# Trasplante de células madre mesenquimales derivadas de médula ósea en el tratamiento de la fibrosis pulmonar experimental inducida por *Paracoccidioides brasiliensis*

**Por**

**Julián Camilo Arango Rincón**

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**Escuela de Microbiología**
**Universidad de Antioquia**

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**Tutor**

**Ángel González Marín, MSc., PhD.**

Profesor Titular
Escuela de Microbiología
Universidad de Antioquia

Grupo de Microbiología Básica y Aplicada (MICROBA)
Escuela de Microbiología
Universidad de Antioquia
Medellín - Colombia

## Comité Tutorial

**David Arboleda, MSc., PhD.**
Profesor
Jefe Centro de investigación y Extensión
Facultad de Odontología
Universidad de Antioquia

**Juan Álvaro López, MSc., PhD.**
Profesor
Coordinador de Posgrados
Escuela de Microbiología
Universidad de Antioquia

**Orville Hernández Ruíz, PhD.**
Profesor
Jefe Departamento de formación Académica
Escuela de Microbiología
Universidad de Antioquia

## Resumen

La paracoccidioidomicosis (PCM) es una micosis sistémica endémica producida por el hongo del género *Paracoccidioides*. Esta enfermedad tiene una progresión generalmente crónica y el órgano blanco es el pulmón, en el que se genera fibrosis pulmonar (FP) en más del 50% de los pacientes. Esta micosis es de gran importancia en América Latina, principalmente en Brasil, Colombia y Venezuela, en donde se estima que hay aproximadamente 10 millones de personas infectadas. El tratamiento de la FP en la PCM está basado en el uso de agentes anti-inflamatorios pero sin ningún éxito. Recientemente, terapias celulares basadas en células madre mesenquimales derivadas de médula ósea (BMMSCs), a nivel experimental, han sido efectivas en el tratamiento de la FP inducida por agentes químicos, gracias a su capacidad inmunomoduladora y regenerativa.

El objetivo de este trabajo fue evaluar el efecto del trasplante de BMMSCs en el desarrollo de la FP en un modelo experimental de PCM pulmonar.

En este modelo se utilizaron ratones BALB/c machos de 8 semanas de edad inoculados con $1.5x10^6$ levaduras de *P. brasiliensis* vía intranasal; luego en la octava semana post-infección los ratones se trasplantaron vía intravenosa con $1x10^6$ BMMSCs, las cuales se aislaron de fémur y tibias de ratones BALB/c hembras de 3 semanas de edad. Un grupo adicional de ratones se trató con itraconazol (ITC) dos semanas antes de la administración de las BMMSCs.

En cada uno de los grupos experimentales los animales fueron sacrificados en la semana 12 pos-infección, y se obtuvieron los pulmones para evaluar los siguientes parámetros: subpoblaciones leucocitarias, carga fúngica, citoquinas y quimioquinas, número de fibrocitos, expresión de genes relacionados con la fibrosis, colágeno soluble y análisis histopatológico. Adicionalmente, cultivos de

fibroblastos humanos se trataron con sobrenadantes de homogeneizados de pulmón (SHP) de los ratones infectados y/o trasplantados para determinar la expresión de colágeno.

Se observó que los ratones infectados con *P. brasiliensis* y trasplantados con BMMSCs tuvieron un aumento significativo en: i) carga fúngica; ii) recuento de neutrófilos, eosinófilos, macrófagos M2 y fibrocitos; iii) niveles de IL-6, IL-9, GM-CSF, CXCL1, CXCL9 y CCL5; iv) colágeno soluble; v) expresión de colágeno-3α1, TGF-ß 3, MMP-8 y MMP-15; y vi) respuesta inflamatoria *in situ* y fibrosis en comparación con los animales infectados y no trasplantados. Así mismo, en los animales infectados con el hongo y trasplantados con BMMSCs se observó disminución de i) macrófagos M1, linfocitos Th9, linfocitos Th17 y células Treg; ii) M-CSF y LIF; y iii) expresión aumentada de MMP-13 en comparación con los animales infectados y no trasplantados.

Adicionalmente, luego del tratamiento con Itraconazol, se evidenció una disminución no solo de la carga fúngica sino también una disminución significativa de la respuesta inflamatoria exacerbada inducida por la administración de las BMMSCs, y una reducción de la expresión de los genes TIMP-1 y MMP-13. Finalmente, los fibroblastos humanos estimulados con SHP de ratones infectados y trasplantados con las BMMSCs mostraron una mayor expresión del gen que codifica para el colágeno 1.

Los resultados de este trabajo, contrario a reportes previos en modelos de FP inducida por agentes no infecciosos, mostraron que el trasplante de BMMSCs exacerba el proceso fibrótico en los animales infectados con *P. brasiliensis*. Este fenómeno podría deberse al constante estímulo inflamatorio inducido por el hongo, que generaría una polarización de las BMMSCs hacia un perfil de células madre

mesenquimales tipo 1 (MSC1), caracterizadas por ejercer un patrón inflamatorio, a la amplificación de la inflamación local por el incremento de las citoquinas, quimioquinas y células inflamatorias, y finalmente a una posible interacción directa de las BMMSCs con el hongo.

Este es el primer estudio enfocado en evaluar el efecto de una terapia celular basada en el uso de BMMSCs en una micosis de tipo crónico como la PCM. En el modelo experimental empleado, las BMMSCs no generaron un efecto inmunomodulador de tipo benéfico; sin embargo, es interesante indicar que la terapia combinada ITC/BMMSCs mostró resultados prometedores. Por lo tanto, el uso de BMMSCs bajo diferentes condiciones o en combinación con otros tratamientos (por ejemplo, ITC) abre la posibilidad de nuevos enfoques en la terapia de la FP en la PCM.

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## Lista de Abreviaciones

| | |
|---|---|
| **BMMSCs** | Bone marrow mesenchymal stem cells |
| **CCL** | CC chemokine ligand |
| **CCR** | CC chemokine receptor |
| **CCXCR** | CCX chemokine receptor |
| **CD** | Cluster of differentiation |
| **CIB** | Corporación para investigaciones biológicas |
| **CXCL** | CXC chemokine ligand |
| **FP** | Fibrosis pulmonar |
| **G-CSF** | Granulocyte colony-stimulating factor |
| **GM-CSF** | Granulocyte-macrophage colony-stimulating factor |
| **Gp** | Glycoprotein |
| **HGF** | Hepatocyte growth factor |
| **HLA-DR** | Human leukocyte antigen - D related |
| **HSC** | Hematopoietic stem cells |
| **ICAM-1** | Intercellular adhesion molecule 1 |
| **IDO** | Indoleamine 2,3-dioxygenase |
| **IFN-γ** | Interferon gamma |
| I**GF** | Insulin-like growth factor |
| **IL** | Interleukin |
| **i.n.** | Intranasal |
| **iNOS** | Inducible nitric oxide synthase |
| **ITC** | Itraconazol |
| **LIF** | Leukemia inhibitory factor |

| | |
|---|---|
| **LPS** | Lipopolysaccharide |
| **MCP** | Monocyte chemoattractant protein |
| **M-CSF** | Macrophage colony-stimulating factor |
| **MEC** | Matriz extracelular |
| **MIP** | Macrophage Inflammatory Protein |
| **MMP** | Matrix Metalloproteinase |
| **MSC** | Mesenchymal stem cells |
| **NK** | Natural killer cell |
| **PAMPs** | Pathogen-associated molecular pattern |
| **PCM** | Paracoccidioidomicosis or Paracoccidioidomycosis |
| **PGE2** | Prostaglandin E2 |
| **PDGF** | Platelet-derived growth factor |
| **PMN** | Polimorfo nuclear neutrófilo |
| **PRRs** | Pattern recognition receptors |
| **ROS** | Reactive oxygen species |
| **SHP** | Sobrenadantes de homogenizados de pulmón |
| **TERMIS** | Tissue engineering and regenerative medicine international society |
| **TGFβ** | Transforming growth factor beta |
| **TLR** | Toll-like receptor |
| **Th** | T helper |
| **TIMP** | Tissue inhibitors of metalloproteinases |
| **LT CD4** | Lymphocytes T CD4 |
| **TNF-α** | Tumor necrosis factor alpha |
| **Treg** | Regulatory T cells |

| | |
|---|---|
| **SIDA** | Síndrome de inmunodeficiencia humana adquirida |
| **UFC** | Unidades formadoras de Colonias |
| **VIH** | Virus de inmunodeficiencia humana |
| **VCAM-1** | Vascular cell adhesion molecule 1 |
| **VEGF** | Vascular endothelial growth factor |
| **v.i.** | Vía Intravenosa |

# Capítulo I

# Introducción general y objetivos

# Capitulo I. Introducción general

## 1. Paracoccidioidomicosis (PCM)

### 1.1. Generalidades

Las micosis endémicas son una de las principales causas de morbilidad y mortalidad en el ámbito mundial y representan un serio problema de salud pública, tanto en individuos inmunocompetentes como inmunocomprometidos (Bonifaz, A. y Col. 2011; Cano, L. E. y Col. 2012; Vallabhaneni, S. y Col. 2016). Entre estas micosis se encuentra la paracoccidioidomicosis (PCM), la cual es producida por el hongo dimórfico del género *Paracoccidioides,* de gran importancia en América Latina; principalmente en Brasil, Colombia, Peru y Venezuela. Países en los cuales se estima que hay aproximadamente 10 millones de personas infectadas, siendo la gran mayoría hombres en edad productiva entre los 30 y 60 años de edad (principalmente agricultores y afines) (Cano, L. E. y Col. 2012; Marques, S. A. 2013). Cerca del 80 % de los casos de PCM se han reportado en Brasil, país con áreas endémicas e hiper-endémicas bien definidas; los casos restantes se presentan principalmente en Venezuela, Argentina y Colombia (Martinez, R. 2015). La incidencia para América Latina es de 1 – 3 casos por cada 100.000 habitantes y la tasa de mortalidad es de 1.4 por millón de habitantes (Bocca, A. L. y Col. 2013; González, A. & Hernandez, O. 2016). En la infección por *P. brasiliensis* se presentan manifestaciones clínicas que van desde una infección asintomática a una enfermedad activa con fases crónicas en las que el pulmón es el principal órgano afectado, y en algunos de los casos hay

diseminación a piel y mucosas, nódulos linfáticos y otros órganos como el hígado y el bazo, entre otros (Bonifaz, A. y Col. 2011; De Oliveira, H. C. y Col. 2015). En toda América Latina la PCM tiene un gran impacto social en áreas de alta endemicidad, esto debido, no solo al alto número de casos sino también a la cronicidad de esta micosis. Así mismo, los largos periodos de tratamiento y las secuelas de la enfermedad llevan a una pobre calidad de vida en los individuos afectados (Martinez, R. 2015).

### 1.2. Agente causal de la PCM

La PCM es causada por las especies del genero *Paracoccidioides,* pertenecientes al filo Ascomycota, orden Onygenales, familia Onygenaceae. En años anteriores se había definido que el género *Paracoccidioides* estaba conformado por dos especies *Lutzii* y *brasiliensis*; y a su vez esta última comprendía cuatro especies filogenéticas a saber: S1, PS2, PS3 y PS4 (Teixeira, M. M. y Col. 2014; González, A. & Hernandez, O. 2016; Muñoz, J. F. y Col. 2016; Camacho, E. & Nino-Vega, G. A. 2017). No obstante, estudios recientes en los que se realizó una rigurosa clasificación taxonómica (basada en aspectos morfológicos y moleculares) sugirieron la presencia de 5 especies diferentes: i) *P. Lutzii,* ii) *P. brasiliensis* especie a la que se le atribuye la mayoría de casos de PCM y ha sido encontrada en casi toda Sur-América (principalmente en el Sureste y Centro Oeste de Brasil, y en Argentina), y la cual se subdivide en los clados S1a y S1b.; iii) *P. americana* presente en el Sudeste de Brasil y Venezuela; iv) *P. restrepiensis,* endémica de Colombia y v) *P.*

*venezuelensis,* descubierta recientemente en Venezuela (Turissini, D. A. y Col. 2017).

*P. brasiliensis,* así como las otras cuatro especies exhiben un dimorfismo térmico en el que las levaduras tienen la capacidad de mantenerse tanto *in-vitro* como en los tejidos del hospedero a temperaturas de 36 – 37ºC, mientras que la forma de moho se mantiene a temperaturas menores de 24ºC. Las levaduras *in-vitro* tardan en crecer una semana a una temperatura de 37ºC y macroscópicamente se caracterizan por la formación de colonias de contextura suave, ligeramente rugosa y de color crema. Al microscopio las levaduras son redondas u ovales, multinucleadas y su pared es delgada y refractaria. La principal característica de las levaduras es la de formar agrupaciones, en las que la célula madre es rodeada por células hijas de igual o diferente tamaño, que se asemejan un timón de barco (característica morfológica patognomónica de este microorganismo); sin embargo, también se pueden observar células madre solitarias o unidas a una sola célula hija; el tamaño de estas estructuras puede variar entre 4 y 40 μm (Restrepo, A. & González, A. 2015; González, A. & Hernandez, O. 2016).

En el micelio el crecimiento, *in-vitro,* puede tardar de 2 a 4 semanas formando colonias blancas e irregulares que poseen un micelio aéreo corto. Microscópicamente, se observan hifas delgadas y septadas con clamidosporas terminales e intercalares. En condiciones nutricionales deficientes, el micelio produce conidias que miden menos de 5 μm, siendo esta la forma infectante del hongo. A temperaturas de $37^0$C se presenta la transformación a levadura que es la forma patogénica del microorganismo

(Marques, S. A. 2013; Restrepo, A. & González, A. 2015; González, A. & Hernandez, O. 2016).

### 1.3. Factores de virulencia de *Paracoccidioides brasiliensis*

Entre los principales mecanismos de virulencia de *P. brasiliensis* se encuentra su dimorfismo térmico, los componentes de su pared, sus moléculas de adhesión y los antígenos extracelulares que produce. De los anteriores, los carbohidratos presentes en su pared celular son de gran importancia; por ejemplo, la forma miceliar tiene una pared compuesta de dos capas, la más externa se compone de ß-1-3 glucanes y la interna de fibras de quitina; mientras que la levadura presenta una pared gruesa compuesta principalmente por α-1-3 glucanes; esta configuración representa un importante factor de virulencia debido a que los macrófagos no poseen alfa-glucanasas. En la tabla 1 se describen los principales factores de virulencia de *P. brasiliensis* reportados hasta el momento.

Tabla 1: Factores de virulencia de *P. brasiliensis*.

| Mecanismos de Patogenicidad | | Función | Referencia |
|---|---|---|---|
| **Dimorfismo** | Cambio de temperatura a 37 $^{0}$C | Adaptación a tejidos del hospedero. | (Cano, L. E. y col. 2012) |
| | SconCp (Proteína relacionada con el metabolismo del sulfuro) | Regulador negativo de las vías de asimilación de sulfuros inorgánicos. | (Menino, J. F. y col. 2013) |
| **Pared** | Quitina<br>α -Glucan<br>β -Glucan<br>Galactomanan | Rigidez estructural.<br>Interacción microorganismo-hospedero.<br>Interacción microorganismo-hospedero.<br>Interacción microorganismo-hospedero. | (Borges-Walmsley, M. I. y col. 2002) |
| **Moléculas de adhesión** | Gp43 | Adherencia a matriz extracelular (MEC). | (Silva Jde, F. y col. 2015) |
| | Proteínas de 19 y 32 kDa | Adherencia a MEC. | (González, A. y col, 2005) |
| | Proteínas 14-3-3 (Proteína 30kDa) | Adherencia a MEC. | (Silva Jde, F. y col. 2015) |
| | 1-6 bifosfato aldolasa | Adherencia a MEC y plasminogeno. | (Chaves, E. G. y col. 2015; De Oliveira, H. C. y col. 2015) |
| | Enolasa (EC 4.2.1.11) | Unión a fibronectina. | (De Oliveira, H. C. y col. 2015) |
| | Hidrolasa HAD-32 | Adherencia a (MEC). | (Hernandez, O. y col. 2010) |
| | Glyceraldehido 3 fosfatasa deshidrogenasa (GADPH) | Adherencia a (MEC). | (Barbosa, M. S. y col. 2006) |
| | Sialo-gliconjungados | Carga negativa que se une a la positiva de los tejidos. | (Tomazett, P. K. y col. 2005) |
| | Triososa fosfato isomerasa | Adherencia a (MEC). | (Pereira, L. A. y col. 2007) |
| **Antígenos extracelulares** | Gp43 | Antígeno inmunodominante, induce apoptosis en neumocitos. | (Silva Jde, F. y col. 2015) |
| | Serina-thiol proteasa | Cliva laminina de los tejidos. | (Parente, J. A. y col. 2010) |
| | Producción de melanina | Protege de la fagocitosis y de la acción de los antifúngicos. | (Restrepo, A. y col. 2016) |

### 1.4 Inmunopatogénesis de la PCM

La PCM se desarrolla principalmente en hombres en una relación de 13:1 (hombre:mujer), y aunque las mujeres se pueden infectar en una proporción igual a los hombres, son menos susceptibles a padecer la PMC debido a que las conidias de *P. brasilensis* expresan receptores de la hormona 17-β-estradiol, de tal forma que cuando las hormonas femeninas interactúan con estos receptores se inhibe su transformación a la forma levadura del hongo (Nosanchuk, J. D. 2016).

La infección por *P. brasiliensis* inicia en la vía respiratoria y llega hasta los pulmones siendo este el órgano blanco. Posteriormente, el microorganismo se disemina vía linfática o sanguínea a otros órganos y tejidos que incluyen la mucosa gastrointestinal, glándulas adrenales, piel, bazo e hígado. Adicionalmente, se ha descrito que el microorganismo puede permanecer quiescente (latente) en los ganglios linfáticos hasta por 30 años (Gould, J. & Aronoff, S. 2016).

La infección en el pulmón inicia con el proceso de adhesión de *P. brasilensis* a las proteínas de matriz extracelular (MEC) mediante las moléculas de adhesión mencionadas en la tabla 1. Entre las principales moléculas se encuentran la glucoproteína Gp43 (el principal antígeno inmunodominante), proteínas de 19, 30 y 32 kDa, malato sintasa, triosafosfato isomerasa, gliceraldehído-3-fosfato deshidrogenasa y enolasa, entre otras. Estas moléculas tienen la capacidad de interactuar con la laminina, el plasminógeno, la fibronectina y el fibrinógeno facilitando la adhesión del microorganismo (Gonzalez, A. y Col. 2005; Tomazett, P. K. y

Col. 2005; González, A. y Col. 2008a; Parente, J. A. y Col. 2010; Cano, L. E. y Col. 2012).

Luego del proceso de adhesión, *P. brasiliensis,* interactúa con las células epiteliales pulmonares y los macrófagos alveolares, células que a su vez se activan y producen citoquinas pro-inflamatorias y quimioquinas. Además, se presenta una interacción con otras células de la inmunidad innata principalmente polimorfo nuclear neutrófilos (PMN), células NK, macrófagos y células dendríticas. Estas células mediante sus receptores de reconocimiento de PAMPs (PRRs, por su sigla en inglés *pattern recognition receptors*), que incluyen los receptores tipo *toll* (TLRs, por su sigla en inglés *toll-like receptors*) y dectina-1 reconocen los patrones moleculares asociados a patógenos (PAMPs, por su sigla en inglés *pathogen-associated molecular pattern*) de *P. brasiliensis* tales como la gp43, quitina y glucanes, entre otros. Como resultado de este reconocimiento se induce la activación de estas células con la subsecuente liberación de citoquinas y quimioquinas de carácter pro-inflamatorio como las IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-17, eotaxina, proteína inflamatoria de macrófagos 2 (MIP-2, por su sigla en inglés *macrophage inflammatory protein 2)*, factor estimulante de colonias de granulocitos (G-CSF, por su sigla en inglés *granulocyte colony-stimulating factor)*, proteína quimo-atrayente de macrófagos 1 *(*MCP1, por su sigla en inglés *monocyte chemoattractant protein* 1), MIP1α, MIP-1β, factor estimulante de colonias de granulocitos y monocitos (GM-CSF, por su sigla en inglés *granulocyte-macrophage colony-stimulating* factor), interferón gamma (IFN-γ) y factor de necrosis tumoral alfa (TNF-α, por su sigla en inglés *tumor necrosis factor alpha*), entre otras (González, A. y Col. 2003;

Lopera, D. y Col. 2011). El microambiente pro-inflamatorio y la expresión de proteínas adhesión leucocitarias como: la molécula de adhesión intercelular 1 (ICAM, por su sigla en *inglés intercellular adhesion molecule 1*) y la molécula de adhesión vascular (VCAM, por su sigla en inglés *vascular cell adhesion molecule*) inducen la llegada de más leucocitos, principalmente PMN y Macrófagos (Restrepo, A. y Col. 2015). En caso de que estas células no sean capaces de controlar la infección, posteriormente participarán en la formación de granulomas cuyo propósito es el de contener la infección; no obstante, durante esta respuesta, estas células activan sus mecanismos efectores que incluyen la liberación de citoquinas pro-inflamatorias y moléculas antimicrobianas, mecanismos que en algunos casos no alcanzan a contener totalmente la infección, y por el contrario inducen mayor inflamación y daño tisular (Cano, L. E. y Col. 2012).

Estudios previos en modelos animales han permitido dilucidar el papel de algunas de estas células, así por ejemplo la depleción de PMN utilizando un anticuerpo específico, evidencia un papel dual de estas células (Pino-Tamayo, P. A. y Col. 2016); en dichos estudios se observó que en las etapas iniciales de la infección, los PMN son fundamentales para controlar la infección, debido a que en su ausencia los animales sucumben en la primera semana post-infección, de igual manera se observa un incremento en el número de eosinófilos, la carga fúngica y altos niveles de citoquinas y quimioquinas pro-inflamatorias (Pino-Tamayo, P. A. y Col. 2016); por el contrario, en las fases crónicas de la enfermedad, la depleción de los PMN generó una disminución de la respuesta inflamatoria, la carga fúngica, fibras

de colágeno todo ello asociado a una recuperación de la arquitectura pulmonar (Puerta-Arias, J. D. y Col. 2016).

Los macrófagos, también representan un componente fundamental en la inmunidad innata frente a *P. brasiliensis*. A pesar de que son células permisivas a la infección si no son activados, éstos participan en la diseminación inicial del hongo. Se ha reportado que cuando son activados por IFN-γ, tienen la capacidad de destruir conidias y levaduras mediante la producción del óxido nítrico sintasa inducible (iNOS, por su sigla en inglés *inducible nitric oxide synthase*). Otro activador importante de los macrófagos es el TNFα que induce una actividad microbicida por mecanismos independientes del óxido nítrico (González, A. y Col. 2008b; Fortes, M. R. y Col. 2011).

Las células NK, también tienen un papel fundamental en las primeras etapas de la infección, estas incrementan en número y despliegan sus mecanismos efectores basados en la liberación de granzimas y perforinas sobre las células infectadas. Otros estudios también han demostrado que estas células pueden tener un papel inmuno-regulador basado en la producción de IFN $\gamma$ y TNF-α ante una estimulación previa con la IL15 recombinante (Longhi, L. N. y Col. 2012; Restrepo, A. y Col. 2016).

En la respuesta inmune adaptativa frente a *P. brasiliensis* hay un predominio de un perfil Th1 y una respuesta inmune celular mediada por LT CD8. En los pacientes con PCM y sus formas más severas se denota el predominio de un patrón de citoqinas Th2 y una deficiente respuesta Th1. Así mismo, estudios en modelos animales confirman la importancia de los

linfocitos T CD8, los cuales participan en la disminución de la carga fúngica y evitan la diseminación de este hongo patógeno (González, A. y Col. 2008b; Restrepo, A. y Col. 2016).

La importancia de la inmunidad celular, también se comprobó al describirse un aumento en la incidencia y mortalidad por PCM en pacientes con VIH-sida en los últimos años. De igual forma se ha descrito que los individuos que reciben esteroides, quimioterapia o pacientes trasplantados tienen un riesgo incrementado de sufrir una forma severa de la micosis (Gould, J. & Aronoff, S. 2016; Restrepo, A. y Col. 2016). Finalmente, se ha descrito un riesgo incrementado en pacientes fumadores, sin embargo se desconoce el mecanismo responsable (Nosanchuk, J. D. 2016).

También se ha descrito tanto en la PCM en el humano como en los modelos experimentales, que la expansión de células Th1 / Th17 concomitante con una cantidad reducida de células Treg representan un cuadro clínico benigno, mientras que la proliferación elevada de células Th2 / Th9 y Treg se asocia con PCM grave (De Castro, L. F. y Col. 2013; Feriotti, C. y Col. 2017). En una respuesta adaptativa en la que predomina un perfil Th1 / Th17, es de gran importancia los componentes de la inmunidad innata como la dectina 1, la Indolamina-prolina 2-3 dioxigenasa (IDO), la producción de citoquinas y la estimulación del receptor similar a NOD P3 (NLRP3, del inglés *NOD-like receptor P3)* (Araujo, E. F. y Col. 2014; Loures, F. V. y Col. 2014; Feriotti, C. y Col. 2017).

Finalmente, el predominio de una respuesta adaptativa de tipo humoral Th2 contra *P. brasiliensis* se ha asociado a un mal pronóstico de la enfermedad

acompañado de una producción de anticuerpos de tipo policlonal con hipergamaglobulinemia y títulos altos de anticuerpos específicos tanto en la forma juvenil como en la crónica. Sin embargo, en adultos con una afección menor se observa una baja producción de anticuerpos (Santos Lda, S. y Col. 2012; De Castro, L. F. y Col. 2013).

### 1.5 Formas Clínicas de la PCM

La infección por *P. brasiliensis* puede llevar a dos estados: i) Infección asintomática o subclínica la cual se puede resolver o generar latencias que pueden perdurar hasta 30 años (Cano, L. E. y Col. 2012); y ii) la enfermedad clínicamente aparente, la cual tiene dos presentaciones clínicas: la forma aguda o juvenil (subaguda) y la forma crónica o del adulto. La forma aguda o juvenil representa menos del 10% de los casos, y afecta principalmente a niños y adultos jóvenes. Esta forma se caracteriza por una sintomatología general de tipo moderado a severo en la que se presenta una diseminación al sistema retículo-endotelial con compromiso de bazo, hígado, nódulos linfáticos y médula ósea (Marques, S. A. y Col. 2016; Restrepo, A. y Col. 2016).

La forma crónica corresponde al 90% de los casos y es diagnosticada en pacientes mayores de 30 años, en la mayoría de los casos se presenta una reactivación endógena tras el contacto inicial con el hongo y se caracteriza por una fuerte afección pulmonar y diseminación multi-orgánica (Queiroz-Telles, F. & Escuissato, D. L. 2011; Restrepo, A. y Col. 2016).

Aproximadamente el 60% de los pacientes con esta presentación clínica de la enfermedad desarrollan secuelas fibróticas en el pulmón (figura 2) (Cano, L. E. y Col. 2012).

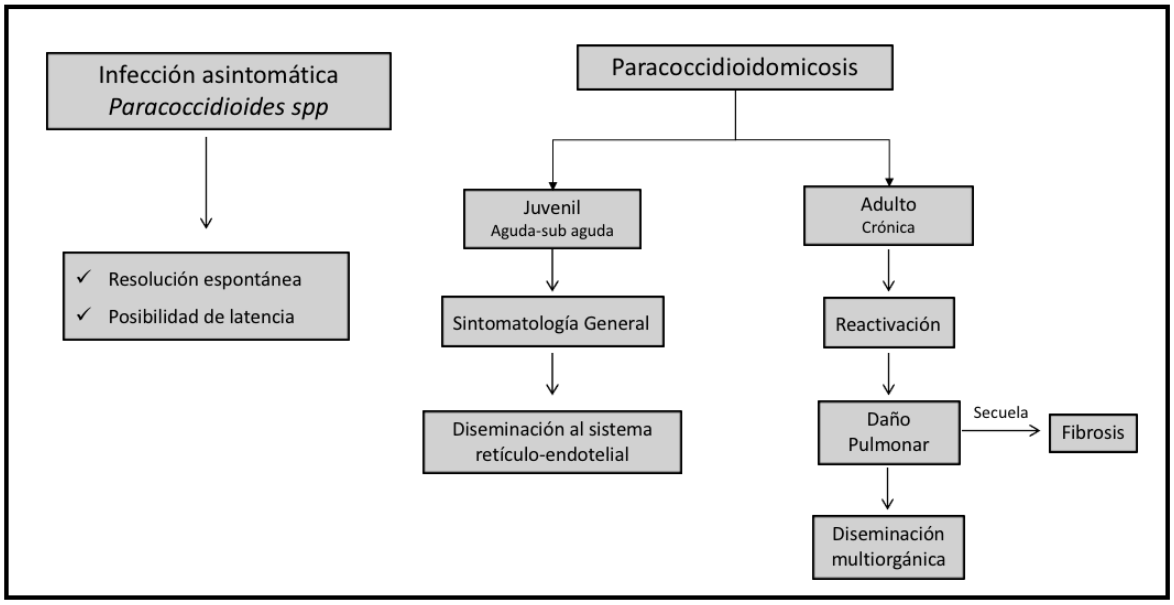


**Figura 1.** Formas clínicas de la paracoccidioidomicosis (Figura elaborada con información proveniente de Queiroz-Telles, F. y Col. 2011)

## 1.6 Fibrosis pulmonar en la PCM

El pulmón es considerado el principal órgano blanco en esta micosis y la mayoría de los pacientes desarrollan lesiones infiltrativas en más del 90% de los casos. Entre los anteriores, el 60% de los pacientes desarrollan FP siendo ésta la principal secuela de la PCM en su forma crónica y la característica que se evidencia al momento del diagnóstico en la mayoría de los pacientes. (Borges-Walmsley, M. I. y Col. 2002). El desarrollo de la FP provoca cambios dramáticos e irreversibles en la arquitectura pulmonar (figura 3), lo que resulta en una insuficiencia respiratoria y deterioro en la calidad de vida de los pacientes, la figura 2 muestra los principales alteraciones pulmonares asociadas al desarrollo de la fibrosis pulmonar (Costa, A. N. y Col. 2013).

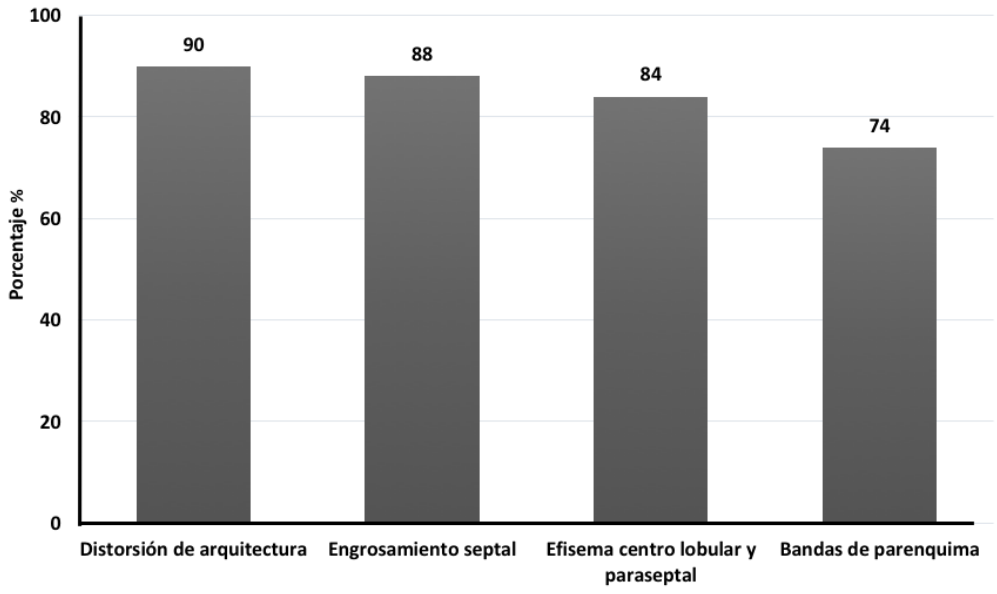


**Figura 2.** Alteraciones pulmonares en una cohorte de pacientes con PCM, figura elaborada con información proveniente de (Costa, A. N. y Col. 2013).

La FP producida en la PCM es un proceso caracterizado por la excesiva acumulación de colágeno y otros componentes de la MEC que conllevan a una remodelación de la arquitectura pulmonar y alteraciones funcionales, clínicas, fisiológicas y radiológicas en el pulmón (Todd, N. W. y Col. 2012). Además, la FP es un evento dinámico en el que participan activamente leucocitos y factores solubles tales como citoquinas: lFNγ, TNFα, IL1, IL4, IL6, IL17; factores de crecimiento que incluyen el factor de crecimiento derivado de plaquetas (PDGF, por su sigla en inglés *platelet-derived growth factor*), factor de crecimiento vascular-endotelial (VEGF, por su sigla en inglés *vascular endothelial growth factor*), factor de crecimiento similar a insulina (IGF, por su sigla en inglés insulin-like growth factor), quimioquinas (CCL2, CCL3, CXCL12) y otros que incluyen metaloproteinasas, prostaglandinas y especies reactivas del oxígeno (ROS, por su sigla en inglés *reactive oxygen species*) (Wynn, T. A. & Ramalingam, T. R. 2012). Del balance entre estos componentes y agentes anti-inflamatorios como la IL10 y las células Treg, depende la diferenciación y activación del fibroblasto, fibrocito y miofibroblasto que, a su vez, producen abundantes cantidades de colágeno que afectan la arquitectura y funcionamiento del tejido (González, A. y Col. 2008a; Wick, G. y Col. 2013). La participación dinámica de todos los componentes anteriores, en la FP, ha sido ampliamente estudiada en modelos animales (Abreu, S. C. y Col. 2011; Todd, N. W. y Col. 2012; Wynn, T. A. & Ramalingam, T. R. 2012). Modelos múridos de PCM inducida con las conidias de *P. brasiliensis* se caracterizan por el desarrollo de una inflamación aguda que compromete entre el 34% y

45% de los pulmones, con presencia de abundantes PMN; posteriormente en etapas crónicas a partir de la cuarta semana post-infección predominan células mononucleares y se observa la formación de granulomas (su consolidación se presenta ente la 8va a 12va semanas post-infección) (Restrepo, S. y Col. 1992; Cock, A. M. y Col. 2000; González, A. y Col. 2003; González, A. y Col. 2008a). En el proceso fibrótico, en la cuarta semana pos-infección, se observa un incremento de fibras de colágeno tipo III y fibras delgadas de reticulina en pulmón. Posteriormente, a partir de la 8ª semana post-infección se observan fibras de colágeno tipo I y fibras gruesas de reticulina con alteraciones en su deposición y organización, así como de otras proteínas de MEC, sugiriendo un proceso fibrótico severo (Cock, A. M. y Col. 2000; González, A. y Col. 2003; González, A. y Col. 2008a; González, A. & Hernandez, O. 2016). Modelos de FP inducidos vía intranasal (i.n) con las levaduras del hongo también muestran fases agudas y crónicas similares a los modelos realizados con las conidias de *P. brasiliensis* (figura 4) (Pino-Tamayo, P. A. y Col. 2016; Puerta-Arias, J. D. y Col. 2016).

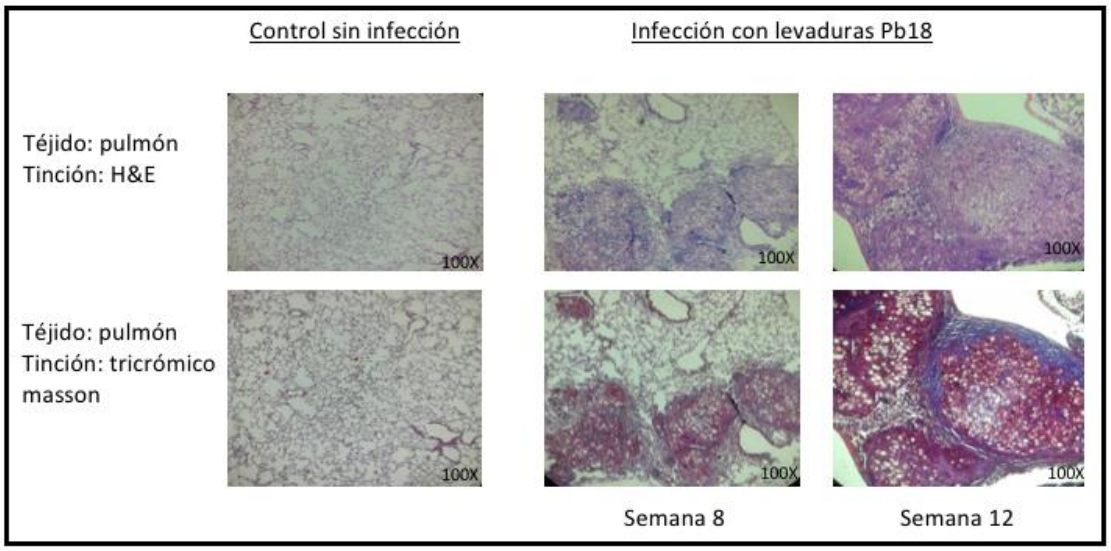


**Figura 3:** Modelo experimental de PCM en ratones BALB/c infectados i.n. con levaduras de *P. brasiliensis* (cepa Pb18). Microfotografías de secciones de pulmón. Se observa la consolidación del proceso fibrótico entre la semana 8 y la semana 12 pos-infección. (Microfotografías propias tomadas en el laboratorio de Micología Médica de la CIB-Universidad de Antioquia).

Estos modelos han permitido estudiar no solo la patogénesis de esta enfermedad, sino también el uso de terapias combinadas entre agentes anti-fúngicos y anti-inflamatorios (Itraconazol + Petoxifilina) (Naranjo, T. W. y Col. 2011; Cano, L. E. y Col. 2012). No obstante, a pesar de que se han logrado importantes avances, se ha observado que la FP no se revierte

completamente; y, además este tipo de estrategia podría generar estados de inmunosupresión y efectos adversos que limitarían su uso.

Teniendo en cuenta lo anterior, en otro trabajo realizado por el mismo grupo, el cual consistió en depletar neutrófilos mediante un anticuerpo específico (mAb-anti-Ly6G) y de forma simultánea con el anti-fúngico ITC, se observó una disminución en la expresión de genes que codifican para algunas proteínas asociadas al desarrollo de fibrosis que incluyeron: la IL-1$\alpha$, IL-6, IL-17, TNF-$\alpha$, factor transformante del crecimiento-β1 (TGF-β1, del inglés *Transforming growth factor*), TGF-$\beta$3, proteína unidora a GATA 3 (GATA-3, por su sigla en inglés *GATA binding protein 3*), receptor huérfano relacionado a RAR gamma (RORc, por su sigla en inglés *RAR-related orphan receptor gamma*), receptor aryl-hidrocarbono (Ahr, por su sigla en inglés *aryl-hydrocarbon receptor),* metaloproteinasa 1α (MMP-1α, por sus siglas en inglés, *metalloproteinases*), MMP-8 MMP-15, inhibidor de metaloproteinasa 1 (TIMP-1, por su sigla en inglés *tissue inhibitors of metalloproteinases* 1) y TIMP-2. Estos hallazgos demuestran que el ITC en combinación con otras biomoléculas (como anticuerpos específicos) podría ser efectivo en el tratamiento de la FP en la PCM (Puerta-Arias, J. D. y Col. 2017).

## 2. Células madre mesenquimales derivadas de médula ósea

### 2.1 Generalidades

En médula ósea se encuentran dos tipos de células madre adultas: i) las células madre hematopoyéticas (HSC, por sus siglas en inglés *hematopoietic stem cells*) y ii) las células madre mesenquimales (BMMSCs,

por sus siglas en inglés *Bone marrow messencymal stem cells*). Las HSC se caracterizan por ser células no adherentes bajo condiciones de cultivo *in vitro,* además expresan marcadores específicos de linaje, y generalmente se diferencian en células de las series hematopoyéticas como los eritrocitos y los diferentes leucocitos (Herzog, E. L. y Col. 2003).

Las BMMSCs son células madre adultas, que en médula ósea pueden tener una proporción del 0.01% del total de las células. Las BMMSCs tienen la capacidad de diferenciarse a células maduras de tejidos mesenquimales incluyendo tejido óseo, graso y cartílago (Herzog, E. L. y Col. 2003; Ortiz, L. A. y Col. 2003); su principal atributo es su capacidad inmunomoduladora, por lo que han sido postuladas como una alternativa terapéutica en el tratamiento de enfermedades autoinmunes e inflamatorias (Abreu, S. C. y Col. 2011; Gebler, A. y Col. 2012). Las células madre mesenquimales (MSCs, por sus siglas en inglés *messencymal stem cells*) también pueden derivarse de tejido adiposo y cordón umbilical. Sin embargo, las obtenidas de médula ósea, son las más empleadas en modelos experimentales y en ensayos clínicos por presentar una mayor capacidad inmunoreguladora y regenerativa en comparación con las MSC de otros orígenes (Dmitrieva, R. I. y Col. 2012; Kyurkchiev, D. y Col. 2013). En 2006 la Sociedad Internacional de terapias celulares (ISCT The International Society for Cellular Therapy) determinó como tres los criterios mínimos para definir las MSCs humanas a saber: i) adherencia a superficies de plástico en condiciones de cultivo estándar; ii) presencia de marcadores de superficie mayor o igual del 95% (CD105, CD73 y CD90) y ausencia o expresión menor o igual al 2% de CD45, CD34, CD11b o CD14, CD79α o CD19 y

HLA-DR; y iii) capacidad de diferenciarse a osteblastos, condroblastos y adipocitos en medios de diferenciación específicos (Dominici, M. y Col. 2006).

## 2.2. Mecanismos inmunoreguladores de las BMMSCs

Se ha descrito que las BMMSCs poseen tres mecanismos de acción los cuales se describen a continuación:

2.2.1. Migración al tejido inflamado. Las BMMSCs expresan receptores de quimioquinas tipo CC (entre ellos CCR1, CCR2, CCR5) y tipo CXC (entre ellos CXCR4, CXCR5 y CXCR6); estos receptores permiten que las BMMSCs puedan seguir gradientes de concentración de quimioquinas hacia los tejidos inflamados, y precisamente se ha descrito que las BMMSCs requieren un ambiente inflamatorio (con predominio de IFNγ, TNFα, IL-1α, IL-1β, y IL-17) para su activación y despliegue de sus mecanismos inmunomoduladores (Stagg, J. 2006; Castro-Manrreza, M. E. & Montesinos, J. J. 2015) (figura 5).

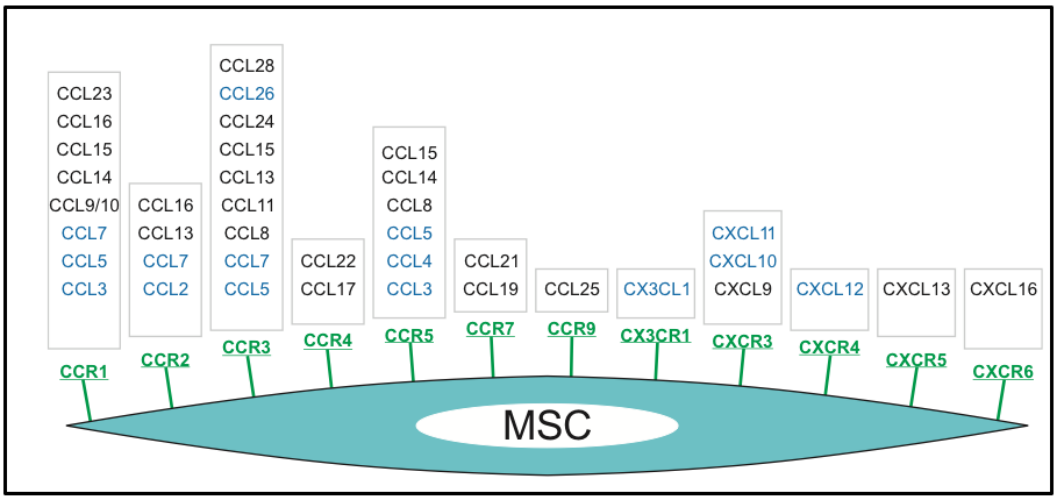

**Figura 4.** Receptores de quimioquinas de las BMMSC. Adaptado de: da Silva Meirelles y col. 2008

2.2.2 Liberación paracrina de moléculas (anti-fibróticas, apoptóticas e inmunoreguladoras) e interacción directa entre membranas celulares.

Se ha demostrado que las BMMSCs humanas producen constitutivamente citoquinas inmunosupresoras, como el factor de crecimiento del hepatocito (HGF, del inglés *hepatocyte growth factor*), IL10, TGF-β1 prostaglandina E2 (PGE2), IDO y óxido nítrico (NO, del inglés *nitric oxide*), entre otras (Gao, F. y Col. 2016).

La PGE2, IDO, el NO y el TGF-β1 tienen la capacidad de inhibir la proliferación de linfocitos T, la producción de citoquinas por LTCD4 y la función citotóxica de LTCD8 (Ren, G. y Col. 2008; Gao, F. y Col. 2016). Adicionalmente, IDO inhibe la activación de linfocitos B y células NK, mediante la conversión del triptófano a kinurenina, metabolito que induce anergia y apoptosis en linfocitos. Adicionalmente, la PGE2 tiene capacidad de afectar la maduración de células dendríticas (figura 5) (Ren, G. y Col. 2008).

Estudios recientes han demostrado que el contacto célula-célula es un factor fundamental en la capacidad inmunomoduladora de las BMMSCs. Han y col. (2011) describieron que las BMMSCs no sólo reducen la supervivencia y proliferación de células T por mecanismos dependientes del contacto, sino que también aumentan la inducción de Treg (Han, K. H. y Col. 2011). En el proceso de interacción célula-célula, las BMMSCs

expresan moléculas tales como CD274 (también conocido como ligando de muerte programada 1), VCAM-1 y galectina-1, estas interacciones inhiben las vías de las moléculas co-estimuladoras en los linfocitos T regulando negativamente su proliferación, adhesión y migración (Ren, G. y Col. 2010; Najar, M. y Col. 2012; Castro-Manrreza, M. E. & Montesinos, J. J. 2015; Gao, F. y Col. 2016).

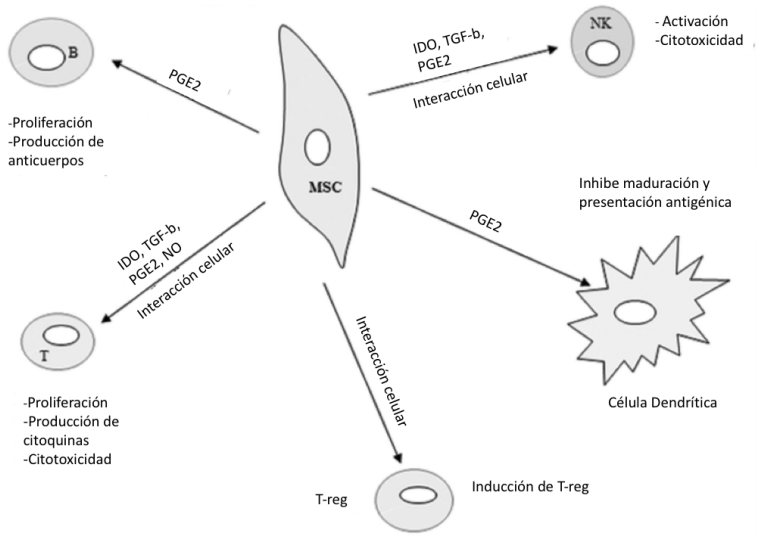


**Figura 5.** Mecanismos inmunoreguladores de las BMMSCs (Adaptado de Gao, F y Col. 2016).

2.2.3. Diferenciación a tejido específico. Las BMMSCs son células madre multipotentes con capacidad de diferenciarse a linajes mesodermales como osteocitos, adipocitos, condrocitos, fibroblastos y miocitos. Adicionalmente, las BMMSCs también tienen la capacidad de trans-diferenciarse en tejidos endodermales y ectodermales tales como el muscular, nervioso, óseo, neuronal y pulmonar (células epiteliales alveolares tipo I y tipo II, células endoteliales, fibroblastos y células epiteliales bronquiales) (Lee, S. H. y Col.

2010; Abreu, S. C. y Col. 2011; Gebler, A. y Col. 2012; Wang, Y. y Col. 2014). Al llegar a los tejidos dañados, se cree que las BMMSCs ejercen sus efectos terapéuticos mediante la sustitución o la fusión con las células alteradas. Varios estudios han intentado aprovechar este potencial para reemplazar así a las células residentes dañadas, como células endoteliales, células de músculo liso, cardiomiocitos o hepatocitos. Actualmente, se desconoce el mecanismo exacto que lleve a la diferenciación de las BMMSCs o a la fusión celular; no obstante, se ha encontrado que este proceso es dependiente de los niveles del TFG-$\beta$ y de señales mecano-transductoras como la flexibilidad de la MEC. En el caso específico de la diferenciación a células del tejido pulmonar se ha descrito que inductores de estrés celular como la hipoxia inducen la expresión del receptor de VEGF en las BMMSCs lo cual favorecería la reparación de dicho tejido (Stagg, J. 2006).

### 2.3. BMMSCs en modelos de fibrosis pulmonar no infecciosa

El potencial inmunomodulador de las BMMSCs ha sido ampliamente estudiado en modelos animales de fibrosis pulmonar inducida por agentes no infecciosos. El tipo de animal más utilizado son los roedores, entre ellos ratones (BALB/C o C57BL/6), hámster y ratas. Uno de los trabajos pioneros y de referencia es el de Rojas y col en 2005, en este estudio se realizó un modelo de fibrosis pulmonar inducido por bleomicina en ratones C57BL/6, en el cual se inocularon por vía intra-traqueal $5 \times 10^6$ BMMSCs, posteriormente 14 días pos-inoculación se evidencio una reducción de la

fibrosis pulmonar en los animales trasplantados, acompañado de una disminución de citoquinas tipo Th1 y Th2 (IL-1β, IFNγ, IL-4 e IL-2) (Rojas, M. y Col. 2005). Posteriormente, Kumamoto M. y Col., evaluaron el efecto de diferentes dosis de BMMSCs (entre 0,5 x $10^6$ y 5 x $10^6$ células) en este modelo de fibrosis inducido por bleomicina, y determinaron que el efecto reparador de estas BMMSCs es independiente de la dosis (Kumamoto, M. y Col. 2009). En otro estudio, en el cual se indujo la fibrosis con bleomicina en ratas, se observó además un efecto inmunomodulador por estas BMMSCs. Efecto que se basó en la disminución de poblaciones leucocitarias en pulmón (PMN, linfocitos y macrófagos) y en la modulación de la producción de citoquinas pro-inflamatorias (IL-1β, IL-6, TNF-α, TGF-β y VEGF) y óxido nítrico (Lee, S. H. y Col. 2010). Posteriormente, en 2014 Sun y Col. trasplantaron 2 x $10^6$ BMMSCs en un modelo de FP aguda inducida por ácido clorhídrico en ratas *Sprague Dawley*, y hallaron una disminución de la fibrosis pulmonar, el área inflamada y la deposición de fibras de colágeno; en este trabajo también se concluyó que la capacidad inmunomoduladora de las BMMSCs se favoreció por la inhibición de las Wnt/β-cateninas (Sun, Z. y Col. 2014).

En otro modelo múrido de injuria severa aguda causada por LPS se demostró que el trasplante con BMMSCs indujo una reducción significativa de la inflamación pulmonar, y llamativamente al trasplantar los animales con BMMSCs modificadas genéticamente con el gen de la angiopoyetina 1 se potenció el efecto anti-inflamatorio sobre la inflamación alveolar y la permeabilidad pulmonar (Mei, S. H. y Col. 2007).

Teniendo en cuenta la importancia de tratar la FP producida en la PCM, y las ventajas que representa el uso de BMMSCs, este trabajo tiene como propósito estudiar el efecto de estas células sobre el desarrollo de la FP en un modelo múrido experimental de PCM inducido por levaduras de *P. brasiliensis*, evaluando su eficiencia, así como su posible mecanismo de acción.

## 2.4 Efecto de las BMMSCs en modelos infecciosos

El potencial terapéutico de las BMMSCs no solo ha sido explorado en modelos de enfermedades autoinmunes o asociadas a fibrosis de órganos (por agentes no infecciosos), sino que además también se ha estudiado su capacidad microbicida y anti-inflamatoria en modelos de enfermedades por microorganismos como *Aspergillus fumigatus*, *Candida albicans*, *Escherichia coli, Porphyromonas gingivalis* y *Mycobacterium tuberculosis* (Krasnodembskaya, A. y Col. 2010; Yang, R. y Col. 2013; Tang, J. y Col. 2015; Cho, S. Y. y Col. 2016; Nenasheva, T. y Col. 2017).

Diferentes trabajos han permitido determinar que la capacidad microbicida de las BMMSCs se desarrolla mediante mecanismos indirectos y directos. Los mecanismos indirectos, son inducidos por la activación de TLRs presentes en la superficie de las BMMSCs y se basan en la producción de moléculas como IFN$\gamma$, TNF$\alpha$ y activina B, que potencian los mecanismos efectores de macrófagos y PMN. Los mecanismos directos se basan en la expresión constitutiva de péptidos antimicrobianos como catelicidina LL-37, β-defensina-2, hepcidina y lipocalina-2. Dada la importancia de estos

mecanismos, ha sido demostrado el potencial terapéutico de las BMMSCs en ensayos preclínicos de sepsis bacteriana, síndrome de dificultad respiratoria aguda (ARDS) y fibrosis quística (Alcayaga-Miranda, F. y Col. 2017).

Con relación al papel inmunoregulador de las BMMSCs en enfermedades infecciosas Yang, R, y Col. determinaron que hay un subtipo especifico de BMMSCs productoras de IL-17, las cuales tienen la capacidad de inhibir el crecimiento de *C. albicans* tanto *in vitro* como *in vivo* (por un mecanismo aún no determinado), además se encontró que este subtipo de BMMSCs produce muy bajas cantidades de TGFβ, lo que conlleva a una alteración en el control de la inflamación en el hígado de los animales infectados (Yang, R. y Col. 2013). Por el contrario, en un modelo múrido de asma desencadenada por hifas de *A. fumigatus*, se observó que las BMMSCs redujeron la inflamación mediada por PMN en las vías respiratorias, a través de la inhibición de la vía de señalización Th17 (Lathrop, M. J. y Col. 2014). Así mismo, en otro estudio *in vitro*, se demostró un efecto inhibidor de las BMMSCs sobre macrófagos estimulados con conidias de *A. fumigatus* asociado a una disminución de TNFα y un aumento de IL-10 (Cho, S. Y. y Col. 2016). Adicionalmente, se demostró que la carga microbiana es un factor crucial que altera el efecto de las BMMSCs. Tang y Col demostraron que un inóculo pequeño de *P. gingivalis* resulta en un incremento de la respuesta anti-inflamatoria y anti-bacteriana, mientras que un inóculo alto de este patógeno conlleva a la inactivación de las BMMSCs (Tang, J. y Col. 2015). Finalmente, Nenasheva, T. y Col en un modelo de daño pulmonar

por *M. tuberculosis*, en el que evaluaron el papel inmunomodulador de las BMMSCs, encontraron un efecto desfavorable en la reducción de la inflamación. En dicho trabajo, inicialmente se determinó que las BMMSCs tenían la capacidad de migrar al tejido afectado (en este caso a pulmón); sin embargo, se logró determinar que las infusiones de las células formaron agregados que no les permitieron atravesar los septos alveolares, por lo tanto a pesar que las BMMSCs llegaron a pulmón, no lograron hacer contacto directo con el foco inflamatorio. Este hecho interfirió en el contacto de las BMMSCs con el micro-ambiente inflamatorio y las células presentes en el mismo, por consiguiente no se generó el perfil anti-inflamatorio esperado, lo que repercutió en un incremento en citoquinas pro-inflamatorias y linfocitos Th1, que a su vez condujo a una exacerbación del proceso inflamatorio (Nenasheva, T. y Col. 2017).

## 3. Objetivos

### Objetivo General

Evaluar el efecto de una terapia celular con células madre mesenquimales derivadas de médula ósea (BMMSCs) en el desarrollo del proceso fibrótico en un modelo de fibrosis pulmonar (FP) experimental en ratón inducido por *P. brasiliensis*.

### Objetivos específicos

1. Comprobar la eficiencia del trasplante de BMMSCs y determinar su localización en hígado, bazo y pulmones en el modelo experimental utilizado.

2. Determinar el efecto del trasplante de BMMSCSs en la carga fúngica en pulmones de ratones infectados y trasplantados.

3. Determinar los efectos del trasplante de BMMSCs en el desarrollo de la FP experimental inducida por la inoculación intranasal de levaduras de *P. brasiliensis.*

4. Determinar el efecto del trasplante de BMMSCs en la expresión de diferentes mediadores moleculares asociados al desarrollo de FP en el homogenizado pulmonar de los animales infectados.

5. Determinar el efecto del trasplante de BMMSCs en las diferentes subpoblaciones celulares en el pulmón de los animales infectados y trasplantados.

# Capítulo II

## Bone marrow-derived mesenchymal stem cells transplantation alters the course of experimental paracoccidioidomycosis to exacerbate the chronic pulmonary inflammatory response

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

**Julián Camilo Arango[1,2], Juan David Puerta-Arias[1], Paula Andrea Pino-Tamayo[1,3], David Arboleda-Toro[4] and Ángel González[2,5,*]**

[1]Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas (CIB), Universidad de Antioquia, Medellin, Colombia, [2]Microbiology School, Universidad de Antioquia, Medellin Colombia, [3]Department of Microbiology and Immunology, Weill Cornell Medical College, New York, USA, [4]Dental Synergy Group, Universidad de Antioquia, Medellin, Colombia and [5]Basic and Applied Microbiology Research Group (MICROBA), Universidad de Antioquia, Medellin, Colombia

*To whom correspondence should be addressed. Ángel González, MSc, PhD, Calle 67 No. 53–108; Office number: 5–103; Medellin, Colombia. Tel: +57-4 219–5489; Fax: +57-4 219–8494; E-mail: angel.gonzalez@udea.edu.co.

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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

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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 $\alpha$ (TNF$\alpha$) 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 $20 \times 10^6$ 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 $1 \times 10^6$ 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 $1.5 \times 10^6$ *P. brasiliensis* yeast cells contained in 60 $\mu$l 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 $\mu$l 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 $1 \times 10^6$ 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 $\mu$g/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 $\mu$M, 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 $\mu$m 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-1$\alpha$, MIP-1$\alpha$), CCL-4 (MIP-1$\beta$), 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), IFN$\gamma$, IL-1$\alpha$, IL-1$\beta$, 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-$\alpha$, 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 IgG2b$\kappa$ | Becton Dickinson |
| Isotype | PE | Rat IgG2a$\kappa$ | Becton Dickinson |
| Isotype | PE | Rat IgG2b$\kappa$ | Becton Dickinson |
| Isotype | PE | Goat IgG | BioLegend |
| Isotype | PerCP-Cy5.5 | Rat IgG2a$\kappa$ | Becton Dickinson |
| Isotype | APC | Rat IgG1$\kappa$ | Becton Dickinson |
| Isotype | APC | Rat IgG2a$\kappa$ | Becton Dickinson |
| Isotype | APC-Cy7 | Rat IgG1$\kappa$ | Becton Dickinson |
| Isotype | APC-Cy7 | Rat IgG2b$\kappa$ | Becton Dickinson |
| Isotype | Pacific blue | Hamster IgG | BioLegend |
| CD3 | PE | Rat IgG2a$\kappa$ | Becton Dickinson |
| CD4 | PerCP-Cy5.5 | Rat IgG2a$\kappa$ | Becton Dickinson |
| CD8 | PerCP-Cy5.5 | Rat IgG2a$\kappa$ | Becton Dickinson |
| CD11b | PE | Rat IgG2b$\kappa$ | Becton Dickinson |
| CD11c | APC | Hamster IgG1, $\lambda$2 | Becton Dickinson |
| CD25 | PE | Rat IgG2b$\kappa$ | Becton Dickinson |
| CD45 | FITC | Rat IgG2b$\kappa$ | Becton Dickinson |
| CD80 | Pacific blue | Hamster IgG1 | BioLegend |
| CD206 | PerCP-Cy5.5 | Rat IgG2a$\kappa$ | BioLegend |
| Siglec-F | PE | Rat IgG2a$\kappa$ | Becton Dickinson |
| Ly-6G | APC | Rat IgG2a$\kappa$ | Becton Dickinson |
| Ly-6 G and Ly-6C | APC-Cy7 | Rat IgG2b$\kappa$ | Becton Dickinson |
| IFN$\gamma$ | PE-Cy7 | Rat IgG1$\kappa$ | Becton Dickinson |
| IL4 | PE-Cy7 | Rat IgG1$\kappa$ | Becton Dickinson |
| IL9 | PE | Hamster IgG2 | Becton Dickinson |
| IL 17a | APC-Cy7 | Rat IgG1$\kappa$ | 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 $1 \times 10^6$ CM-DiI-BM-MSCs. We observed that the $CM\text{-}DiI^+$ cells were located mostly in lungs of infected mice between

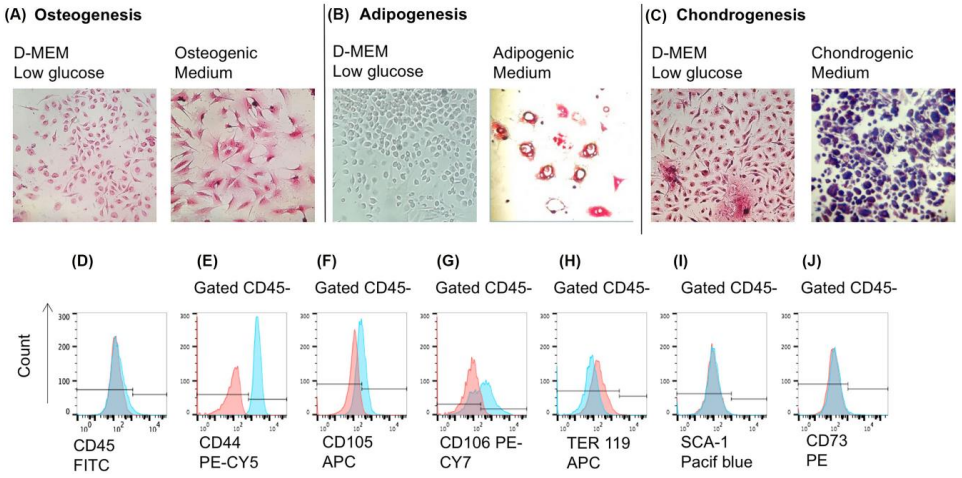


**Figure 1.** Characterization of bone marrow mesenchymal stem cells (BM-MSCs). Linage differentiation of BM-MSCs was induced as described in the Materials and Methods section. A: Osteogenesis at 14th day, stained with Safranin; B: Adipogenesis at 14th day stained with Oil Red; C: Chondrogenesis at 20th 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% ($0.7 \times 10^6$) 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 $1.5 \times 10^6$ cells of *P. brasiliensis* and BM-MSCs ($1 \times 10^6$ 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).

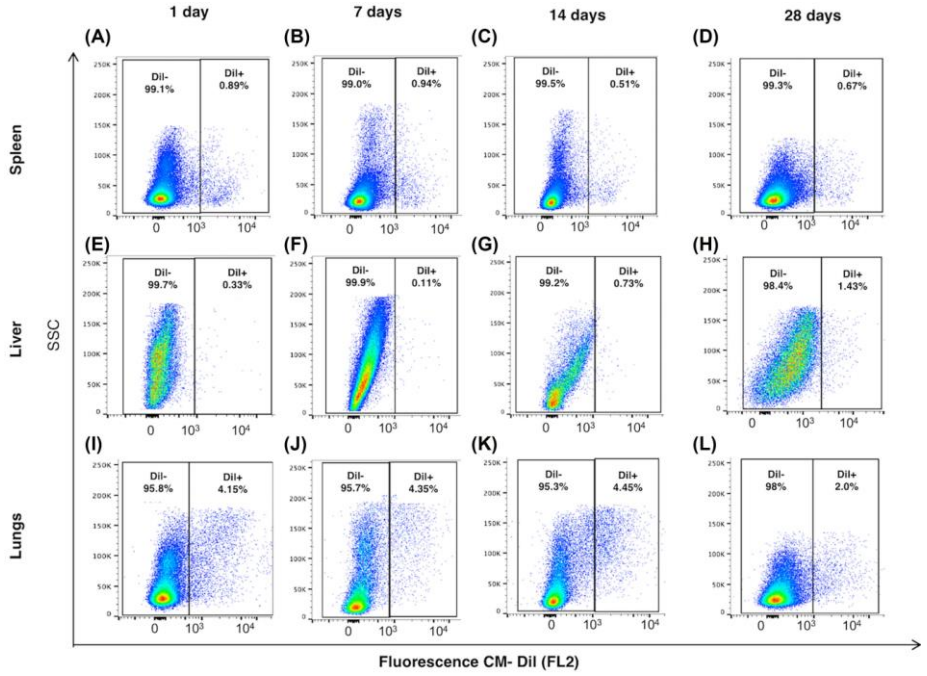


**Figure 2.** Bone marrow mesenchymal stem cells (BM-MSCs) tracking in lungs, livers and spleens of *P. brasiliensis*-infected mice. BM-MSCs were labeled with CM-DiI (1 $\mu$M) CellTracker® and then administered IV in BALB/c mice infected with *P. brasiliensis* after 4 weeks p.i. Tracking of BM-MSCs-CM-DiI 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-DiI 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

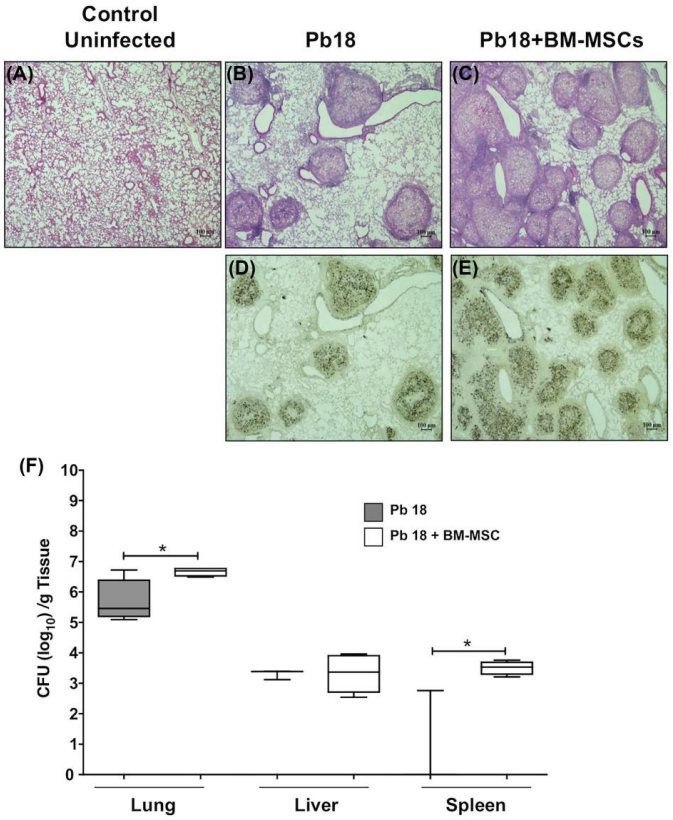


**Figure 3.** Bone marrow mesenchymal stem cells (BM-MSCs) administration increases the chronic inflammatory response and fungal load. Microphotographs are representative of lungs from uninfected mice (A); *P. brasiliensis* (Pb18) - infected control mice (B, D); BM-MSCs administration at 8th week p.i. (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

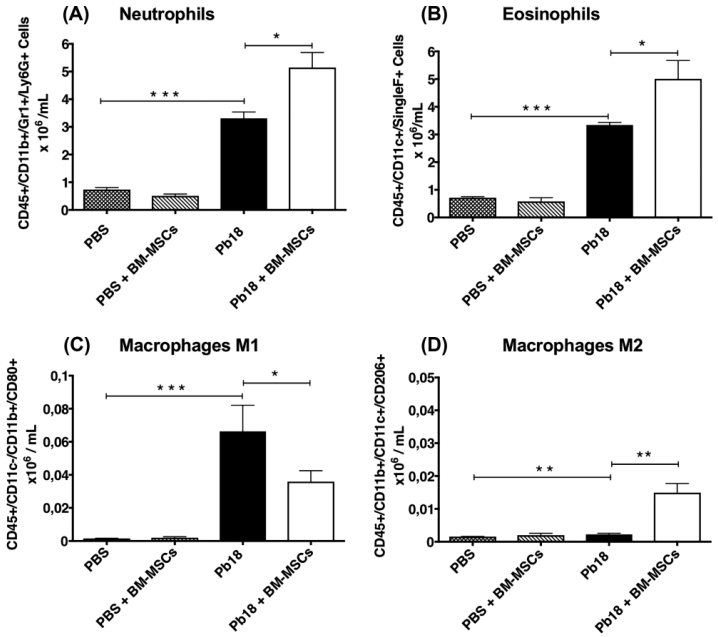


**Figure 4.** Effect of bone marrow mesenchymal stem cells (BM-MSCs) on recruitment of inflammatory cells. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in the Materials and Methods section. Cell populations were assessed by flow cytometry. Significant 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

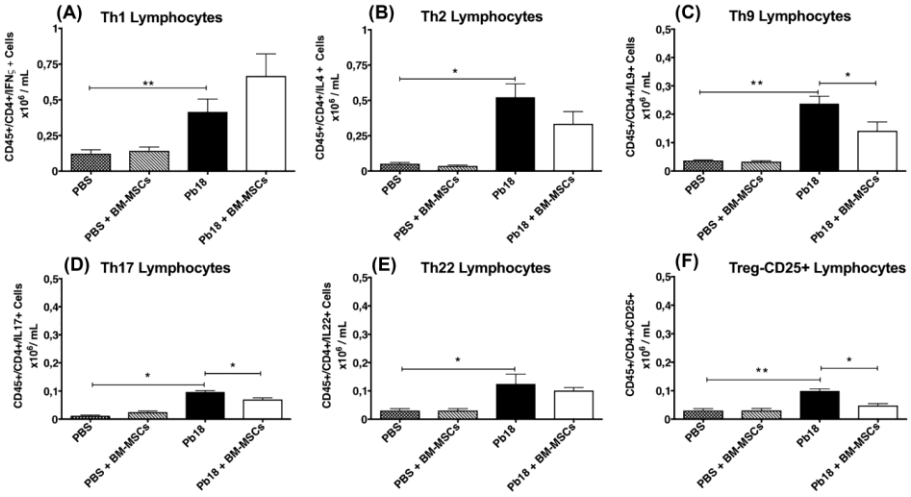


**Figure 5.** Effect of bone marrow mesenchymal stem cells (BM-MSCs) on recruitment of T helper subsets. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in Materials and Methods. Cell populations were assessed by flow cytometry. A) Th1 ($CD45^+CD4^+IFN\text{-}\gamma^+$), 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.

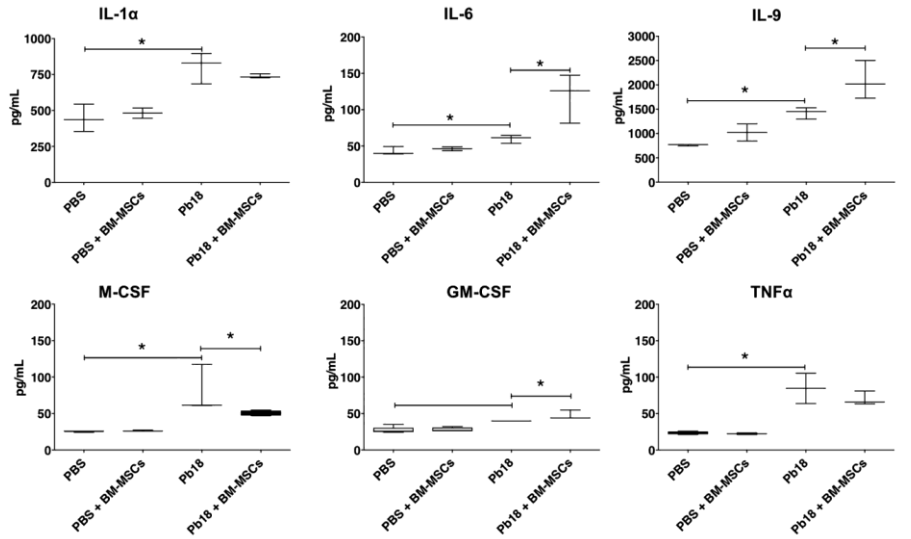


**Figure 6.** Effect of bone marrow mesenchymal stem cells (BM-MSCs) on cytokine levels. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in Methods. Pro-inflammatory cytokines were assessed with Luminex 200 system. Statistical analysis was performed 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.

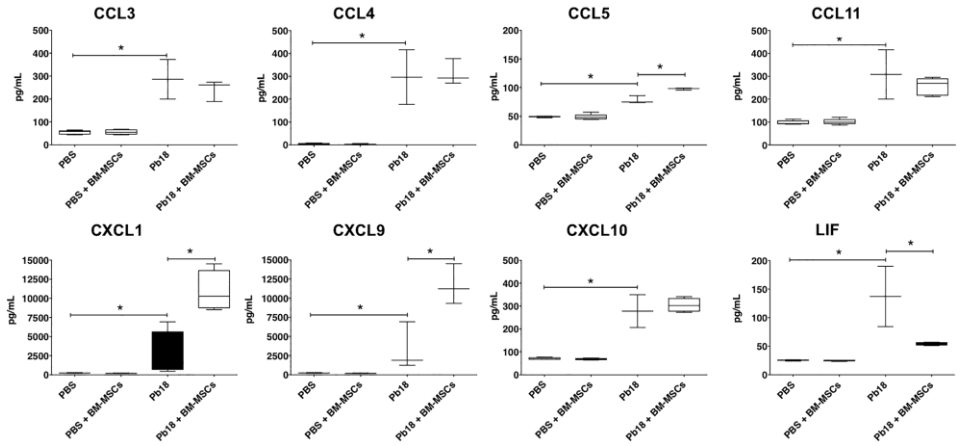


**Figure 7.** Effect of bone marrow mesenchymal stem cells (BM-MSCs) on chemokine levels. BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC as described in Methods. Chemokines were assessed with Luminex 200 system. Statistical analysis was performed using Mann–Witney 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.

## Acknowledgment

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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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# Capítulo III

## Impaired anti-fibrotic effect of bone marrow-derived mesenchymal stem cells in a mouse model of pulmonary paracoccidioidomycosis

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

# Impaired anti-fibrotic effect of bone marrow-derived mesenchymal stem cell in a mouse model of pulmonary paracoccidioidomycosis

**Julián Camilo Arango[1,2], Juan David Puerta-Arias[1], Paula Andrea Pino-Tamayo[1,3], Lina María Salazar-Peláez[4], Mauricio Rojas[5], Ángel González[2,6]***

**1** Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas (CIB)–Universidad de Antioquia, Medellín, Colombia, **2** School of Microbiology, Universidad de Antioquia, Medellín, Colombia, **3** Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York, Unites States of America, **4** Basic Sciences Group, Universidad CES, Medellín, Colombia, **5** Dorothy P. & Richard P. Simmons Center for Interstitial Lung Disease, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, Unites States of America, **6** Basic and Applied Microbiology Research Group (MICROBA), Universidad de Antioquia, Medellín, Colombia

* angel.gonzalez@udea.edu.co

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**Data Availability Statement:** All relevant data are within the paper.

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## Abstract

Bone marrow-derived mesenchymal stem cells (BMMSCs) have been consider as a promising therapy in fibrotic diseases. Experimental models suggest that BMMSCs may be used as an alternative therapy to treat chemical- or physical-induced pulmonary fibrosis. We investigated the anti-fibrotic potential of BMMSCs in an experimental model of lung fibrosis by infection with *Paracoccidioides brasiliensis*. BMMSCs were isolated and purified from BALB/c mice using standardized methods. BALB/c male mice were inoculated by intranasal infection of $1.5x10^6$ *P. brasiliensis* yeasts. Then, $1x10^6$ BMMSCs were administered intra venous at $8^{th}$ week post-infection (p.i.). An additional group of mice was treated with itraconazole (ITC) two weeks before BMMSCs administration. Animals were sacrificed at $12^{th}$ week p.i. Histopathological examination, fibrocytes counts, soluble collagen and fibrosis-related genes expression in lungs were evaluated. Additionally, human fibroblasts were treated with homogenized lung supernatants (HLS) to determine induction of collagen expression. Histological analysis showed an increase of granulomatous inflammatory areas in BMMSCs-treated mice. A significant increase of fibrocytes count, soluble collagen and collagen-3α1, TGF-β3, MMP-8 and MMP-15 genes expression were also observed in those mice. Interestingly, when combined therapy BMMSCs/ITC was used there is a decrease of TIMP-1 and MMP-13 gene expression in infected mice. Finally, human fibroblasts stimulated with HLS from infected and BMMSCs-transplanted mice showed a higher expression of collagen I. In conclusion, our findings indicate that late infusion of BMMSCs into mice infected with *P. brasiliensis* does not have any anti-fibrotic effect; possibly because their interaction with the fungus promotes collagen expression and tissue remodeling.

analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Author summary

This is the first study that evaluates the effect of BMMSCs therapy for lung fibrosis induced by the fungal pathogen *Paracoccidioides brasiliensis*, the causative agent of paracoccidioidomycosis, one of the most important systemic endemic mycosis diagnosed in South America and Central America. Our findings showed an impaired anti-fibrotic effect of BMMSCs transplantation. This effect could be triggered by either the chronic inflammatory microenvironment induced by *P. brasiliensis* or by a direct interaction between BMMSCs and the fungus, resulting in an exacerbation of the pulmonary fibrosis. In fact, the pro-fibrotic effect exerted by BMMSCs was toned-down by the usage of the antifungal ITC.

## Introduction

Bone marrow-derived mesenchymal stem cells (BMMSCs) are adult stem cells capable of both renew themselves and differentiate in vitro into multiple cell lineages [1]. These cells can also modulate the inflammatory response and induce tissue regeneration through release of cytokines, chemokines, growth factors and genetic material (e.g. miRNAs) [2–6]. Likewise, their microbicidal properties have been already described [7–10]. Cell-based therapies in regenerative medicine using syngeneic or autologous BMMSCs are considered a promising approach, because they do not induce tissue rejection and exert a localized effect than the systemic classical pharmacological strategies [11].

BMMSCs have also been evaluated in animal models of acute lung injury induced by chemicals, such as bleomycin [12, 13] and hydrochloric acid (HCl) [14]. These studies have shown that BMMSCs secrete cytokines, chemokines, growth factors and extracellular matrix proteins, and that can influence the magnitude and quality of the immune response (e.g. modulating the inflammatory response), and promote tissue repair. Likewise, BMMSCs can differentiate into pulmonary stromal cells (e.g. lung fibroblasts and myofibroblasts) [12, 14, 15].

Pulmonary fibrosis (PF) is a process characterized by excessive deposition of collagen and extracellular matrix components that results in a pathological remodeling of the pulmonary architecture. Thus, patients with PF exhibit radiographic, but also functional and clinical alterations in the lung [16]. From the pathological perspective, PF is a dynamic process involving immune system cells and soluble factors including leukotrienes, cytokines (IFNγ, TNFα, IL1, IL4, IL6, IL17), chemokines (CCL2, CCL3, CXCL12), reactive oxygen species (ROS), growth factors [platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)], and membrane-bounded and soluble molecules such as prostaglandins, metalloproteinases (and their tissue inhibitors), among others [17]. An imbalance between pro-fibrotic responses and anti-inflammatory and pro-tissue repair agents, results in the differentiation and activation of myofibroblasts which once activated produce abundant amounts of collagen, thus inducing fibrosis of the pulmonary parenchyma [18]. The participation of all the previous components in the PF has been extensively studied in animal models [16, 18, 19].

Pulmonary fibrosis can be induced by microbial agents including the dimorphic fungal pathogen *Paracoccidioides brasiliensis*, the causative agent of paracoccidioidomycosis (PCM), disease that is considered one of the most important endemic systemic mycosis in South America and Central America [20–23]. Brazil, Colombia and Venezuela are the countries with the highest number of cases reported so far, with an estimated of 10 million people infected [20, 21]. The chronic form of PCM is the most frequent clinical presentation (90% of the

cases), and it is characterized by a granulomatous inflammatory response with fibrosis development and loss of respiratory function, which is observed in 60% of the patients [22]. Itraconazole (ITC) is the treatment of choice in PCM [23]. Nonetheless, it exerts a fungistatic effect against *P. brasiliensis in vivo*, and it does not attenuate the pulmonary alterations induced by the fungal infection, including fibrosis [24, 25]. Animal models of PCM have allowed characterization of the mechanisms involved in the development of pulmonary fibrosis, and evaluate diverse strategies to treat it. Thus, combined therapies including pentoxifylline plus ITC [26], or an anti- neutrophil monoclonal antibody alone or in combination with ITC [27, 28], have showed that such treatment strategies reduced substantially the PF. However, the potential risk for using immunosuppressive drugs or biological agents, mainly in host with other unknown latent infections, should be considered [29].

The objective of this study was to investigate the regenerative effect of BMMSC on PF induced by the fungal pathogen *P. brasiliensis* in an *in vivo* experimental model of PCM; nonetheless, our findings indicated that these cells impaired the anti-fibrotic effect and by the contrary, an exacerbated fibrotic process was observed.

## Materials and methods

### BMMSCs isolation, purification and characterization

BMMSCs were obtained from four weeks-old BALB/c mice from the breeding colony maintained at the *Corporación para Investigaciones Biológicas* (CIB, Medellín-Colombia). The BMMSCs isolation and purification protocols were adapted from a protocol previously described by Rojas *et al* [12]. Briefly, mice were anesthetized with a solution of ketamine (80 mg/kg) and Xylazine (8 mg/kg) via intramuscular. Femurs and tibias were removed and bone marrow cells were isolated by flushing with Dulbecco's Modified Eagle Medium (DMEM)-low glucose (GIBCO, Invitrogen Corporation, Carlsbad, CA, USA) containing penicillin/streptomycin1% (vol/vol) (GIBCO). Cells were transferred to cell culture flasks (Eppendorf, Hamburg, Germany) with DMEM-low glucose supplemented with 10% (vol/vol) fetal bovine serum (FBS) (GIBCO) and nonessential amino acids 1% (vol/vol) (GIBCO), followed by incubation at 37˚C in 5% $CO_2$. Non-adherent cells were removed after 48 hours, and maintained in standard culture media for 7 days.

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 used. The BMMSC surface markers expression profile was determined by flow cytometry. The following antibodies were used: 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 (Biolegend, San Diego, CA, USA). 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). In addition, a differentiation assay to demonstrated the BMMSCs plasticity (differentiation to chondrogenic, adipogenic and osteogenic lineages) was performed using a differentiation commercial kit, and following the manufacturer's instructions [StemPro (Waltham, MA, USA)]. Finally, the purified cells were kept in standard culture media until the day of transplant.

### Ethical statement

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

## Mouse model of chronic pulmonary paracoccidioidomycosis

A highly virulent strain of *P. brasiliensis* (Pb18) was used in order to develop the experimental pulmonary fibrosis model as described previously [28]. Briefly, BALB/c male mice (8 weeks old) were intranasally infected with 1.5 x $10^6$ *P. brasiliensis* yeast cells contained in 60 μl of phosphate-buffered saline (PBS). The total inoculum was split into two equal doses, which were instilled within a 5–10 minutes (min) period. Non-infected (control) mice were inoculated with 60 μl of PBS.

## BMMSCs transplant

Infected and non-infected mice were intravenously injected with 1x$10^6$ BMMSCs at $8^{th}$ week post-challenged given in a single dose. Six week post-inoculation, an additional group of infected animals was treated with 100 μl of Itraconazole (ITC) oral solution (Sporanox, Janssen-Cilag S.A., Mexico) administered at a dose of 1mg/day in order to achieve serum levels equal to 1 μg/mL. The above treatment was administrated daily and uninterruptedly for 6 weeks by gavage. All animals included in the various experimental groups were sacrificed at week $12^{th}$ p.i. and their lungs harvest for further studies.

## Fibrocytes count by flow cytometry

Lungs of mice were removed, homogenized and sequentially filtered through 70 and 40μm sterile cell strainers (Thermo Fisher Scientific Inc, Waltham, MA, USA) in RPMI cell culture medium plus 1% (vol/vol) FBS (Sigma-Aldrich, Saint Louis, MO, USA). Cells suspension were centrifuged at 500 G, 10˚C for 10 min, and red blood cells were lysed using the ACK Lysing Buffer (GIBCO). Viability of the cells was determined by trypan blue exclusion test with samples being used if they were 95% of viable. Cells were resuspended in RPMI plus 10% FBS and counted using a hemocytometer. Fc receptors were blocked using a purified rat anti-mouse CD16/CD32 (BD Pharmigen, San Diego, CA, USA). Then, cells were treated with Cytofix/Cytoperm and Perm/Wash solution (BD Pharmigen, San Diego, CA, USA) [28]. Fibrocytes were determined using FITC anti-collagen I (Rocklad inc Limerick USA), PE anti-CD45 (Biolegend San Diego USA), and APC anti-CD34 (BD Pharmingen, San Diego USA). Anti-mouse IgG-FITC (Rocklad), anti-mouse IgG2aκ-PE (Biolegend) and anti-mouse IgG1κ-APC (BD) were used as isotype controls. The stained cell suspensions were fixed with FACS buffer/1% (vol/vol) PFA (Carlo Erba, Barcelona, Spain). Assays were performed using a FACS Canto II system (BD Biosciences, San Jose, CA, USA), while information analysis were done using FlowJo V10 (FlowJo, LLC, Data Analysis software, Ashland, OR, USA). Fibrocyte population was 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 to determine fibrocytes by collagen 1+ (intracellular) and CD34+ (surface). The number of fibrocytes was determined by multiplying the percentage of the gated population by the total number of leukocytes (CD45+ population).

## Histopathological analysis

Lungs were processed and analyzed as described by Puerta-Arias *et al* [28]. Briefly, lungs were perfused with 1X PBS to wash out red blood cells. Tissue fixation was completed in a 4% buffered formalin solution. Then, fixed tissues were embedded in paraffin and sections stained with Masson trichrome, and examined using a Nikon Eclipse Ci-L microscope—Nikon DS-Fi2 digital camera. A morphometric analysis was performed using NIS Elements 4.30.02

Laboratory Image Software (Nikon Instruments Inc., Melville, USA). The percentage of occupied area by the inflammatory response was calculated by dividing the total inflamed area, which includes cellular infiltrates and granulomatous lesions by the total area of the lung.

## Soluble collagen determination

Homogenized lung suspensions were treated with acid neutralizing reagent (0.5M acetic acid, 0.1 mg/ml pepsin) (Sigma-Aldrich, Saint Louis, MO, USA). Then, colorimetric detection of soluble collagen content was performed according to the manufacturer's protocol of a sircol collagen assay kit (Biocolor, Northern Ireland, U.K.). A calibration curve was constructed using bovine collagen-I in the range of 1–10 μg.

## Determination of collagen expression by human fibroblasts stimulated with homogenized lung supernatants from experimental animals

Human lung fibroblasts were obtained from Rojas' Lab repository, collected under an established protocol from the University of Pittsburgh Center for Organ Research Involving Decedents (CORID). Cultures of human fibroblasts ($2x10^4$ cells/200uL, pass 4) were treated with soluble lungs supernatants (protein concentration 10ug/mL) from all experimental groups, for 24h at 37˚C. Then, fibroblast activity was determined by measuring the expression of collagen type-I gen using reverse transcriptase real-time-PCR (RT-qPCR) assays, as previously described [29]. As controls, we used PBS and TGF-β [(5ng/ml final concentration) Peprotech Rocky Hill, United States].

## Real time PCR analysis

All real time PCR assays were performed as previously described [28]. Briefly, RNA was obtained from lungs of mice using Trizol (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase I (Thermo Fisher Scientific Inc, Waltham, MA, USA), and cDNA was synthesized using 500ng of total RNA using cDNA synthesis kit for RT-qPCR according to the manufacturer's instructions (Thermo Fisher Scientific Inc, Waltham, MA, USA). Real-time PCR was done using Maxima EVAGreen/Fluorescein qPCR Master Mix according to the manufacturer's instructions (Applied Biological Materials ABM Inc, Richmond, Canada). The CFX96 Real-Time PCR Detection System (Bio-Rad, Headquarters Hercules, California, USA) was employed to measure gene expression levels. Melting curve analysis was performed after the amplification phase of real time PCR assays to eliminate the possibility of non-specific amplification or primer-dimer formation. Validation of housekeeping genes for normalization mRNA expression was performed before gene expression analysis. Expression of fibrosis-related genes encoding for collagen, transforming growth factor beta (TGF-β), matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP) were evaluated. Fold changes in the target gene mRNA expression were quantified relative to glycer-aldehyde-3-phosphate dehydrogenase (GAPDH the housekeeping gene previously defined) [28]. Each experiment was repeated twice using 5 mice per each one of the groups with gene expression analysis being conducted by triplicate.

## Statistical analysis

Data analysis was performed using Graph Pad Prism software version 7 (GraphPad Software, Inc., La Jolla, CA, USA). Normality for all values was calculated by the Shapiro-Wilk test and when comparisons between three or more groups were required, the ANOVA test was employed. On the other hand, comparisons between two specific groups were determined by

Student-t test. Mean and standard error of the mean (SEM) were calculated for all analyses. We considerate $P<0.05$ values as significant.

## Results

### BMMSCs therapy induced an increase in pulmonary inflammation and fibrosis in the experimental model of paracoccidioidomycosis

We determined the granulomatous inflammatory areas through histopathological analysis in lung of experimental mice. We observed that lungs of *P. brasiliensis* infected-mice developed a granulomatous inflammatory response with collagen fibers surrounding granulomas (Fig 1A). Interestingly, administration of BMMSCs in infected mice showed an exacerbation of the inflammatory process, with a higher granulomatous inflammation and fibrosis areas with loss of parenchyma (Fig 1B). In contrast, the infected animals treated with ITC alone showed inflammatory and fibrotic responses similar to those infected and non-transplanted mice (Fig 1C). Moreover, the combined administration of BMMSCs/ITC in *P. brasiliensis*-infected mice considerably decreased the inflammatory response and fibrosis (Fig 1D), in comparison with those infected animals that only received cell-based therapy. A morphometric analysis revealed that occupied area by granulomatous inflammation in infected and transplanted mice was twice higher when compared with infected non-treated mice ($p<0.001$), or three time that infected and BMMSCs/ITC-treated animals ($p<0.001$) (Fig 1E). There was statistically significant difference in the average of occupied area by granulomas between infected and ITC-treated mice and those that received combined therapy ($p<0.005$).

### BMMSCs administration increases the number of fibrocytes in lungs

Fibrocytes are bone marrow-derived fibroblast progenitor cells that have been implicated in tissue remodeling or repairing process, including the development of fibrosis. Following flow cytometry analysis we found a significantly increased number of fibrocytes ($CD45^+/CD34^+/Collagen1^+$) in lungs from infected mice ($p<0.005$) (Fig 2) relative to PBS instilled controls. Moreover, infected and BMMSCs-treated animals showed almost twice the number of fibrocytes when compared with infected non-treated mice ($p<0.005$) (Fig 2). Interestingly, ITC treatment, in combination with BMMSCs, reduced the fibrocytes counts, versus *P. brasiliensis* infected mice ($p<0.001$) or infected BMMSCs-treated animals ($p<0.001$) (Fig 2).

### BMMSC therapy increases lung soluble collagen content

Collagen is considered the most important extracellular matrix protein involved in fibrosis. Accordingly, we determined the effect of BMMSCs therapy on lung soluble collagen content. Significantly increased levels of collagen in lungs from mice infected with *P. brasiliensis* were observed when compared with PBS instilled animals ($p<0.005$) (Fig 3). Infected and BMMSCs-treated mice exhibited an increased significantly in collagen content relative to infected non-treated animals ($p<0.001$) (Fig 3). Remarkably, ITC treatment reduced the amount of soluble collagen in the lungs from both, *P. brasiliensis* infected-mice ($p<0.005$), or those with BMMSCs transplantation ($p<0.005$) (Fig 3).

### Human fibroblasts stimulated with homogenized lung supernatants from experimental animals show increased collagen gene expression

To assessment the capability of lung homogenized to activate human lung fibroblasts, we performed *in vitro* assays stimulating fibroblasts with lung supernatants from experimental animals. We observed that lung supernatants from infected and BMMSCs-treated mice induced a

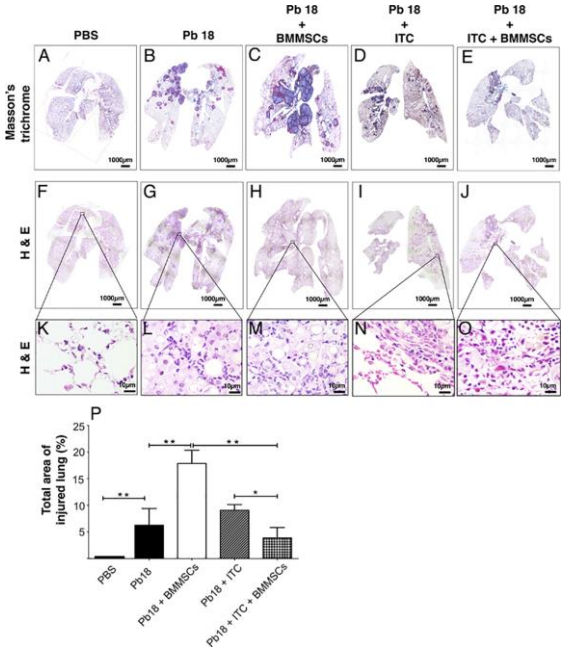


**Fig 1. Bone marrow mesenchymal stem cells (BMMSCs) induced an increase in pulmonary inflammation and fibrosis in lungs of mice infected with *P. brasiliensis*.** Microphotographs A, F, K represent lungs from uninfected mice; B, G, L corresponded to mice infected with *P. brasiliensis* (Pb18); C, H, M were taken from infected and BMMSCs-treated animals at 8th week post-challenge; infected and Itraconazole (ITC) treated-mice at 6th week p.i. in D, I, N and samples from infected mice treated with ITC at 6th week p.i. and transplanted with BM-MSCs at 8th week post-challenge as shown in E, J, O. Lungs were fixed in formalin, embedded in paraffin, cut and stained with Masson's trichrome stain to determine injured and fibrotic lungs areas (A-E) and H&E was used to evaluate the inflammatory response *in situ* (F-O). Lungs stained sections were scanned using a Nikon Eclipse Ci-L microscope—Nikon DS-Fi2 digital camera and analyzed using NIS Elements 4.30.02 Laboratory Image Software. Magnification 4X (A-J) and 100X (K-O). The percentage of injured area was calculated by dividing the total inflamed area, which includes cellular infiltrates and inflammatory lesions (granulomas) by the total area of the lung (P). Data shown represent mean and SEM (n = 4–5 mice/group, representative of two independent experiments). *, $P<0.05$ comparing *P. brasiliensis*-infected and BM-MSCs- and ITC-treated mice versus *P. brasiliensis*-infected and ITC-treated mice; and **, $P<0.001$ comparing uninfected versus *P. brasiliensis*-infected mice, or *P. brasiliensis*-infected and BM-MSCs-treated mice with either *P. brasiliensis*-infected control mice or *P. brasiliensis*-infected and BM-MSCs- and ITC-treated mice.

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higher expression of the gene encoding for collagen type-I in human fibroblast, in comparison with the respective homogenized lung supernatants from the infected non-treated mice ($p<0.005$) (Fig 4). The homogenized lung supernatants from mice infected with *P. brasiliensis* and treated with ITC, alone or in combination with BMMSCs transplantation, in human fibroblast also induced a collagen gene expression similar to that found in human fibroblast stimulated with lung homogenates from infected non-treated-mice (Fig 4).

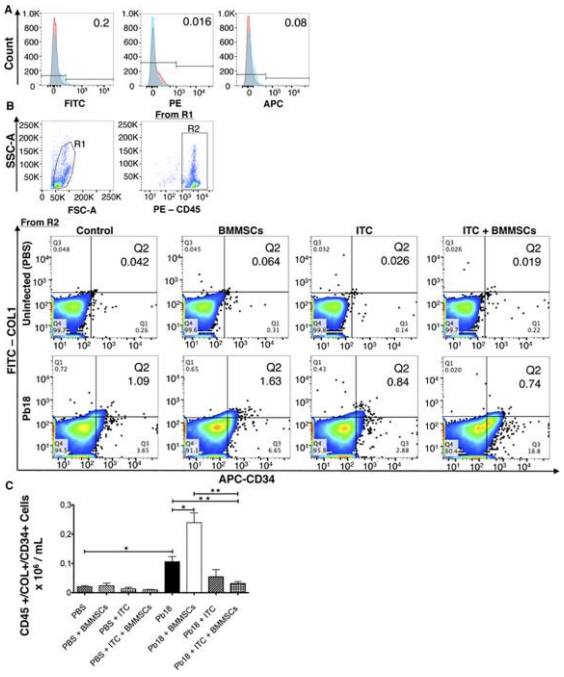


**Fig 2. BMMSCs therapy increases fibrocytes in lungs of *P. brasiliensis* infected mice.** BALB/c mice were infected with *P. brasiliensis* yeast (Pb18) and treated with BM-MSC and/or ITC as described in Materials and Methods section. Fibrocytes were assessed by flow cytometry as follows: A) correspond to representative plots of unstained cells (red) and of those treated with the isotype controls (blue). B) correspond to representative plots of gating strategy to determine fibrocytes; R1: represents forward scatter areas (FSC-A) versus side scatter areas (SSC-A) gated cell events; R2: CD45+ pictures cell events gated from R1 by SSC-A versus PE-CD45; finally, fibrocytes were gated from R2 employing FITC-collagen 1+ versus APC-CD34+ (Q2). C) corresponds to absolute number of fibrocytes (CD45+/COL1+/CD34+). Data shown represent mean and SEM of 4–5 mice/group representing two independent experiments. *, $P < 0.05$, when comparing infected untreated mice with uninfected mice, or comparing *P. brasiliensis*-infected untreated mice with *P. brasiliensis*-infected and BM-MSCs-treated mice; **, $P < 0.001$ comparing *P. brasiliensis*-infected and BM-MSCs- and ITC-treated mice with either *P. brasiliensis*-infected untreated mice or with *P. brasiliensis*-infected and BM-MSCs-treated mice.

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## BMMSCs administration alters the expression of genes related with fibrosis development

Our next step was to determine if the BMMSCs administration affects the related-fibrotic response genes expression in lungs from mice infected with *P. brasiliensis*. We observed that *P. brasiliensis* infected-mice showed a higher expression of almost all genes evaluated (Col1α3, Col3α1, TGF-β1, TGF-β3, MMP-8, MMP-12, MMP-13, MMP14, TIMP-1 and TIMP-2) when compared with uninfected mice. Moreover, after BMMSCs treatment, a significantly higher expression of Col3α1, TGF-β3, MMP-8, and MMP-15 was observed in comparison with those *P. brasiliensis* infected-mice, while a reduction on MMP-13 gene expression was also observed

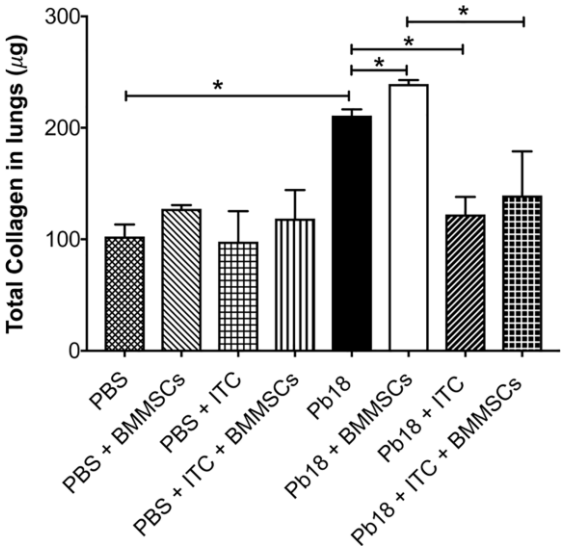


**Fig 3. BMMSCs administration increases the total collagen levels in lungs of mice infected with *P. brasiliensis*.** Total collagen was performed using Sircol Dye reagent as described in Materials and Methods section. Data shown represent mean and SEM (n = 4–5 mice/group; representative of two independent experiments). *, $P < 0.05$, comparing infected untreated mice with uninfected mice; or comparing *P. brasiliensis*-infected untreated mice with either *P. brasiliensis*-infected and BM-MSCs-treated mice or *P. brasiliensis*-infected and ITC-treated mice; or comparing *P. brasiliensis*-infected and BM-MSCs-treated mice with *P. brasiliensis*-infected and BM-MSCs and ITC-treated mice.

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(Fig 5). Infected mice that received the ITC treatment showed a slight but higher expression of the MMP-15 and TIMP-2 genes relative to infected non-treated animals. Remarkably, the combined therapy BMMSCs/ITC induced a synergistic reduction of Col3α1, TGF-β-3, MMP-8, MMP-12, and TIMP-1, as well as an increase of TIMP-2 gene expression, when compared to infected mice that received cell transplantation (Fig 5).

## Discussion

Pulmonary fibrosis (PF) is a serious disease triggered by chemical, physical, or infectious agents, but also it could be an idiopathic or cryptogenic process [16, 18]. The fungus *P. brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM), a disease endemic in Latin America. In the chronic form of the disease, more than 60% of patients develop fibrotic sequelae compromising lung parenchyma, even after the completion of treatment [22]. In fact, the current therapeutic strategy for PCM is based on azole compounds as itraconazole (ITC), an antimycotic that reduces fungal load but not PF development [20]. Therefore, in recent years, we had been focused to evaluate alternative therapies in an attempt to reduce the fibrotic response in this disease [22, 28]. In the present study, we investigated for the first time the effect of a BMMSCs-based cellular therapy on PF in a murine model of PCM. Nonetheless, contrary to other reports showing a beneficial effect of BMMSCs transplants in PF due to

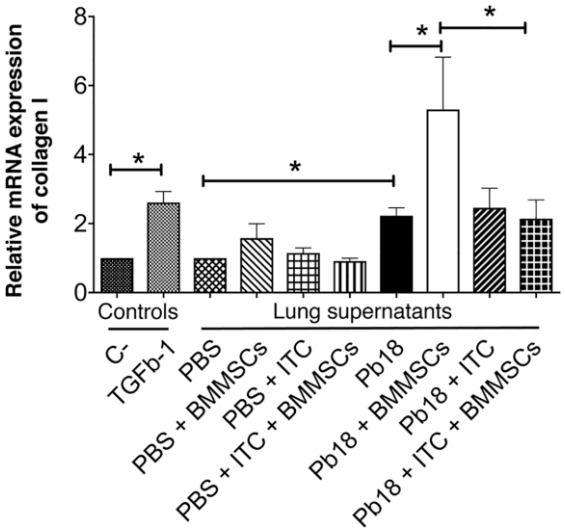


**Fig 4. Lung supernatants from mice infected with *P. brasiliensis* and treated with BMMSCs induces high expression of collagen I gen by human fibroblasts.** Lung human fibroblasts were stimulated with soluble lung supernatants from *P. brasiliensis*-infected mice treated with either BMMSCs and/or ITC, for 24h at 37˚C. Fibroblast activation was determined by measuring the expression of collagen I gen by real-time PCR as described in Materials and Methods section. Data shown represent mean and SEM (n = 4–5 mice/group; representative of two independent experiments). *, $P < 0.05$, comparing TGFβ-1-stimulated fibroblast with unstimulated cells; or comparing infected untreated mice with either uninfected mice or *P. brasiliensis*-infected and BM-MSCs-treated mice; or comparing *P. brasiliensis*-infected and BM-MSCs-treated mice with *P. brasiliensis*-infected and BM-MSCs and ITC-treated mice.

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chemical or physical agents [30–32], we observed that this type of cellular therapy exacerbated the fibrotic response.

BMMSCs are considered as promising in the development of cellular therapies due to their capacity to regenerate tissue, as well as for their immunomodulatory properties, which include the release of paracrine or endocrine signaling molecules [5, 32, 33]. Moreover, different studies have shown that BMMSCs are able to sense the microenvironment and respond to both physical (i.e. mechanical) and chemical stimulus [34, 35]. Namely, it has been recognized that extracellular matrix (ECM) influences stem cell lineage commitment [35]. As an example, Li *et al.* [34, 35] described that fibronectin, a glycoprotein that connects integrins in cell surface with collagen fibers in the ECM, playing a role as mechanotransducer signal that regulate human mesenchymal stem cells (hMSC) differentiation [34]. Our group have previously shown that lungs of mice infected with *P. brasiliensis* exhibit an increased expression and re-arrangement of ECM components (e.g. collagen, fibronectin, laminin, proteoglycans), even after two days post-infection, but fully established after 4$^{th}$ week post-infection [36, 37]. Accordingly, that early deposition could favors the BMMSCs differentiation to fibrocytes, who in turn can proliferate and undergo phenotypic conversion to fibroblast or myofibroblast, the cellular culprits of fibrosis [38]. In fact, after BMMSCs administration, we observed a rise of fibrocytes counts and soluble collagen content in lungs from infected-mice.

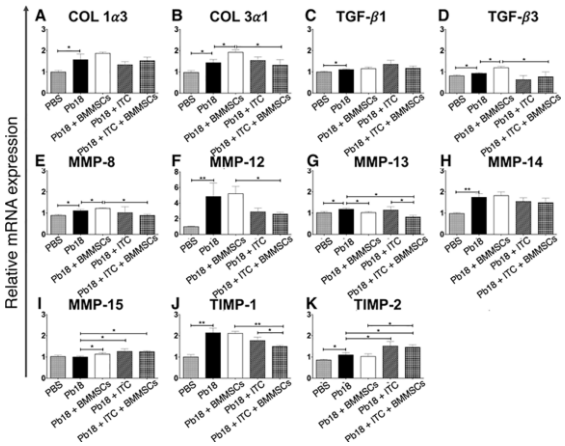


**Fig 5. BMMSCs therapy alters the expression of genes related with the fibrosis process in lungs of mice infected with *P. brasiliensis*.** Relative quantification of the mRNA expression of fibrosis related genes were performed in lungs of mice. A) COL 1α3; B) COL 3α1; C) TGF-β1; D) TGF-β3; E) MMP-8; F) MMP-12; G) MMP-13; H) MMP-14; I) MMP-15; J) TIMP-1; and K) TIMP-2. Data shown represent mean and SEM (n = 4–5 mice/group; representative of two independent experiment). *, $P< 0.05$, **, $P<0.001$ comparing infected untreated mice with uninfected mice, or comparing *P. brasiliensis*-infected untreated mice with *P. brasiliensis*-infected and BM-MSCs-treated mice; or comparing *P. brasiliensis*-infected untreated mice with *P. brasiliensis*-infected and ITC-treated mice; or comparing *P. brasiliensis*-infected and BM-MSCs-treated mice with *P. brasiliensis*-infected and ITC-treated mice; or comparing *P. brasiliensis*-infected and BM-MSCs-treated mice with *P. brasiliensis*-infected and BM-MSCs and ITC-treated mice, respectively. COL, collagen; TGF-β, transforming growth factor beta; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases.

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In addition to their ability to interact with ECM proteins, BMMSCs may also recognize microbial compounds through pattern recognition receptors (PRRs), interactions that may induce a pro-inflammatory response [35, 38]. Bernardo *et al* [39], just as Waterman *et al* [40], have demonstrated that the activation of Toll-like receptors (TLRs) in mesenchymal stem cells promotes their polarization into a pro-inflammatory phenotype, named *MSC1*, which can fuel inflammation and subsequent fibrosis [39, 40]. In this sense, it has been reported that the interaction between *P. brasiliensis* and TLR4 lead to a severe fungal infection, associated with an enhanced exacerbated proinflammatory response [41]. All these reports support both the fungal proliferation and tissue damage observed after BMMSCs administration, and suggest an immunoregulatory role of these stem cells; thus, the deleterious effect observed maybe triggered by the interaction of BMMSCs with either *P. brasiliensis* compounds, extracellular matrix and the inflammatory microenvironment developed during the chronic course of PCM. However, more studies are needed to evaluate the interaction between BMMCSs and this fungal pathogen, as well as its implications not only for the immune response, but also for tissue repair.

Besides that, it is worth to highlight that myofibroblasts might be derived from other cellular sources beyond bone marrow fibrocytes. In fact, these collagen-producing cells, and main effectors of fibrosis, may arise from resident fibroblast, or as a result of epithelial/endothelia mesenchymal transitions [42]. In this study, the administration of BMMSCs was associated with an increase in the number of fibrocytes. Although, the origin of these cells could not be

confirmed, we may suppose that they could come from bone marrow or pericytes, which subsequently differentiate into fibroblasts and then into myofibroblasts collagen-producer cells, thus contributing to the increase of PF in those *P. brasiliensis* infected- and BMMSCs treated-mice. Accordingly, we evaluated the collagen I gene expression in human fibroblast stimulated with homogenized lung supernatants from *P. brasiliensis* infected mice. We found that supernatants from infected and BMMSCs-transplanted mice induced a higher production of type I collagen transcript in human fibroblast, in comparison with those cells stimulated with infected non-treated animals. These results clearly indicate the presence of molecules from the lung microenvironment able to stimulate the collagen production in human fibroblast. Among the major stimuli to activate fibroblasts, IL6, TGFβ, IL13 and FGF are found, and once activated, these cells differentiate into myofibroblasts or could produce pro-fibrotic molecules such as IL1, VEGF, Insulin-like growth factor 2 (IGFII), Insulin-like growth factor-binding protein (IGFBP), IL6 and IL33 [42]. In fact, we observed an increased expression of Col 3α1 and TGFβ-3 genes in lungs of *P. brasiliensis*–infected mice treated with BMMSCs. In concurrence with these reports, more recently we have observed a significant increase of IL-1α, IL-1β, IL-6, TNF-α and IL17 levels in homogenized lung supernatants from mice infected with *P. brasiliensis* [28]. All these cytokines have also been implicated in the pathogenesis of PF. Nonetheless, a direct activation of fibroblast by *P. brasiliensis* compounds could not be ruled out.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases (around 25 members are know so far) that are either secreted or membrane-bound enzymes [43]. MMPs have long been considered to be essentials for ECM remodeling, which is critical in embryonic development and tissue homeostasis, including inflammatory response and tissue repair [43]. In this context, as stated by Pardo et al, MMPs not only degrade ECM components, but also release, cleave and active a wide range of growth factors, cytokines, chemokines and cell surface receptors affecting numerous cell functions (e.g. adhesion, proliferation, differentiation, migration, cell death) [44]. Thus, MMPs and their tissue inhibitors (TIMPs) play a central role in the extracellular pathways of ECM degradation and, therefore, in fibrosis development or resolution [44]. Namely, MMP8, a collagenase, can also cleave the chemokines CXCL8 and LIX, resulting in enhanced chemoattractant activities, which could be associated with fibrosis development [43, 44]. These results show that after BMMSC administration there was not only an increase in the expression MMP8 gene but also elevated neutrophils counts, findings noticed before in association with development of fibrosis as observed in our PCM model previously reported [28]. BMMSCs transplantation also induced a decrease in gene coding for MMP-13, another collagenase, but not changes in the expression of TIMP-1 and TIMP2 genes were observed. Meanwhile, the ITC/BMMSC transplantation therapy decreased synergistically the expression of TIMP-1 and MMP-13. Over expression of TIMP-1 is associated whit liver fibrosis [45], while MMP-13 cleaves and inactivates CCL2, CCL7 and CXCL12 leading to reduction in chemotaxis, as well as to a decrease in the fibrosis process [43, 45]. However, our data interpretation relative to MMPs or TIMPs gene expression is limited, as the current knowledge concerning pathological tissue repair in PMC is scarce. In addition, although most of the fibrosis-related genes analyzed showed small fold-changes with statistical significant, a possible meaningful biological effect cannot be ruled-out.

ITC is the antifungal treatment of choice in PCM. Of note, additionally to its antifungal effect, it has been recently documented that this antifungal medication also exhibits immunomodulatory properties [46]. Moreover, in a previous work, we found that ITC reduces the expression of certain genes encoding for pro-inflammatory cytokines (IFN-γ, IL-6, IL-17, TGF-β1, TNF-α), transcriptional factors (T-bet, GATA-3, Spi-1, RoRc, Ahr, FoxP3), and fibrosis development (MMP-1, MMP-8, MMP-13, and Col3α1). Additionally, it also diminished the number of inflammatory cells—including neutrophils—in the lungs of mice infected with

*P. brasiliensis* [27]. In the present study, it was observed that the ITC regulates the expression of the MMP-15 and TIMP-2 genes that have been recognized as inducers of pulmonary fibrosis [27].

## Conclusions

Overall, our results demonstrated an exacerbating effect of the BMMSCs therapy on pulmonary fibrosis induced by *P. brasiliensis* infection. We hypothesized that this outcome could be triggered by either the interaction with *P. brasiliensis* compounds or by the inflammatory microenvironment induced by this process. Nonetheless, the combined therapy ITC/BMMSCs showed promising results since synergistically it reduced TIMP-1 and MMP-13. Thus, the use of BMMSC under different conditions or combined with other treatments (e.g. ITC) opens the possibility to new therapeutic approaches for this type of fibrosis resulting from an infectious disease.

PCM is considered a neglected tropical disease mostly affecting low income individuals who live in underdeveloped Latin American rural regions where the technology and the resources needed to administer the immunotherapeutic measures here suggested would probably not be available. Nonetheless, the implementation of cellular therapies is progressing and the prospects are to arrive in a few years to the administration of autologous bone marrow or stem cells obtained from adipose tissues even in these regions.

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## Author Contributions

**Conceptualization:** Lina María Salazar-Peláez, Ángel González.

**Data curation:** Julián Camilo Arango, Juan David Puerta-Arias, Paula Andrea Pino-Tamayo.

**Formal analysis:** Julián Camilo Arango, Juan David Puerta-Arias, Paula Andrea Pino-Tamayo, Lina María Salazar-Peláez, Mauricio Rojas, Ángel González.

**Funding acquisition:** Ángel González.

**Investigation:** Julián Camilo Arango, Juan David Puerta-Arias, Paula Andrea Pino-Tamayo, Lina María Salazar-Peláez, Mauricio Rojas, Ángel González.

**Methodology:** Julián Camilo Arango, Juan David Puerta-Arias, Paula Andrea Pino-Tamayo, Mauricio Rojas.

**Project administration:** Ángel González.

**Resources:** Mauricio Rojas, Ángel González.

**Supervision:** Ángel González.

**Visualization:** Julián Camilo Arango, Juan David Puerta-Arias, Paula Andrea Pino-Tamayo, Mauricio Rojas, Ángel González.

**Writing – original draft:** Julián Camilo Arango, Lina María Salazar-Peláez, Mauricio Rojas, Ángel González.

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# Capítulo IV

# Discusión General y Perspectivas

## Discusión General

Esta tesis se enfocó en determinar el potencial terapéutico de las BMMSCs en el tratamiento de la fibrosis pulmonar en un modelo experimental de paracoccidioidomicosis pulmonar. Entre las principales características de este trabajo se destacan:

1. Se utilizó un modelo de fibrosis pulmonar previamente estandarizado, en el cual se controlaron variables tales como la cepa del hongo (Pb18), el inoculo (1,5 x $10^6$ levaduras) y los tiempos de desarrollo de la fibrosis pulmonar (FP), por lo tanto, es un modelo apropiado para evaluar diferentes estrategias terapéuticas, entre ellas las terapias celulares. Adicionalmente, las características de este modelo animal han sido validadas en varios trabajos previamente reportados (Pino-Tamayo, P. A. y Col. 2016; Puerta-Arias, J. D. y Col. 2016).

2. El uso de las BMMSCs como agentes inmunomoduladores de la fibrosis pulmonar ha sido evaluado en otros modelos de FP inducida por agentes no infecciosos que causan daño en el tejido como LPS, bleomicina o ácido clorhídrico (Rojas, M. y Col. 2005; Wecht, S. & Rojas, M. 2016). Dado el potencial inmunoregulador de las BMMSCs, se evaluó el papel de estas células en el desarrollo de la FP en el modelo en ratón de PCM pulmonar, en el cual se evaluaron diferentes componentes inmunológicos que participan en este proceso tales como las células del sistema inmune,

mediadores solubles y la expresión de genes asociados al desarrollo de la FP.

3. El modelo empleado es de tipo crónico. Hasta ahora el efecto de las BMMSCs, en los modelos de FP tanto en ratas como en ratones se ha determinado en periodos relativamente cortos (de 1 a 4 semanas), mientras que, en nuestro modelo experimental, la evaluación del efecto de las BMMSCs, se realizó en tiempos más prolongados (crónicos) de hasta 12 semanas pos-infección. Este modelo de PCM de tipo crónico se asemeja al contexto de la PCM en humanos.

Este es el primer estudio enfocado a evaluar el efecto de una terapia celular basada en BMMSCs sobre una micosis crónica como la PCM. Los resultados expuestos en el capítulo II y III permiten esbozar que la terapia con BMMSCs en el modelo experimental de PCM pulmonar exacerbó la respuesta inflamatoria y el desarrollo de FP, resultados que contrastan a los descritos en modelos de FP aguda producida por agentes no infecciosos.

En la figura 1 se describe el modelo propuesto de los eventos que suceden en los animales infectados y trasplantados con BMMSCs. En la representación se pueden observar dos etapas, una etapa de incremento o exacerbación de la respuesta inflamatoria en pulmón descrita en el capítulo II y aumento de la fibrosis pulmonar descrita en el capítulo III.

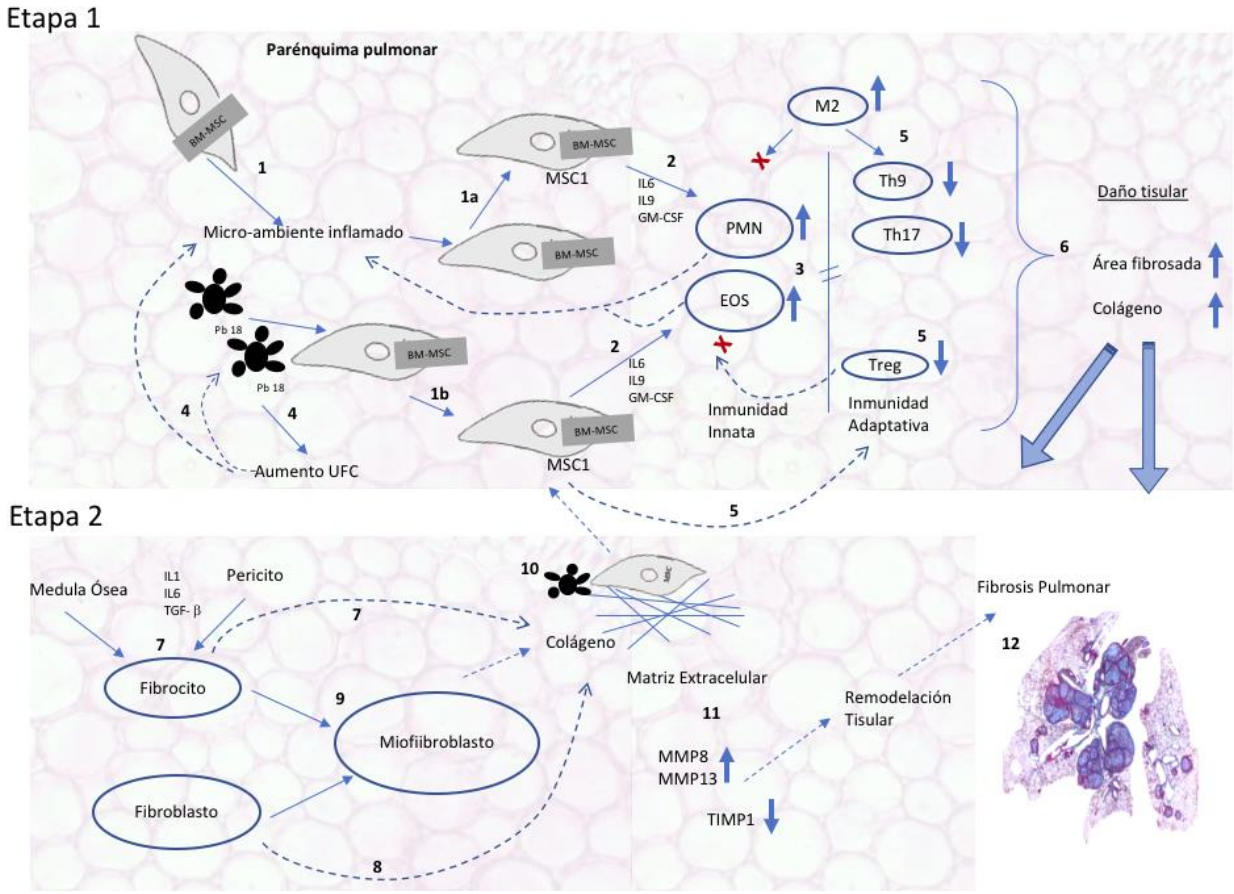


**Figura 1.** Mecanismos asociados a la exacerbación de la inflamación y la fibrosis pulmonar en ratones infectados con *P. brasiliensis* y tratados con BMMSCs.

En la etapa uno, las BMMSCs trasplantadas migran al microambiente inflamatorio en el pulmón **(1)**. Este proceso de migración inicial se corroboró en un experimento independiente en el que las BMMSCs teñidas con el colorante Dil

llegan en su mayoría al pulmón y permanecen en este órgano hasta por 28 días (Capitulo II, Figura II). En el modelo propuesto las BMMSCs se exponen a un micro-ambiente inflamatorio característico del proceso causado por *P. brasiliensis* en el que participan la IL-1, IL-6, IL-9, GM-CSF y TNF-$\alpha$, entre otras moléculas (Benard, G. 2008). Estas citoquinas pueden inducir la polarización de las BMMSCs a un perfil inflamatorio, es decir células MCS1(**1a)**. De forma paralela las BMMSCs, podrían interactuar con el hongo mediante receptores tipo *toll* (TLRs) lo que también podría explicar la polarización a MSC1 (Waterman, R. S. y Col. 2010) (**1b)**. Posteriormente, estas células generan aún más moléculas de carácter pro-inflamatorio; en este medio se encontró un importante incremento de IL-6, IL-9 y GM-CSF (**2)**. El incremento de dichas citoquinas induce la migración de neutrófilos y eosinófilos a través de la producción de quimioquinas y/o expresión de moléculas de adhesión; a su vez, estas células inflamatorias retroalimentan el proceso inflamatorio (**3)**. Las condiciones de este micro-ambiente, potencian la diseminación del hongo a otros tejidos y el incremento de la carga fúngica (UFC) en el pulmón **(4)**. Investigaciones previas demuestran la importancia de los neutrófilos en la progresión de la infección (Pino-Tamayo, P. A. y Col. 2016; Puerta-Arias, J. D. y Col. 2016) y de los eosinófilos en la diseminación de *P. brasiliensis* (Shikanai-Yasuda, M. A. y Col. 1992). Simultáneamente, durante el desarrollo de la inmunidad adaptativa se presenta una disminución de linfocitos Th17 y Th9, lo cual puede asociarse con la polarización de macrófagos a un perfil M2 **(5)**. Adicionalmente, en el presente estudio, también se observó una disminución de linfocitos Treg lo cual ha sido asociado al perfil de MSC1 (Bernardo, M. E. & Fibbe, W. E. 2013). Los fenómenos anteriores evidencian un proceso inflamatorio amplificado por la inmunidad innata, en los cuales los

mecanismos de regulación ejercido por los macrófagos M2 y los linfocitos Treg no controlan este proceso. Los resultados anteriores son similares a los obtenidos por Nenasheva, T. y col 2017 (referenciado en el capítulo 1) en un modelo de daño pulmonar e inflamación por *Mycobacterium tuberculosis,* en este trabajo se evidenció un incremento en citoquinas pro-inflamatorias y linfocitos Th1 (Nenasheva, T. y Col. 2017).

En la etapa dos, los eventos anteriores favorecen el daño tisular en el que se evidencia un incremento en el área inflamatoria con aumento de fibras de colágeno **(6)**. Durante el proceso fibrótico se observa un incremento de fibrocitos, que a su vez, pueden provenir de médula ósea o de pericitos (Células contráctiles que se envuelven alrededor de las células endoteliales) (Barron, L. y Col. 2016) **(7)**. Aunque no hay evidencia directa que demuestre que los fibrocitos pueden diferenciarse a partir de BMMSCs, se ha descrito que estas células pueden diferenciarse a pericitos (De Souza, L. E. y Col. 2016). Entre las moléculas que favorecen la llegada de fibrocitos al tejido están la IL-1, IL-6 y el TGF-β que se encuentran notablemente incrementadas **(7)**. Adicionalmente, el TGF-β induce la producción de proteínas de matriz extracelular (MEC) por parte de los fibrocitos, los cuales pueden liberar grandes cantidades de colágeno (Wynn, T. A. 2011) (**7).** Los fibroblastos residentes en el microambiente pulmonar pueden activarse y contribuir a la síntesis de proteínas de MEC generando más fibrosis **(8)**. Posteriormente, los fibrocitos, fibroblastos, células en transición endotelio-mesénquima y células en transición epitelio-mesénquima se pueden diferenciar a miofibroblastos los cuales, a su vez, producen abundante colágeno (Wynn, T. A. 2011; Wynn, T. A. & Ramalingam, T. R. 2012; Barron, L. y Col. 2016) **(9).** Luego en el tejido pulmonar ya fibrosado se pueden presentar interacciones MEC –

hongo o MEC – BMMSCs, este tipo de interacciones también pueden polarizar las BMMSCs a un perfil inflamatorio MSC1 (Somaiah, C. y Col. 2015; Prockop, D. J. 2016) **(10).** El desarrollo de la fibrosis va acompañado de un proceso de remodelación en el que participan tanto metaloproteinasas de MEC (MMP) como sus inhibidores (TIMP) **(11)**. Las MMP8 y MMP13 tienen la capacidad de generar más cicatrices fibróticas, y al presentarse una disminución en TIMP1 se ve favorecida su función. Los mecanismos anteriores llevan a una exacerbación del proceso de fibrosis pulmonar **(12).**

En el modelo experimental empleado en el presente trabajo, las BMMSCs no generaron un efecto inmuno-modulador de tipo benéfico como se ha descrito previamente en modelos de FP inducidos por agentes químicos. En nuestro diseño experimental se incluyó un microorganismo vivo con capacidad de replicarse y estimular constantemente los componentes del sistema inmune. Por lo tanto, con el ánimo de controlar la proliferación del hongo, se empleó el antimicótico ITC, el cual es usado en pacientes con PCM. Es llamativo que la terapia combinada ITC/BMMSCs mostró un efecto fungistático acompañado de una disminución en la expresión de TIMP1 y MMP-13, moléculas que se asocian con progresión de la fibrosis en pulmón e hígado. Por lo tanto, el uso de BMMSCs bajo diferentes condiciones o en combinación con otros tratamientos (por ejemplo, ITC) abre la posibilidad de nuevos enfoques en la terapia de PF en la paracoccidioidomicosis.

## Perspectivas

Los resultados de este trabajo de investigación abren las puertas a nuevos estudios que permitan comprender el potencial terapéutico de las BMMSCs en los procesos asociados al desarrollo de la FP tanto de carácter infeccioso como no infeccioso. Como perspectivas se plantean las siguientes:

1. **Aumentar el número de dosis de BMMSCs.** En este trabajo se tomó como referencia los procedimientos de trasplante de BMMSCs en modelos de fibrosis pulmonar inducida por bleomicina y otros agentes químicos en los que se empleó una sola dosis. Teniendo en cuenta que el modelo utilizado en este estudio fue de tipo infeccioso y crónico, un trabajo a futuro consistiría en incluir una o dos dosis adicionales antes del sacrificio de los animales.

2. **Entender la interacción de *P. brasiliensis* con las BMMSCs.** Es posible que en el modelo de PCM pulmonar aquí analizado, las BMMSCs interactúen directamente con las levaduras de *P. brasiliensis*. Una interesante propuesta consistiría en determinar si hay interacciones vía PAMPs – PRRs, y si las BMMSCs podrían llegar a tener un efecto microbicida directo o indirecto sobre las levaduras del hongo o por el contrario son permisivas y permiten el crecimiento del hongo, o si ésta interacción altera el patrón de expresión de genes asociados a la capacidad anti-inflamatoria de las BMMSCs.

3. **Células madre pre-activadas.** Otra posible estrategia consistiría en pre-activar las BMMSCs con citoquinas como TNF-α, IL-1β; lo cual se ha descrito en células humanas. De igual manera, también se podría pre-activar con LPS, buscando una respuesta mediada por receptores tipo *toll* y la activación de un perfil anti-inflamatorio (MSC2) en estas células.

4. **Células modificadas genéticamente.** Recientes estudios demuestran que las BMMSCs pueden ser modificadas genéticamente con el propósito de inducir la expresión de genes que mejoren su capacidad de migración a un tejido específico o de sobre-expresar moléculas con características anti-inflamatorias como la IL-10.

## Publicaciones y comunicaciones derivadas de la tesis

### 1. Artículos en revistas internacionales

**Medical Mycology (Diciembre, 2017)**

**Bone marrow-derived mesenchymal stem cells transplantation alters the course of experimental paracoccidioidomycosis to exacerbate the chronic pulmonary inflammatory response**

Julián Camilo Arango1,2, Juan David Puerta-Arias1, Paula Andrea Pino-Tamayo1,3, David Arboleda-Toro4, Ángel González2,5

1Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas (CIB) – Universidad de Antioquia, Medellín, Colombia; 2Microbiology School, Universidad de Antioquia, Medellín Colombia; 3Department of Microbiology and Immunology, Weill Cornell Medical College, New York, USA; 4Dental Synergy Group, Universidad de Antioquia, Medellín, Colombia; 5Basic and Applied Microbiology Research Group (MICROBA), Universidad de Antioquia, Medellín, Colombia.

**PLoS Neglected Tropical Disease (Octubre, 2017)**

**Impaired anti-Fibrotic effect of bone marrow-derived mesenchymal stem cells in a mouse model of pulmonary paracoccidioidomycosis**

Julián Camilo Arango[1,2], Juan David Puerta-Arias[1], Paula Andrea Pino-Tamayo[1,3], Lina María Salazar-Peláez[4], Mauricio Rojas[5], Ángel González[2,6]

1. Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas (CIB) – Universidad de Antioquia, Medellín, Colombia; 2. School of Microbiology, Universidad de Antioquia, Medellín, Colombia; 3. Department of Microbiology and Immunology, Weill Cornell Medical College, New York, USA; 4. Basic Sciences Group, Universidad CES, Medellín, Colombia; 5. Dorothy P. & Richard P. Simmons Center for Interstitial Lung Disease, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; 6. Basic and Applied Microbiology Research Group (MICROBA), Universidad de Antioquia, Medellín, Colombia.

**2. Presentación en eventos**

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; Arboleda-Toro, D.; González, A. 2014.** Trasplante de células madre mesenquimales derivadas de médula ósea como terapia para el control del proceso de fibrosis pulmonar experimental inducido por *Paracoccidioides brasiliensis*. **V Seminario Corporación Ciencias Básicas Biomédicas** – Universidad de Antioquia. Ruta N, Medellín –Colombia. (Presentación Oral).

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; Arboleda-Toro, D.; González, A. 2015.** Trasplante de células madre mesenquimales derivadas de médula ósea como terapia para el control del proceso de fibrosis pulmonar experimental inducido por *Paracoccidioides brasiliensis*. **VI Seminario Corporación Ciencias Básicas Biomédicas** – Universidad de Antioquia. Ruta N, Medellín –Colombia. (Poster).

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; Arboleda-Toro, D.; González, A. 2016.** Bone marrow derived mesenchymal stem cells transplantation exacerbates the chronic inflammatory response and pulmonary fibrosis in mice infected with *Paracoccidioides brasiliensis*. **Congreso internacional de la Sociedad Americana de Microbiología ASM.** Boston – USA. (Poster).

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; Arboleda-Toro, D.; González, A. 2016.** El trasplante de células madre mesenquimales derivadas de médula ósea exacerba la respuesta inflamatoria y la fibrosis pulmonar en ratones infectados con *Paracoccidioides brasiliensis*. **Asociación Colombiana de Infectología ACIN** Medellín- Colombia. (Presentación Oral).

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; Arboleda-Toro, D.; Salazar-Peláez L.M.; González, A. 2016.** El trasplante de células madre mesenquimales derivadas de médula ósea no es efectivo en el control de la fibrosis pulmonar inducida por *Paracoccidioides brasiliensis*. **IX Jornadas de Investigación y Extensión de la Escuela de Microbiología** "Conociendo la Investigación y la Extensión hecha en casa". Medellín – Colombia. (Presentación Oral).

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; David Arboleda-Toro, D.; Salazar-Peláez L.M.; González, A. 2017.**
Evaluación del efecto anti-fibrótico de la terapia con células madre mesenquimales derivadas de médula ósea en un modelo en ratón de paracoccidioidomicosis

pulmonar. **Seminario MICROBA**. Universidad de Antioquia. Medellín – Colombia. (Presentación Oral).

**Arango, J.C.; Puerta-Arias, J.D.; Pino-Tamayo, P.A.; Salazar-Peláez L.M.; González, A. 2017.** Impaired anti-fibrotic effect of bone marrow-derived mesenchymal stem cell in a mouse model of pulmonary paracoccidioidomycosis. **Congreso internacional de la Federación Española de Microbiología FEMS**. Valencia – España. (Poster).

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