and

their lungs, livers, and spleens were removed, weighted, and homogenized to determine fungal burdens by cfu counting, cytokine and chemokine levels, and cellularity. In each group, lungs were collected for histopathological analysis.

Determination of cfu counts

Lungs, livers, and spleens were weighed after removal and homogenized in 2 ml sterile PBS/ 1% penicillin–streptomycin solution (GIBCO Invitrogen Corporation, Carlsbad, CA, USA) to a final concentration of 100 U/ml penicillin–100 μg/ml streptomycin using a gentleMACS Dissociator (Miltenyi Biotec, Teterow, Germany). Homogeneous suspensions were diluted (1:100, 1:1000 and 1:10 000) and 0.5 ml of each dilution was plated on petri dishes with brain heart infusion (BHI) agar medium (BD BBL, Franklin Lakes, NJ, USA) supplemented with D-(+)-Glucose (Sigma-Aldrich, Saint Louis, MO, USA) to a final concentration of 0.5%, horse serum (GIBCO) previously heat-inactivated at 56°C for 30 min to a final concentration of 4%, and EDTA (Sigma-Aldrich, Saint Louis, MO, USA) to a final concentration of 300 μM, followed by incubation at 36°C, 5% CO_2. The cfu counts were assessed 11 days after culture; cfu counts were calculated as previously reported.[17,23]

Lung leukocyte populations

Lung tissue was homogenized, resuspended and filtered through 40 and 70 μm sterile cell strainers (Thermo Fisher Scientific Inc, Waltham, MA, USA) in Roswell Park Memorial Institute medium (RPMI) plus 1% FBS (Sigma-Aldrich, Saint Louis, MO, USA) previously heat-inactivated at 56 °C for 30 min. Cell suspensions were centrifuged at 500 *G*, 10°C for 10 min, and red blood cells were lysed using ACK Lysing Buffer (GIBCO). Cells were resuspended in RPMI plus 10% FBS and counted using a hemocytometer. Cell viability was detected by trypan blue exclusion (over 95% in all samples). Fc receptors were blocked using a purified rat anti-mouse CD16/CD32 (BD Pharmigen, San Diego, CA, USA). Cell populations such as neutrophils, eosinophils, macrophages, and T CD4 cells were determined by flow cytometry using fluorescent mAbs against murine surface molecules and isotype controls. All fluorescent antibodies employed in this study are listed in Table 1. For analysis of Treg cells and intracellular cytokines analysis, cells were treated with Cytofix/Cytoperm™ solution and Perm/Wash™ solution (BD Pharmigen, San Diego, CA, USA); stained cell suspensions were fixed with FACS buffer/1% fixing paraformaldehyde (PFA) solution (Carlo Erba, Barcelona, Spain). The analysis was performed using a FACS Canto II system (BD Biosciences, San Jose, CA, USA) and FlowJo V10 software (FlowJo, LLC, Data Analysis software, Ashland, OR, USA). Cell populations were analyzed as follows: (a) cell events in region 1 (R1) were gated by forward scatter versus side scatter areas; (b) CD45+ events were gated from R1 by side scatter area versus CD45 staining to establish the R2 region, from which (c) cell events were gated according to the specific surface marker to determine specific population and intracellular markers. The total number of leukocytes was obtained after multiplying the cell suspension count by the percentage of CD45+ cells. The number of each leukocyte subpopulation was determined by multiplying the percentage of each gated subpopulation by the total number of leukocytes (CD45+ population). Leukocytes subpopulations were defined as described elsewhere.[17]

Cytokine and chemokine measurement

Lung tissue was homogenized as mentioned above. Protease inhibitor cocktail 1X (Roche Applied Science, Mannheim, Germany) was added to the homogeneous suspensions and centrifuged at 800 G, 4°C for 5 min. Aliquots of supernatants were stored at −70°C until use. Cytokines, CC chemokine ligand (CCL) and CXC chemokine ligand (CXCL) were determined by a multiplex assay using a commercial kit (MPXCYTO-70 K, Milliplex ®) and the Luminex 200 system (EMD Millipore, Billerica, MA, USA). The molecules analyzed were: CCL-2 (monocyte chemoattractant protein 1, MCP-1), CCL-3 (macrophage-inflammatory protein-1α, MIP-1α), CCL-4 (MIP-1β), CCL-5 (regulated on activation, normal T cell expressed and secreted, RANTES), CCL-11 (Eotaxin), CXCL-1 (keratinocyte chemoattractant, KC), CXCL-2 (MIP-2), CXCL-5 (lipopolysaccharide induced CXC chemokine, LIX), CXCL-9 (monokine induced by gamma interferon [MIG]), CXCL-10 (interferon-inducible protein-10 [IP-10]), Leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), monocyte colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, TNF-α, and vascular endothelial growth factor (VEGF).

Histopathological analysis

Lungs of mice were removed and analyzed as described by Puerta-Arias et al.[17] In brief, the lungs were intracardially perfused with 1X PBS using a 10 ml syringe with a 21 G x 1,5-inch needle through left ventricle until lungs cleared of blood, followed by formalin solution [Formaldehyde solution (EM Science, Gibbstown, NJ) to a final concentration

Table 1. Fluorescent antibodies against murine surface molecules used to determine leukocyte populations by flow cytometry assay.

Target	Fluorochrome	Source	Brand
Isotype	FITC	Rat IgG2bκ	Becton Dickinson
Isotype	PE	Rat IgG2aκ	Becton Dickinson
Isotype	PE	Rat IgG2bκ	Becton Dickinson
Isotype	PE	Goat IgG	BioLegend
Isotype	PerCP-Cy5.5	Rat IgG2aκ	Becton Dickinson
Isotype	APC	Rat IgG1κ	Becton Dickinson
Isotype	APC	Rat IgG2aκ	Becton Dickinson
Isotype	APC-Cy7	Rat IgG1κ	Becton Dickinson
Isotype	APC-Cy7	Rat IgG2bκ	Becton Dickinson
Isotype	Pacific blue	Hamster IgG	BioLegend
CD3	PE	Rat IgG2aκ	Becton Dickinson
CD4	PerCP-Cy5.5	Rat IgG2aκ	Becton Dickinson
CD8	PerCP-Cy5.5	Rat IgG2aκ	Becton Dickinson
CD11b	PE	Rat IgG2bκ	Becton Dickinson
CD11c	APC	Hamster IgG1, λ2	Becton Dickinson
CD25	PE	Rat IgG2bκ	Becton Dickinson
CD45	FITC	Rat IgG2bκ	Becton Dickinson
CD80	Pacific blue	Hamster IgG1	BioLegend
CD206	PerCP-Cy5.5	Rat IgG2aκ	BioLegend
Siglec-F	PE	Rat IgG2aκ	Becton Dickinson
Ly-6G	APC	Rat IgG2aκ	Becton Dickinson
Ly-6 G and Ly-6C	APC-Cy7	Rat IgG2bκ	Becton Dickinson
IFNγ	PE-Cy7	Rat IgG1κ	Becton Dickinson
IL4	PE-Cy7	Rat IgG1κ	Becton Dickinson
IL9	PE	Hamster IgG2	Becton Dickinson
IL 17a	APC-Cy7	Rat IgG1κ	Becton Dickinson
IL-22	PE	Goat IgG	BioLegend

APC, allophycocyanin; Cy, Cychrome; FITC, Fluorescein isothiocyanate; PE, phycoerythrin; PerCP: Peridinin chlorphyll protein.

of 4%, sodium dihydrogen phosphate (Merck, Darmstadt, Germany) to a final concentration of 0.15 M and sodium hydroxide (Sigma-Aldrich, Saint Louis, MO, USA) to a final concentration of 0.11 M] to fix tissues. Then, the fixed tissues were embedded in paraffin, cut and stained with Hematoxylin and Eosin (H&E) to determine the lung inflammatory response, and Methenamine Silver to identify *P. brasiliensis* yeast cells. Tissue images were captured using a Nikon Eclipse Ci-L microscope - Nikon DS-Fi2 digital camera (Nikon Instruments Inc., Melville, USA).

Statistical aAnalysis

Data analysis was performed using the Graph Pad Prism software version 7 (GraphPad Software, Inc., La Jolla, CA, USA). For all values, normality was checked by the Shapiro-Wilk test. Mean and standard error of the mean (SEM) were calculated to analyze flow cytometry by Student *t* test; medians and interquartile range (IQR) were employed to analyze cfu counts, cytokines, and chemokines, respectively, using Mann–Whitney test for comparison between two groups. Values of $P < .05$ were considered to be significant.

Results

Isolation and characterization of BM-MSCs

To determine if isolated cells corresponded to BM-MSCs, they were grown in a specific differentiation medium. Adipogenic differentiation was detected approximately at day 14, while osteogenic and chondrogenic differentiation were observed at day 20 in their corresponding medium. These tests demonstrated that isolated cells corresponded to BM-MSCs (Fig. 1A–C). In addition, the phenotype of these BM-MSCs was determined and the following profile was found: $CD44^+/CD105^+/CD106^+/CD45^-/CD73^-/Sca\text{-}1^-/TER119^-$ (Fig. 1D–J).

BM-MSCs localized in lungs after transplant

Previous to main experiments, the localization of the injected BM-MSCs in organs had been proven by the identification of donor cells stained with CM-DiI; after 4 weeks p.i. with *P. brasiliensis*, mice were injected IV with 1×10^6 CM-DiI-BM-MSCs. We observed that the $CM\text{-}Dil^+$ cells were located mostly in lungs of infected mice between

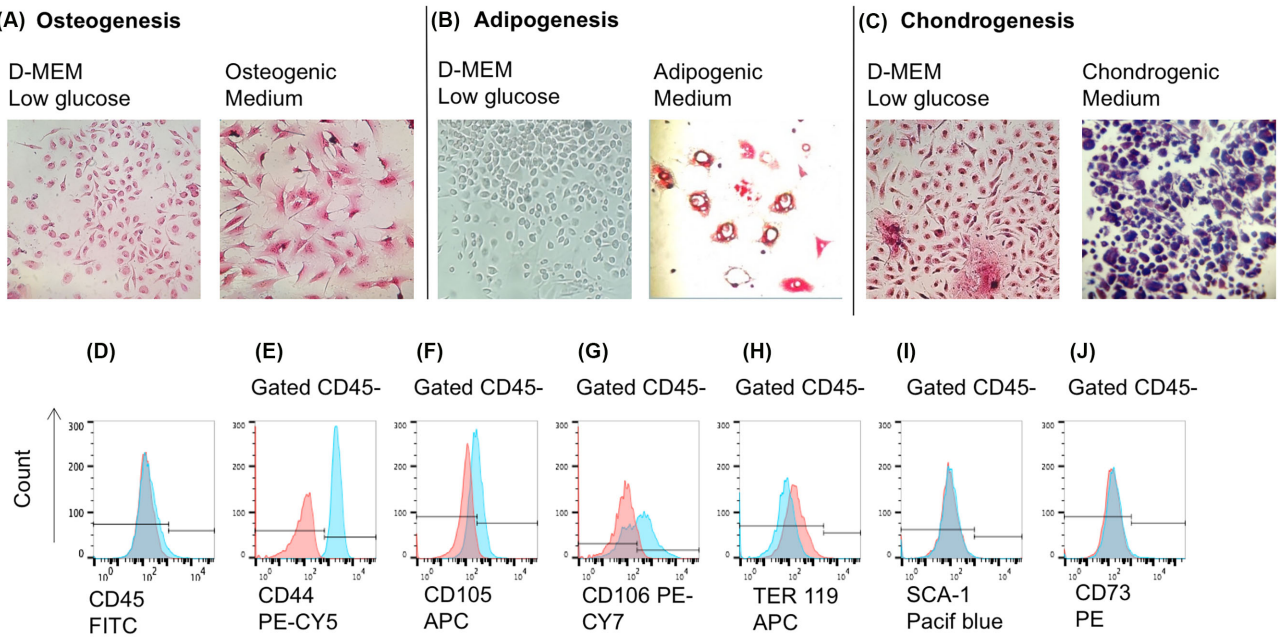

Figure 1. Characterization of bone marrow mesenchymal stem cells (BM-MSCs). Linage differentiation of BM-MSCs was induced as described in the Materials and Methods section. A: Osteogenesis at 14th day, stained with Safranin; B: Adipogenesis at 14th day stained with Oil Red; C: Chondrogenesis at 20th day, stained with Masson's trichrome. D–J: Phenotype cells were analyzed using a FACS Canto II system and FlowJo V10 software. FITC-CD45− cells were gated from forward scatter (FSC-A) versus side scatter (SSC-A) areas. Blue: Specific antibody, Red: Isotype control. For all analysis 50 000 events were counted.

1st and 14th day post-administration; approximately 4% of the cells were positive for CM-Dil, which represented 70% (0.7×10^6) of the total BM-MSCs administered (Fig. 2).

BM-MSCs transplant increases both chronic inflammatory response and fungal load

BALB/c male mice were infected with 1.5×10^6 cells of *P. brasiliensis* and BM-MSCs (1×10^6 cells) were administered intravenously at 8th week post-infection in a single dose. The animals were sacrificed at 12th week post-infection to assess the pulmonary tissue for histopathological analysis. Compared to infected- and non-BM-MSCs-transplanted mice (Fig. 3B), infected and BM-MSCs-transplanted mice showed an increased inflammatory reaction with marked congestion, granulomas, edema, and leukocyte infiltration (Fig. 3C).

To determine whether the BM-MSCs had any effect on the fungal load, we assessed fungal cfu in lungs, livers, and spleens. We observed a higher and significant number of cfu in lungs and spleens of mice transplanted at 8th week p.i. (Fig. 3F). Similar findings were observed in the histopathological analysis using Methenamine Silver stain, which allowed *in situ* identification of fungal structures in lungs (Fig. 3D, E).

BM-MSCs increase number of inflammatory cells: neutrophils, eosinophils and type-2 macrophages

We decided to evaluate some populations of leukocytes that participate in the inflammatory response against *P. brasiliensis*. We observed a significant increase of neutrophils, eosinophils and type-2 macrophages and a decrease of type-1 macrophages in the group of animals infected and BM-MSCs-transplanted (Fig. 4).

Th9, Th17, and Treg cells decrease in lungs of BM-MSCs-transplanted mice

T helper (Th) lymphocyte subpopulations are important in developing immune response, since they are the source of different profiles of cytokines that may define the course of infection. We thus evaluated different Th subsets (Th1, Th2, Th9, Th17, Th22, and Treg) using intracellular flow cytometry. We observed a significant decrease of Th9, Th17, and Treg cells in *P. brasiliensis*-infected and BM-MSCs-transplanted mice in comparison with infected and non–BM-MSCs transplanted animals (Fig. 5C, D, F).

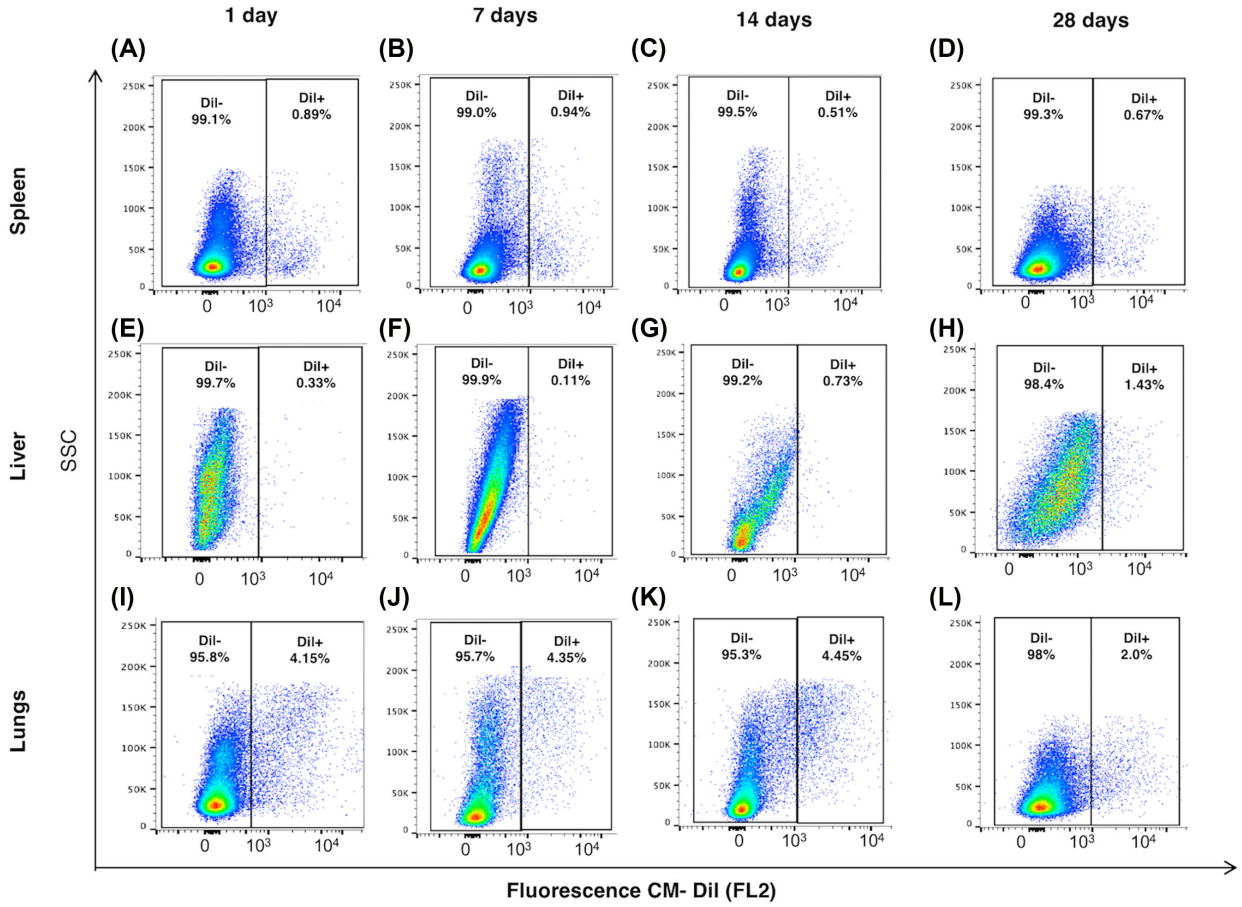

Figure 2. Bone marrow mesenchymal stem cells (BM-MSCs) tracking in lungs, livers and spleens of *P. brasiliensis*-infected mice. BM-MSCs were labeled with CM-Dil (1 μM) CellTracker® and then administered IV in BALB/c mice infected with *P. brasiliensis* after 4 weeks p.i. Tracking of BM-MSCs-CM-Dil was evaluated in spleens (A–D), livers (E–H), and lungs (I–L) at different periods (1, 7, 14, and 28 days) after cell administration using FACS, fluorescence of CM-Dil cells were collected in FL-2 red spectra 553–570 nm.

Regarding Th1, Th2, and Th22 subsets, we observed that these cell populations were significantly higher in *P. brasiliensis*-infected mice than in uninfected animals; of note, BM-MSCs treatment did not affect these T-cell subsets (Fig. 5A, B, E).

BM-MSCs increase levels of pro-inflammatory cytokines, CCL5 and CXCL chemokines

The levels of chemokines and cytokines were also determined in lungs. We observed that the levels of some cytokines (IL6, IL-9, and GM-CSF) and chemokines (CCL5, CCXL1, and CXCL9) were significantly higher in infected and BM-MSCs-transplanted mice compared to infected-untreated animals (Figs. 6 and 7, respectively).

Discussion

Lungs are organs exposed to many harmful agents, whether chemical or infectious. Several studies have employed MSCs in the treatment of lung diseases.[24] Animal models and clinical trials have been focused on noninfectious threats,[24–26] but to our knowledge, there are no studies focused on the effects of MSCs on chronic pulmonary infections. This document describes, for the first time to our knowledge, a therapy based on administering BM-MSCs in an animal model of the chronic pulmonary disease caused by the fungal pathogen *P. brasiliensis*, which is of special importance in Latin American countries. We showed that BM-MSCs induced an exacerbated inflammatory response and worsened the course of infection in *P. brasiliensis*–infected mice.

It is noteworthy that although human BM-MSCs have been well characterized, there is no consensus concerning the BM-MSC phenotype in mice. We confirmed isolation of BM-MSCs since they differentiated into adipocytes, chondrocytes, and osteocytes *in vitro*. Besides, BM-MSCs expressed typical phenotype markers such as CD44, CD105, and CD106; nonetheless, the expression of other specific markers such as CD73 and SCa1 were negative, probably due to mouse strain differences.[27] Further studies are

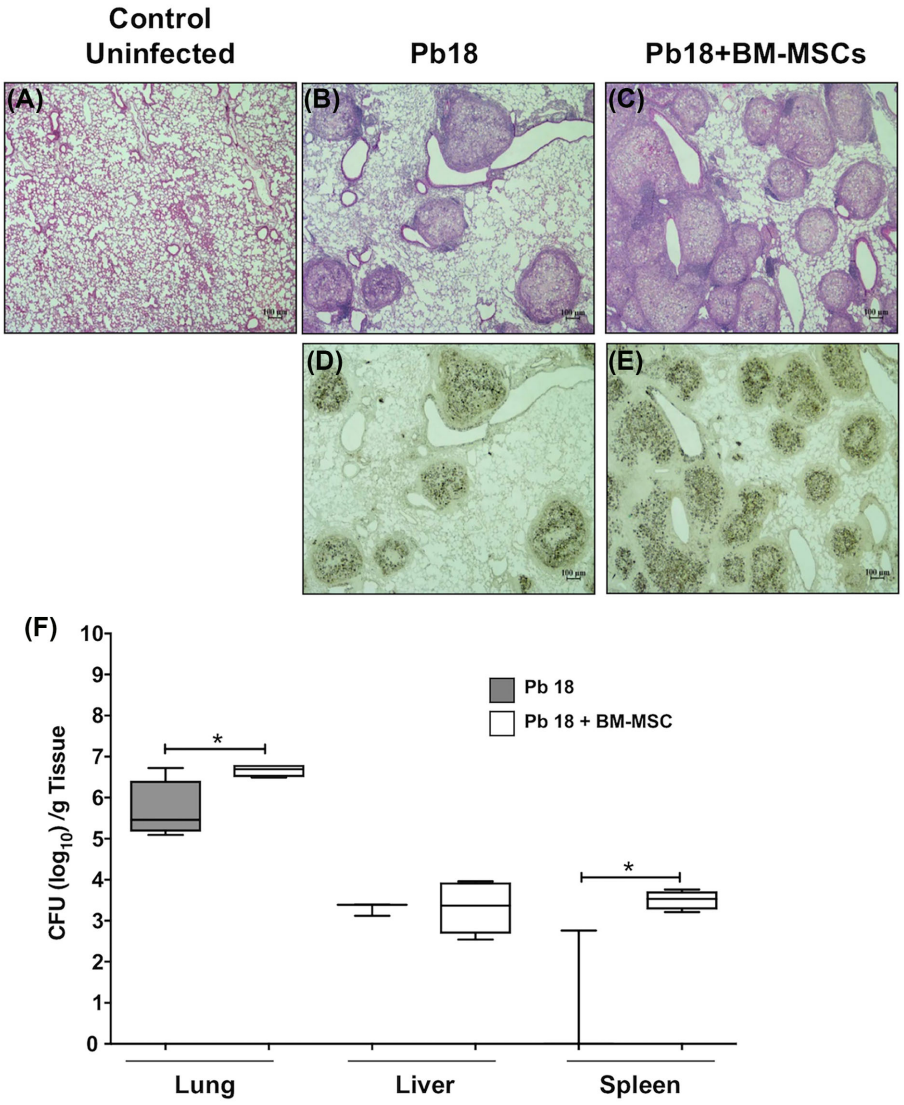

Figure 3. Bone marrow mesenchymal stem cells (BM-MSCs) administration increases the chronic inflammatory response and fungal load. Microphotographs are representative of lungs from uninfected mice (A); *P. brasiliensis* (Pb18) - infected control mice (B, D); BM-MSCs administration at 8th week p.i. (C, E). Lungs were fixed, embedded in paraffin, cut, and stained using H&E staining to determine lung inflammatory response (A–C) and Methenamine Silver staining (D, E) to identify fungal structures. Magnification 40 ×. Fungal load measurement was performed in lungs, livers, and spleens of infected mice at 12th week p.i. (F). The cfu dates were analyzed using the Mann-Whitney test and values are presented as median (IQR). (Pb18-infected $n = 4$; Pb18-infected + BM-MSCs $n = 6$). *, $P < .05$ comparing *P. brasiliensis*-infected and BM-MSCs-treated mice versus *P. brasiliensis*-infected control mice.

required to identify or confirm more specific markers for these cells.

During interaction, MSCs and microbial agents may be modulated by several factors including the microbial burden and the immune cells present in the inflammatory environment. As an example, it has been described that low numbers of *Porphyromonas gingivalis* result in an enhancement of the anti-inflammatory and antibacterial response by MSCs, while high concentrations of this bacterial pathogen render MSCs inactive.[28] Therefore, MSCs may exert an appropriate antimicrobial response if they are used early in the course of infection or as preventive measure, but they could be deleterious if they are administered in a more advanced infectious process with both higher inflammatory response and microbial burden.[10,28–30] In accordance to these reports, we observed that administration of BM-MSCs during chronic courses of infectious processes, characterized by abundant inflammation with high fungal burdens, caused deleterious effects such as increased lung inflammation and edema, recruitment of leukocytes, disruption of lung parenchyma with large confluent granulomas and higher fungal burdens in lungs and spleens.

It has been described that MSCs express several Toll-like receptors (TLRs) on their surface, including TLR2 and

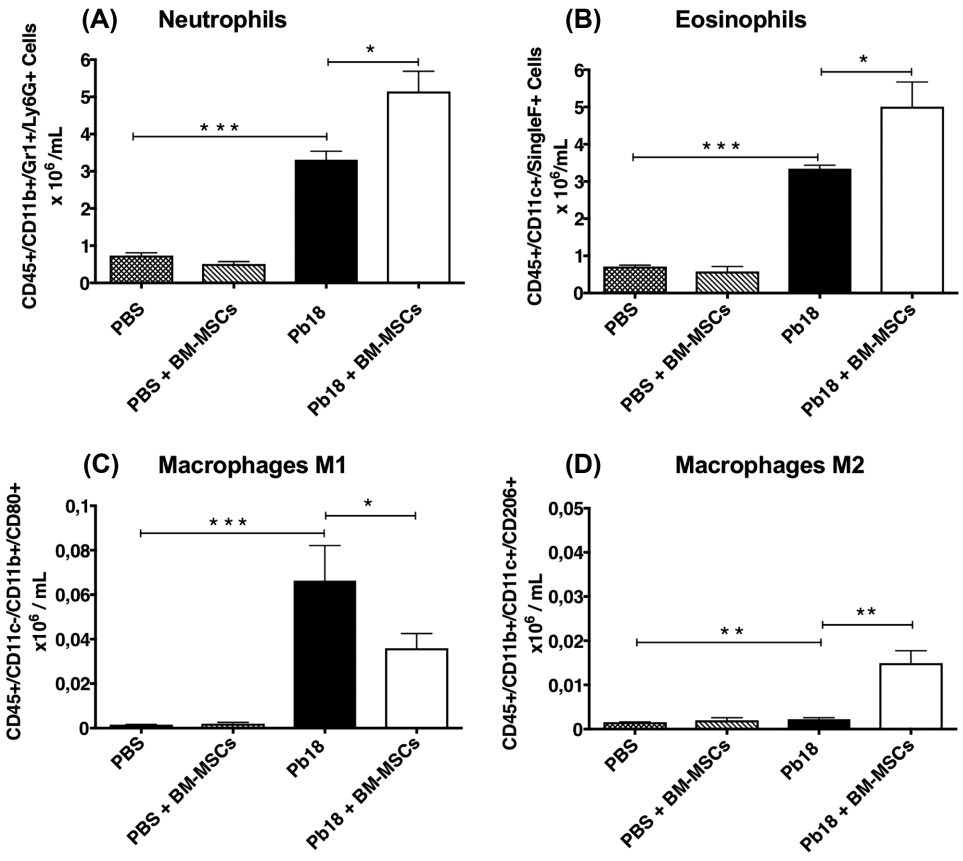

Figure 4. Effect of bone marrow mesenchymal stem cells (BM-MSCs) on recruitment of inflammatory cells. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in the Materials and Methods section. Cell populations were assessed by flow cytometry. Significant increase in neutrophils (A), eosinophils (B), and type-2 macrophages (D), and significant decrease in type-1 macrophage (C) numbers were observed. Data shown represent mean and SEM (n = 4–5 mice/group) and were analyzed by student-*t* test. *, $P < .05$ and **, $P < .001$ comparing infected untreated mice versus uninfected mice; or comparing *P. brasiliensis*-infected untreated mice versus *P. brasiliensis*-infected and BM-MSCs-treated mice ***, $P < .0001$ comparing infected untreated mice versus uninfected mice.

TLR4. Once MSCs interact with pathogens, they become capable of producing pro-inflammatory cytokines and antimicrobial peptides, or inducing T-cell activation, proliferation or suppression.[10,11,31] *In vitro* and *in vivo* studies have allowed the evaluation of the interaction between TLRs and *P. brasiliensis*; it has thus been described that TLR2 signaling appears to have a negative regulatory effect on Th17 immunity and tissue pathology, while TLR4 signaling leads to severe fungal infections associated with enhanced pro-inflammatory response and impaired proliferation of Treg cells.[32,33] Although we did not evaluate the involvement of TLRs expressed by BM-MSCs and their interaction with *P. brasiliensis* in this report, such participation should not be ruled out.

We have recently reported that neutrophils and eosinophils appear to have a detrimental effect during the chronic course of PCM. Depletion of neutrophils was thus associated with better control of the infection, with decrease of inflammatory cell numbers including eosinophils, and low levels of pro-inflammatory cytokines and chemokines.[17] In agreement with such previous results, in the present study we observed that the exacerbated inflammatory response in infected and BM-MSCs–transplanted mice was accompanied by higher numbers of pro-inflammatory cells including neutrophils, eosinophils, and type-2 macrophages, as well as increased levels of cytokines such as IL-6, IL-9, and GM-CSF, and chemokines including CXCL1 and CXCL9. Therefore, we hypothesize that the increased number of neutrophils may be correlated with the increased levels of CXCL1 observed after transplant of BM-MSCs. Furthermore, eosinophils have been described as a predictor of spread of *P. brasiliensis*, and they are associated with poor outcomes of PCM.[29,34]

M1 and M2 are important subpopulations of macrophages since they influence the course of infectious diseases;[30] thus, the balance between anti-inflammatory (M2) and pro-inflammatory (M1) macrophages is determinant in controlling inflammation and preventing excessive tissue damage.[35] Kim et al. demonstrated that human MSCs antagonize the M1 phenotype and promote

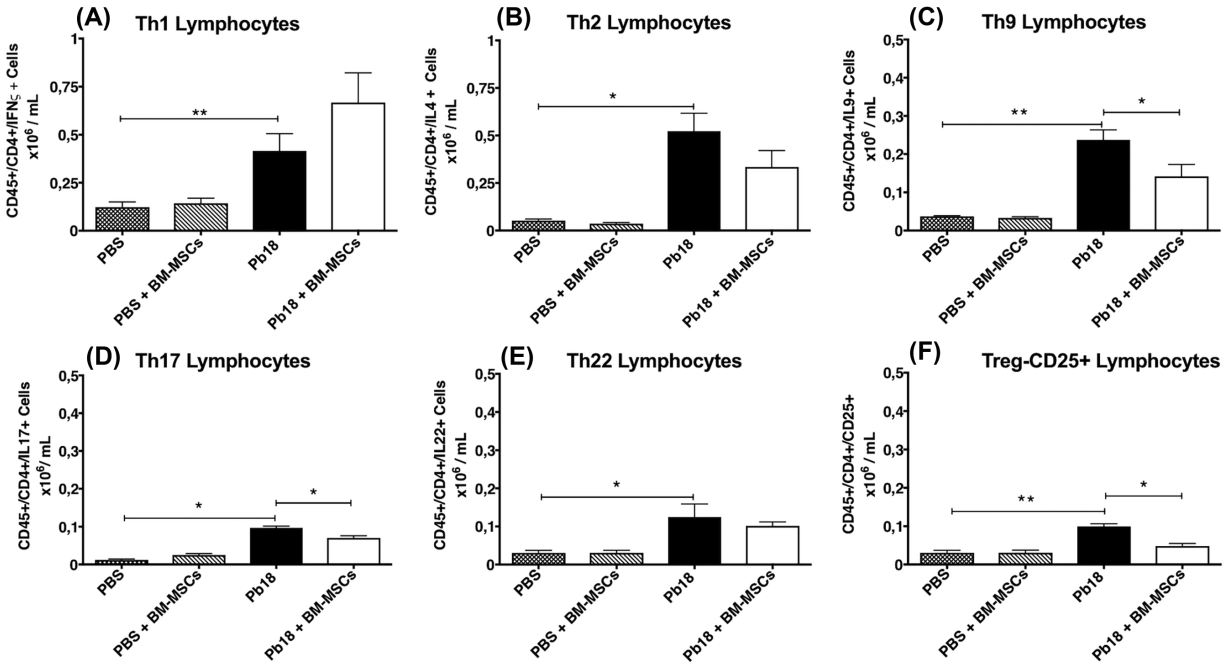

Figure 5. Effect of bone marrow mesenchymal stem cells (BM-MSCs) on recruitment of T helper subsets. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in Materials and Methods. Cell populations were assessed by flow cytometry. A) Th1 ($CD45^+CD4^+IFN\text{-}\gamma^+$), B) Th2 ($CD45^+CD4^+IL\text{-}4^+$), C) Th9 ($CD45^+CD4^+IL\text{-}9^+$), D) Th17 ($CD45^+CD4^+IL\text{-}17^+$), E) Th22 ($CD45^+CD4^+IL\text{-}22^+$), and F) Treg ($CD45^+CD4^+CD25^+$). Data shown represent mean and SEM (n = 4–5 mice/group) and were analyzed by Student *t* test. *, $P < .05$; comparing infected untreated mice versus uninfected mice, or comparing *P. brasiliensis*-infected untreated mice versus *P. brasiliensis*–infected and BM-MSCs-treated mice; **, $P < .001$; comparing infected untreated mice versus uninfected mice.

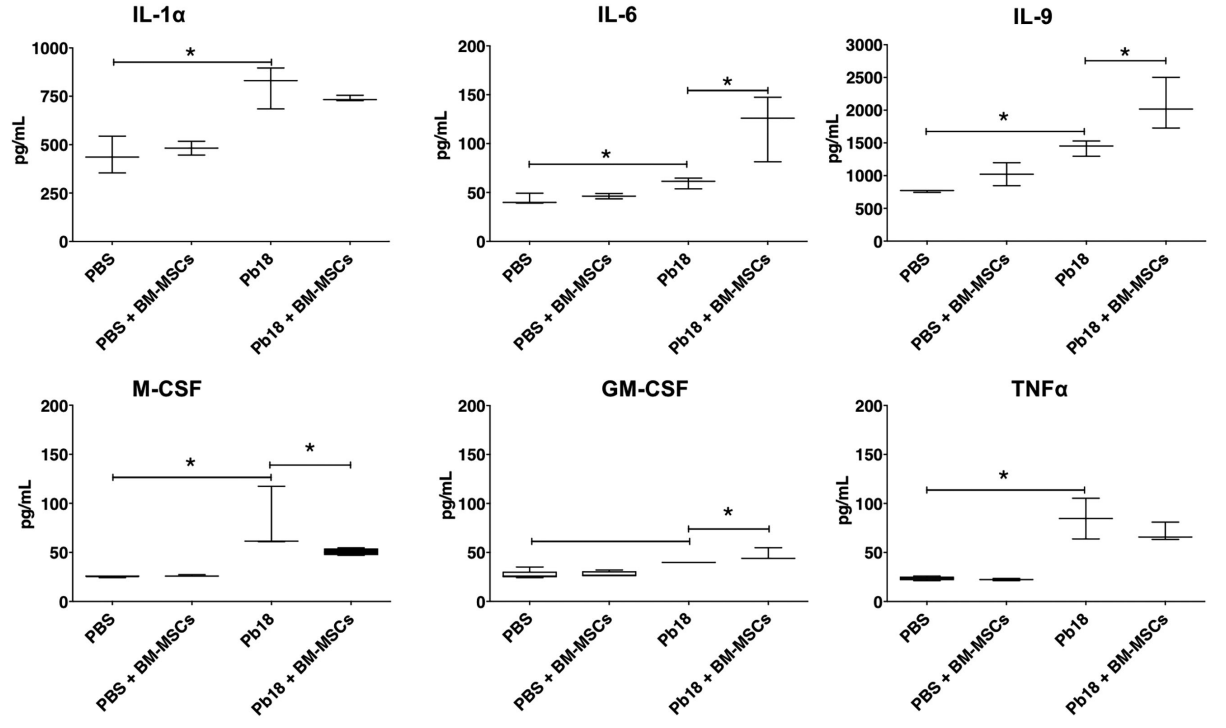

Figure 6. Effect of bone marrow mesenchymal stem cells (BM-MSCs) on cytokine levels. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in Methods. Pro-inflammatory cytokines were assessed with Luminex 200 system. Statistical analysis was performed using Mann–Witney test; data shown represent medians and IQR ($n = 4$–5 mice/group). *, $P < .05$ comparing infected untreated mice versus uninfected mice, or comparing *P. brasiliensis*-infected untreated mice versus *P. brasiliensis*-infected and BM-MSCs-treated mice.

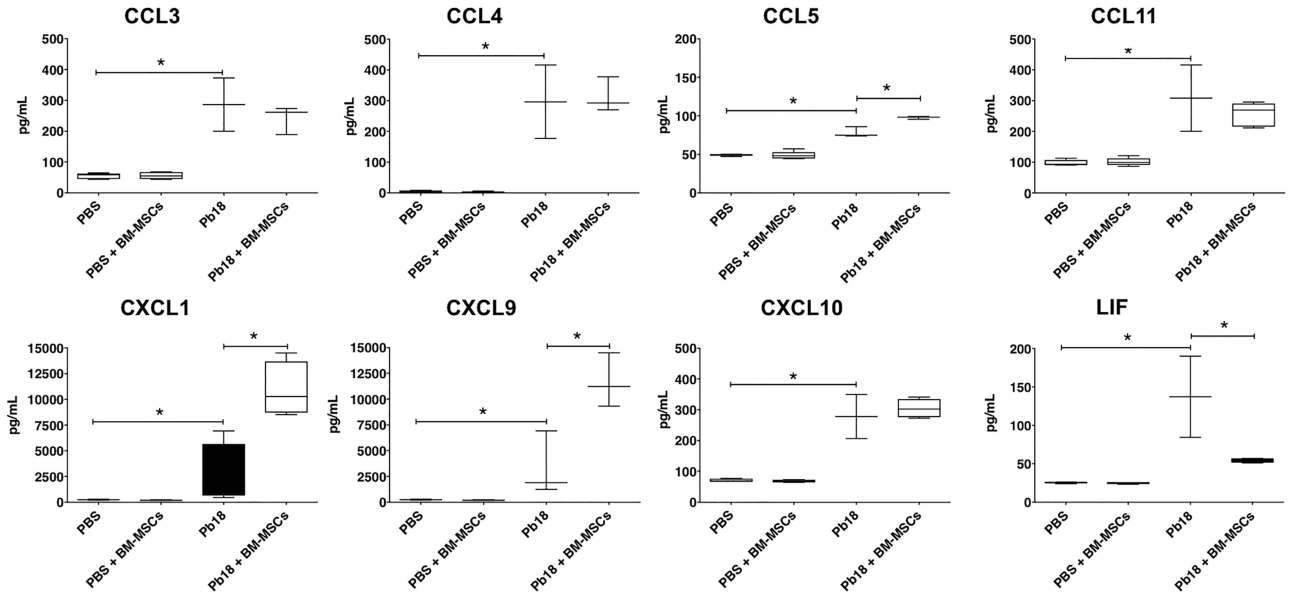

Figure 7. Effect of bone marrow mesenchymal stem cells (BM-MSCs) on chemokine levels. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in Methods. Chemokines were assessed with Luminex 200 system. Statistical analysis was performed using Mann–Witney test; data shown represent medians and IQR (n = 4–5 mice/group). *, $P < .05$ comparing infected untreated mice versus uninfected mice, or comparing *P. brasiliensis*-infected untreated mice versus *P. brasiliensis*-infected and BM-MSCs-treated mice.

M2 polarization.[36] This antagonism was also observed in the present study, since M2 macrophages increased while M1 macrophages decreased in number upon transplant of BM-MSCs; nonetheless, a poor control of the inflammatory process was noticed.

All things considered, these results showed that BM-MSCs exert a detrimental effect during experimental PCM. These cells may polarize toward a pro-inflammatory phenotype influenced by the inflammatory microenvironment or by the interactions of fungal components with pattern recognition receptors (PRRs) present on the surface of BM-MSCs. More studies are needed to understand the interaction between BM-MSCs and *P. brasiliensis* yeast, and how this fungal morphotype, or the microenvironment found in infected lungs, could modify BM-MSCs to function as either pro-inflammatory (MSC1) or immunosuppressive (MSC2) cells, or to induce their potential antifungal mechanisms.

In conclusion, and contrary to previous reports, our results indicated a deleterious effect of BM-MSCs therapy on *P. brasiliensis*–infected animals. These findings suggest that BM-MSCs induce an immunosuppressive effect on the immune response and do not exert an antifungal mechanism against *P. brasiliensis*, since the inflammatory microenvironment polarizes the stem cell toward an inflammatory cell type.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for content and writing of the paper.

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