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Veruska Cintia Alexandrino de Souza

AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DE MONÓCITOS E VIAS DE ATIVAÇÃO DE MACRÓFAGOS EM MODELOS EXPERIMENTAIS DE HEPATOPATIAS CRÔNICAS

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Tese apresentada ao curso de doutorado em Biociências e Biotecnologia em Saúde, do Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, como parte dos requisitos para a obtenção do grau de Doutor em Ciências.

Orientadoras:

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Aos meus pais que tanto amo, Vera e Hélio.

Ao meu amor, Victor Vasconcelos.

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Mahatma Gandhi

SOUZA, Veruska Cintia Alexandrino de. **Avaliação do potencial terapêutico de monócitos e vias de ativação de macrófagos em modelos experimentais de hepatopatias crônicas.** 2017. Tese (Doutorado em Biociências e Biotecnologia em Saúde) - Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, 2017.

RESUMO

As doenças crônicas do fígado são caracterizadas por alterações no processo de reparo tecidual, resultando na deposição excessiva de tecido fibroso e inibição da dinâmica de regeneração. Atualmente não há métodos terapêuticos eficazes para as hepatopatias avançadas, e a terapia celular tem aberto novas perspectivas de tratamento. Nesse contexto, os monócitos têm se destacado como potenciais candidatos ao transplante celular, devido à sua plasticidade e envolvimento nos processos de inflamação e reparo. Assim, o presente estudo avaliou os efeitos terapêuticos de monócitos derivados de medula óssea em modelos experimentais de lesão hepática crônica. Para isso**,** Camundongos C57BL/6 foram utilizados para a obtenção de dois modelos de injúria hepática: 1) Modelo tóxico, induzido por tetracloreto de carbono (CCl_4) e etanol, e 2) Modelo parasitário, induzido por infecção pelo *Schistosoma mansoni*. Após o estabelecimento dos modelos de lesão hepática, monócitos isolados por separação imunomagnética, a partir da medula óssea de camundongos doadores, foram administrados via endovenosa nos animais com hepatopatias. Os efeitos da terapia foram avaliados por meio de análises morfológica, morfometria, avaliação bioquímica, imunológica e de expressão gênica. A terapia com monócitos promoveu redução significativa da fibrose hepática e do estresse oxidativo. Além disso, citocinas pró-inflamatórias e fatores pró-fibróticos foram reduzidos, bem como fatores anti-inflamatórios e anti-fibrogênicos tiveram seus níveis aumentados. O transplante de monócitos induziu alterações significativas na expressão hepática de alfa-actina de músculo liso (α-SMA), além de mudança nos níveis de expressão de marcadores de perfis de ativação de macrófagos. Pôde-se concluir que a terapia com monócitos foi capaz de promover diminuição do quadro de fibrose hepática em modelos murinos de lesão crônica de fígado, reduzindo o estresse oxidativo e inflamação e atuando na regulação de mediadores da fibrogênese.

Palavras-chave: Terapia Baseada em Transplante de Células e Tecidos. Monócitos. Macrófagos. Tetracloreto de Carbono – toxicidade. Esquistossomose mansônica - complicações.

SOUZA, Veruska Cintia Alexandrino de. **Evaluation of therapeutic potential of monocytes and macrophage activation pathways in experimental models of chronic hepatopathy.** 2017. Tese (Doutorado em Biociências e Biotecnologia em Saúde) - Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, 2017.

ABSTRACT

Chronic liver diseases are characterized by changes in the tissue repair process, such as an excessive deposition of fibrous tissue and inhibition of regeneration dynamics. Currently, there are no effective therapeutic methods for advanced liver disease. In this context, monocytes have emerged as potential candidates for cell transplantation because of its plasticity and involvement in inflammation and repair processes. The present study evaluated the therapeutic effects of bone marrow-derived monocyte in experimental models of liver fibrosis. C57BL/6 mice were used to obtain two models of liver fibrosis: 1) Toxic model, induced by administration of CCl_4 and ethanol; 2) Parasitic model, induced by *Schistosoma mansoni* infection. After the establishment of liver injury models, monocytes isolated by immunomagnetic separation from bone marrow of donor mice were administered were administered intravenously in the animals with hepatopathies. The effects to therapy were evaluated by morphological, morphometric, biochemical, immunological and gene expression analysis. monocyte therapy promoted a significant reduction of liver fibrosis and oxidative stress. In addition, pro-inflammatory cytokines and pro-fibrotic factors were reduced, as well as the anti-inflammatory and anti-fibrogenic factors had their levels increased. monocyte transplantation induced significant alterations in liver expression of alpha-smooth muscle actin (α-SMA), as well as changes in the expression levels of macrophage activation profile markers. monocyte transplantation was able to promote reduction of liver fibrosis in murine models of chronic liver injury, reducing oxidative stress and inflammation, and acting on the regulation of fibrogenesis mediators

Key words: Cell and Tissue Transplantation Based Therapy. Monocytes. Macrophages.

Carbon Tetraclorhide; Schistosomiasis.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanina aminotransferase
Arg-1	Arginase-1
AST	Aspartato aminotransferase
α-SMA	Alpha-Smooth Muscle Actin
Cl_3^-	Íon Triclorometil
CCl_4	Tetrachloromethane
CCL5	C-C motif chemokine ligand 5
CCR2	C-C motif chemokine receptor 2
cDNA	Complementary Deoxyribonucleic Acid
CD206	Receptor de manose 1 tipo-C
CECAL	Centro de Criação de Animais de Laboratório
CEUA	Comissão de ética no uso de animais
CEH	Célula Estrelada Hepática
CMMO	Células Mononucleares de Medula òssea
CMO	Células da medula óssea
CTE	Controle
CYP2E1	Citocromo p450 2E1
DAB	3,3'diaminobenzidina
DMN	D-galactosamina
EDTA	Ethylenediamine tetraacetic acid/ ácido etilenodiamino tetra-acético
ELISA	Enzyme Linked ImmunonoSorbent Assay
EtOH	Etanol
Fizz1	Molécula alfa 1 semelhante a resistina
GFAP	Glial fibrillary acidic protein
GSH	Glutationa
Gal-3	Galectina-3
IL	Interleucina
Ly6C	Antígeno de linfócito 6C
MEC	Matriz Extracelular
MCP-1	Monocyte Chemoattractant Protein 1
MMP	Matrix metalloproteinase

MMPC	Células progenitoras mesenquimais multipotentes do mesoderma
MON	Monócitos
NaCl	Cloreto de Sódio
NO	Nitric oxide / Óxido Nítrico
OPN	Osteopontina
PDGF	Platelet-derived growth factor
qPCR	Quantitative Polimerase Chain Reaction
RNA	Ribonucleic acid / Ácido Ribonucléico
ROS	Reactive oxygen species / Espécies Reativas de Oxigênio
RT-qPCR	Transcriotase Reverse-Quantitative Polimerase Chain Reaction
TAA	Tioacetamida
TGF-β	Transforming growth factor-beta1
TLR	Tool-like receptor
TIMP	Tissue Inhibitor of Metalloproteases
TNF-α	Tumor Necrosis Factor-alpha
Tris-HCl	Trisaminometano - Ácido clorídrico
VEGF	Vascular Endothelial Growth Factor
YM-1	Proteína selmelhante a chitinase 3

SUMÁRIO

1 INTRODUÇÃO

As enfermidades hepáticas vêm representando um grave problema de saúde pública em todo o mundo. Essas doenças podem ser secundárias a diferentes etiologias a exemplo das infecções virais dos tipos A, B e C, infecções parasitárias, uso excessivo de álcool e de substâncias tóxicas e até mesmo disfunções metabólicas e auto-imunes.

De uma forma geral, quando o tecido hepático sofre algum dano e o equilíbrio funcional do órgão é prejudicado, podem ocorrer alterações no processo de reparo, resultando em uma exacerbação do tecido de cicatrização. O desenvolvimento contínuo e difuso do tecido de reparo no parênquima, juntamente com as alterações secundárias do sistema vascular, leva ao desenvolvimento da fase crônica da doença, podendo evoluir, em alguns casos, à insuficiência hepática. Nos casos de insuficiência hepática descompensada o transplante de fígado passa a ser o tratamento com melhor resultado, no qual cerca de 75% dos indivíduos apresentam um índice de sobrevida de cinco anos.

A principal dificuldade encontrada nos centros que realizam transplantes hepáticos é o número limitado de doadores, em função do número crescente de pacientes que aguardam transplante. Além disso, deve-se considerar que o transplante hepático é um procedimento cirúrgico com riscos e que leva o paciente ao uso de drogas imunossupressoras para o resto da vida. Diante destes aspectos torna-se importante o desenvolvimento de novas estratégias terapêuticas para as doenças hepáticas crônicas.

Na busca de substitutos biológicos que mantenham, melhorem ou restaurem as funções de órgãos e tecidos do corpo humano, a perspectiva do uso da terapia celular pela medicina regenerativa vem demonstrando importantes resultados nas doenças hepáticas. Investigações acerca do potencial terapêutico de determinadas populações celulares têm mostrado que células da medula óssea (CMO) podem contribuir para o processo de reparo tecidual. Estudos pré-clínicos têm demonstrado que as CMO são capazes de se diferenciar em células epiteliais hepáticas, com melhora das funções do fígado. A redução do índice de mortalidade de camundongos com lesão hepática também foi evidenciada no modelo experimental de hepatite fulminante, após transplante de CMO. A diminuição dos níveis de tecido fibroso também contribui para a melhora do tecido hepático. Um menor percentual de fibrose após terapia celular pode estar associado a um aumento da produção de metaloproteinases e diminuição dos níveis do Fator de Crescimento e Transformação-beta (TGF-β), uma das principais citocinas envolvidas na fibrogênese.

Com base nos estudos experimentais, ensaios clínicos de fase I utilizando transplante

autólogo de medula óssea em pacientes portadores de hepatopatias crônicas foram iniciados. Pacientes portadores de cirrose hepática foram submetidos à infusão de células-tronco mononucleares autólogas da medula óssea através da artéria hepática. Nenhum efeito adverso da terapia com células da medula óssea foi relatado. Além disso, este e outros estudos indicam melhora da função hepática por terapia celular.

Nossos estudos vêm demonstrando que dentre populações celulares presentes na medula óssea, células da linhagem monocítica podem apresentar relevante potencial terapêutico frente às hepatopatias, devido a sua plasticidade e conhecida participação em diversos processos, tais como inflamação e reparo tecidual. Os macrófagos são a forma madura dos monócitos e distribuem-se amplamente nos tecidos, podendo ser ativados por vias distintas e assumindo assim diferentes fenótipos. Estes desempenham funções específicas, que vão desde fagocitose e apresentação de antigeno, até mesmo ação direta na resposta imune associada à infecções parasitárias, processos alérgicos e no reparo tecidual, através da secreção de citocinas anti-inflamatórias e anti-fibróticas.

Pesquisas recentes têm investigado o comportamento dos monócitos/macrófagos frente às doenças crônico-degenerativas, em diferentes modelos pré-clínicos, bem como em estudos com pacientes. Os resultados obtidos até então permitem considerar essas células como potenciais ferramentas terapêuticas para o tratamento das doenças crônicas do fígado.

2 REFERENCIAL TEÓRICO

2.1 Reparo Hepático

As células do tecido hepático estão suscetíveis a agressões oriundas de diferentes etiologias. A morte celular (necrose ou apoptose), resultante de estímulos agressores, sejam eles de natureza química, física ou biológica, induz a liberação de mediadores químicos que tentam manter a homeostase, iniciando assim o processo de reparo tecidual (PELLICORO et al., 2014). O reparo hepático é caracterizado por dois eventos importantes: a cicatrização ou fibrogênese e a regeneração.

2.1.1 Fibrogênese Hepática

A fibrose hepática pode ser definida como um processo tecidual, celular e molecular dinâmico e altamente integrado, consequência de uma reação de cicatrização que ocorre no fígado em resposta às lesões teciduais crônicas (DRANOFF; WELLS, 2010; NOVO et al., 2014; ZHANG et al., 2016). Essa condição patológica está presente na maioria das disfunções crônicas do fígado, e é considerado um evento essencial diretamente associado à severidade das lesões hepáticas (SCHUPPAN; KIM, 2014). Todavia, a deposição continua do tecido fibroso é capaz de comprometer as funções normais do fígado, podendo levar o paciente ao estágio de cirrose e insuficiência hepática (PINZANI, 2013; ROSSELLI; MACNAUGHTAN; WEISKIRCHEN; TACKE, 2016). Interações entre diferentes vias de sinalização e diversos tipos celulares conduzem a progressão da fibrose hepática (SUN; KISSELEVA, 2015).

De um modo geral, a fibrogênese hepática é desencadeada por resposta inflamatória e induzida por células da imunidade inata, principalmente macrófagos e neutrófilos (BATALLER; BRENNER, 2005). Essas células liberam citocinas e quimiocinas pró-fibrogênicas, que estimulam e intensificam a produção e secreção de proteínas da matriz extracelular (MEC – colágeno, proteoglicanas, fibronectina e elastina), que são depositadas no parênquima hepático, com o objetivo de substituir as células mortas (FRIEDMAN et al., 2013). Esta etapa é de grande importância para a recuperação do fígado, que terá sua estrutura mantida até que novas células hepáticas sejam formadas e o parênquima seja restaurado, fenômeno esse denominado regeneração (PELLICORO et al., 2014).

No fígado, quando um estímulo agressor torna-se contínuo e persistente, muitos hepatócitos são perdidos e a capacidade regenerativa desse órgão fica comprometida (POPOV

et al., 2011). Além disso, o acúmulo contínuo de MEC rica em colágeno I e III tem como consequência uma deposição excessiva de tecido de cicatrização e fibrose hepática, com substituição de um tecido íntegro (parênquima composto de hepatócitos) por tecido com função, apenas, de sustentação (DRANOFF; WELLS, 2010; VAN DIJK et al., 2015).

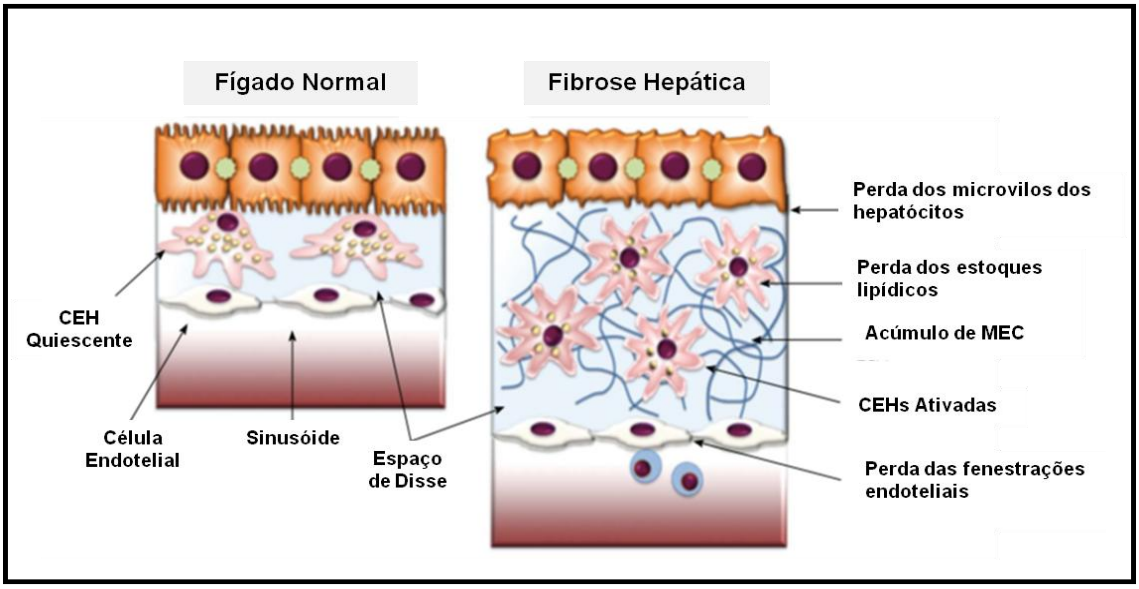
2.1.2 Células Estreladas Hepáticas

A produção de MEC e formação de tecido fibroso é estabelecida por populações celulares heterogêneas (ELPEK, 2014). Em geral, fibroblastos e miofibroblastos são as principais células envolvidas no processo de cicatrização tecidual (PELLICORO et al., 2014). No fígado, as células estreladas hepáticas (CEHs) são consideradas a principal população celular com capacidade fibrogênica, por serem grandes produtoras de componentes da MEC e por atuarem diretamente na regulação da fibrogênese hepática, independente da etiologia (KISSELEVA; BRENNER, 2011).

As CEHs, conhecidas também por células de Ito, são células de origem ainda não definida, localizadas nos espaços perisinusoidais de Disse (FRIEDMAN; SCHWABE, 2012; LEPREUX; DESMOULIÈRE, 2015). No fígado normal, as CEHs encontram-se quiescentes, armazenam grandes quantidades de ácido retinóico (Vitamina A) e sintetizam Proteína Ácida Fibrilar Glial (GFAP), o que reforça a hipótese de que essas células tenham origem a partir de precursoras de células neurais, as células progenitoras mesenquimais multipotentes derivadas do mesoderma (MMPC) (BRENNER et al, 2012; KORDES et al., 2014; VISHNUBALAJI et al., 2012). Em resposta à lesão hepática, mediadores inflamatórios de origem parácrina e autócrina estimulam a transdiferenciação das CEHs (Figura 1), que perdem seus estoques de vitamina A e de GFAP, e assumem fenótipo de miofibroblasto, altamente proliferativo, migratório e contráctil, produzindo e secretando grandes quantidades de proteínas da MEC, principalmente colágeno tipo I (PUCHE; SAIMAN; FRIEDMAN, 2013; SHAH et al., 2013).

Durante o processo de ativação, as CEHs passam a expressar, em seu citoplasma, grandes quantidades da proteína citoesquelética alfa-actina de músculo liso (α-SMA), adquirindo capacidade de contractilidade (COHEN FTALY; FRIEDMAN, 2011; PUCHE; SAIMAN; FRIEDMAN, 2013; SHI; ROCKEY, 2010;). Esse comportamento tem como consequência a constricção perisinusoidal e hipertensão portal, impedindo o fluxo sanguíneo portal, o que agrava ainda mais o quadro de fibrose hepática (XU et al., 2012).

Figura 1- Ativação das células estreladas hepáticas no fígado.



Fonte: Adaptado de Elpek (2014).
Nota: Em condições normais, as CEHs encontram-se quiescentes, e estão localizadas no espaço perisinusoidal de Disse, atuando como células armazenadoras de ácido retinóico. Essas células são ativadas mediante estímulos inflamatórios decorrentes, e sofrem transdiferenciação, assumindo fenótipo de miofibroblastos, passando a produzir e secretar grandes quantidades de proteínas da MEC.
Legenda: CEH-célula estrelada hepática; MEC-matriz extracelular

A ativação das CEHs é um evento primordial no estabelecimento da fibrose hepática (KORDES et al., 2014), uma vez que estas células são responsáveis pela secreção de cerca de 80% do colágeno fibrilar tipo I (LEPREUX; DESMOULIÈRE, 2015). Essa ativação é induzida por mediadores químicos, produzidos por células inflamatórias e também por células residentes do fígado (hepatócitos, células de Kupffer e células endoteliais) (ELPEK, 2014).

2.1.3 Síntese e degradação de tecido fibroso

O remodelamento do tecido fibroso é crucial para a manutenção da homeostase tecidual durante a lesão hepática. O processo de cicatrização é eficientemente regulado em condições normais, por um grupo de enzimas envolvidas na degradação do tecido fibroso, conhecidas com metaloproteinases de matriz (MMP), que controlam e mantém a relação fibrogênese-fibrólise estável (GAO; BATALLER, 2011). No entanto, lesões contínuas ao tecido induzem maior deposição e menor degradação de matriz cicatricial, que se acumula no tecido hepático (DAS; VASUDEVAN, 2008; SCHUPPAN, 2015).

MMPs são colagenases produzidas por vários tipos celulares, e estão diretamente associadas à degradação de tecido de cicatrização (IREDALE; BATALLER, 2014). No fígado, as enzimas MMP-2, MMP-9 e MMP-13 desempenham papel importante na resolução

da fibrose hepática, e têm sido consideradas potentes alvos terapêuticos (PHAM VAN et al., 2008).

A regulação da resposta fibrogênica também envolve moléculas denominadas Inibidores Teciduais de Metaloproteinases (TIMPs), que têm por função inibir a atividade enzimática das MMPs (KESSENBROCK; PLAKS; WERB, 2010). Na lesão hepática crônica, esses inibidores encontram-se em quantidades elevadas, contribuindo para o estabelecimento do quadro de fibrose hepática e inibição da regeneração do fígado (GIELING; BURT; MANN, 2008; MOHAMMED et al., 2005). Além disso, o TIMP-1 também tem efeitos anti-apoptóticos nas CEHs, estmulando assim a fibrogênese por promover a sobrevivência dessas células fibrogênicas (ELPEK, 2014).

2.1.4 Moléculas de sinalização na fibrose hepática

O estabelecimento da fibrose hepática é precedido por uma resposta inflamatória, com envolvimento de elementos tanto da imunidade inata quanto adaptativa (ZHANG et al., 2016). Diferentes moléculas de sinalização, com origem e função distintas, são fundamentais para a regulação do processo fibrogênico (ZHOU; ZHANG; QIAO, 2014).

A maioria dos tipos de agressão ao tecido hepático provoca dano aos hepatócitos, evento que estimula a liberação de mediadores inflamatórios, e consequente recrutamento de leucócitos para as áreas de lesão (JIANG; TOROK, 2013). A fagocitose das células mortas amplifica a resposta inflamatória, através da produção das citocinas fator de necrose tumoral-alfa (TNF-α), interleucina (IL)-6 e IL-1β (MA et al., 2016). Tais mediadores pró-inflamatórios estimulam a secreção de moléculas envolvidas no processo de fibrogênese, dentre as quais se destaca o fator de crescimento e transformação-Beta (TGF-β), considerado o principal mediador pró-fibrogênico conhecido (YOSHIDA; MATSUZAKI, 2012).

No fígado, o TGF-β é sintetizado principalmente por células hepáticas residentes, apresentando-se em três isoformas - TGF-β1, TGF-β2 e TGF-β3 (DOOLEY; DIJKE, 2012). O TGF-β1 se destaca por ter um papel fundamental na iniciação e na manutenção da fibrogênese hepática (DOOLEY; DIJKE, 2012; LEE; WALLACE; FRIEDMAN, 2015), e está diretamente envolvido na ativação das CEHs e na síntese de componentes da MEC, tais como colágeno tipo I, fibronectina e proteoglicanas (INAGAKI; OKAZAKI, 2007). Tem participação na inibição da degradação da MEC, através da diminuição da síntese de MMPs e do aumento da produção de TIMPs, levando à deposição excessiva de fibras colágenas e promovendo o estabelecimento da fibrose hepática (KISSELEVA et al., 2011).

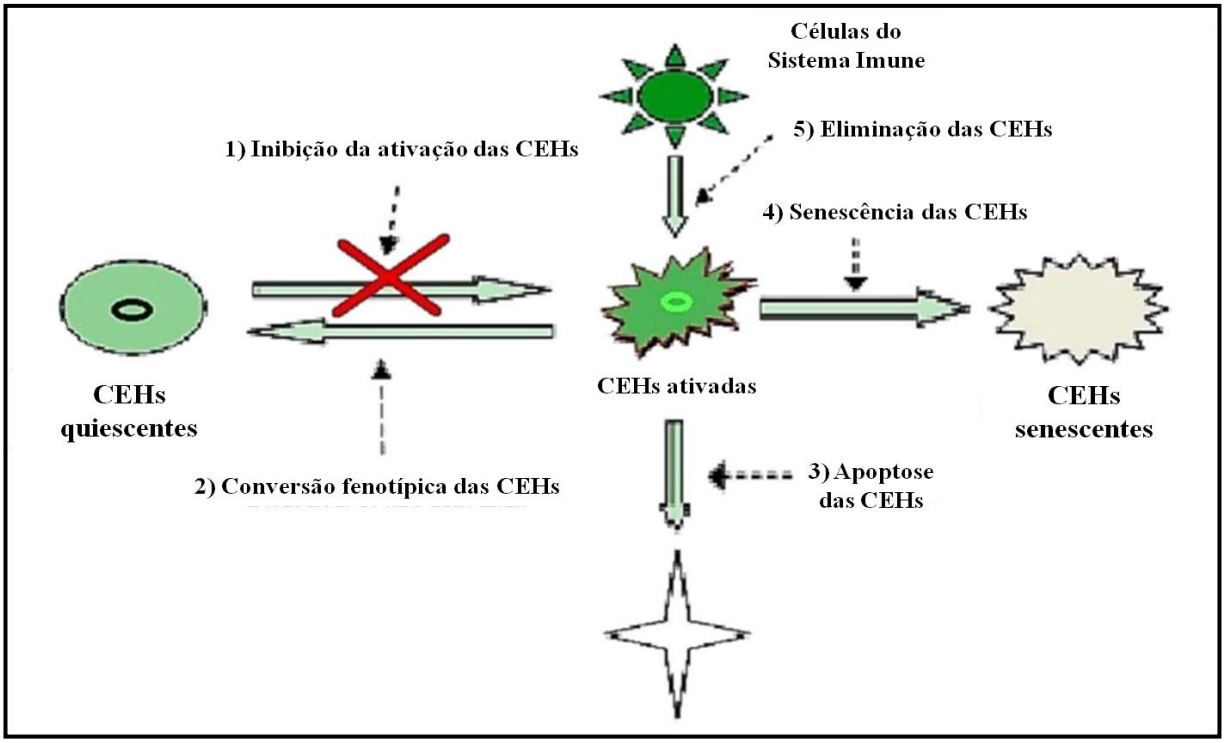
Outros mediadores também estão envolvidos na patogênese da fibrose hepática. Dentre estes, pode-se destacar: galectina-3 (gal-3), uma proteína que tem sido associada à ativação das CEHs e de outras células fibrogênicas (HENDERSON et al., 2006; TRABER; ZOMER, 2013); IL-13, envolvida na regulação da síntese de TGF- β1 pelas células do fígado (KAVIRATNE et al., 2004); osteopontina (OPN), citocina pró-inflamatória que modula o fenótipo pró-fibrogênico de CEHs e está envolvida em muitos processos patológicos incluindo inflamação, fibrose e angiogênese (PEREIRA et al., 2015). Pesquisas demonstram que a inibição desses fatores pode contribuir para a regressão da fibrose hepática (OLIVEIRA et al., 2012; TRABER et al, 2013).

2.1.5 Reversibilidade da fibrose hepática

Atualmente, não existe nenhum tratamento efetivo para fibrose hepática e dessa maneira, diversos estudos têm buscado alternativas terapêuticas seguras e eficazes. Nos estágios iniciais de lesão hepática, é possível restabelecer o microambiente hepático e a fibrose ser potencialmente reversível. A deposição prolongada de fibras colágenas torna o tecido fibroso mais estável e resistente à degradação (POPOV et al., 2011). Uma possível reversibilidade no quadro de fibrose hepática vai depender da remoção da exposição ao agente etiológico associada a uma terapia anti-fibrogênica (SCHUPPAN; PINZANI, 2012). Estudos têm associado à diminuição da fibrose hepática à degradação de tecido fibroso, através da ação de MMPs, e inibição da ativação das CEHs (TROEGER et al., 2012).

Pesquisas estão sendo conduzidas, considerando as CEHs como alvo bastante promissor no desenvolvimento de estratégias terapêuticas para as doenças crônicas do fígado (TANIMOTO et al., 2013; ZHANG et al., 2016). A reversibilidade da fibrose hepática tem sido associada à: 1) Inibição da ativação das CEHs (HUANG; DENG; LIANG, 2017); 2) Conversão fenotípica das CEHs para o estado quiescente (TROEGER et al., 2012); 3) Indução da apoptose das CEHs (MALAT; LOTERSZTAJN, 2013); 4) Indução à senescência das CEHs (KRIZHANOVSKY et al., 2008); 5) Eliminação das CEHs pela imunidade inata (MALAT; LOTERSZTAJN, 2013) e adaptativa (WYNN; RAMALINGAM, 2012) (Figura 2).

Figura 2- Mecanismos de reversibilidade da fibrose hepática, com atuação nas células estreladas hepáticas



Fonte: Adaptado de Huang; Deng e Liang (2017).
Nota: Novas propostas terapêuticas podem ter como alvo a inibição da ativação das CEHs (1), a conversão fenotípica das CEHs para o estado quiescente (2), a iIndução da apoptose das CEHs (3), a indução à senescência das CEHs (4), ou a eliminação das CEHs pela imunidade inata e adaptativa (5).
Legenda: CEH-célula estrelada hepática

2.2 Modelos experimentais de lesão hepática crônica

A utilização de modelos experimentais de doenças é de extrema importância para desenvolvimento de pesquisas nas áreas da saúde humana. Esses modelos permitem investigar alterações imunopatológicas e patofisiológicas resultantes de diferentes etiologias, ou ensaiar o uso de novas ferramentas para tratamento e cura de enfermidades que acometem o homem. Esses estudos devem ser realizados de forma ética, obedecendo a critérios estabelecidos por comissões que garantam o bem-estar animal durante toda a experimentação (ZHANG et al., 2016).

O estabelecimento de modelos experimentais que mimetizam as doenças humanas do fígado tem contribuído enormemente para o entendimento dos mecanismos envolvidos na patologia da doença, bem como para o desenvolvimento de novas estratégias de tratamento para as hepatopatias (STARKEL; LECLERCQ, 2011). Dentre a variedade de espécies utilizadas, os camundongos (*Mus musculus*) são a preferência, por serem animais de baixo custo, fácil reprodução e manutenção no laboratório. Além disso, apresentam forte similaridade genética com a espécie humana e permitem o desenvolvimento de pesquisas em

diferentes estágios da lesão hepática, bem como a possibilidade de utilizar animais geneticamente modificados (LIU et al., 2012).

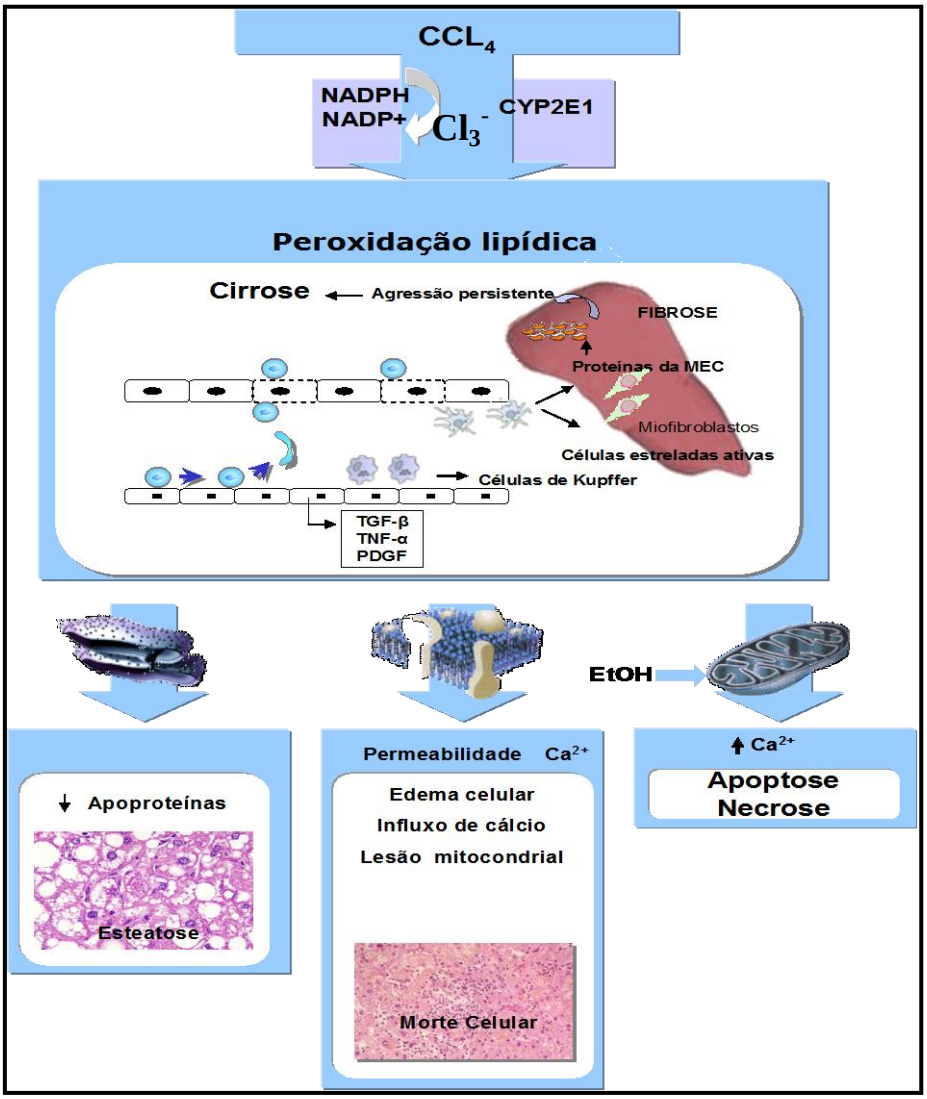
Os modelos experimentais podem ser obtidos por métodos tóxicos (Tetracloreto de Carbono – CCl_4, Etanol, Tioacetamida –TAA, Dietilnitrosamina – DMN, D-galactosamina), métodos cirúrgicos (ligação do ducto biliar), por modificações genéticas (camundongos knouckout Abcb4$^{-/-}$) e por uso de agentes infecciosos (esquistossomose mansônica) (CRESPO YANGUAS et al., 2016).

2.2.1 Modelo tóxico induzido pelo tetracloreto de carbono

O tetracloreto de carbono (CCl_4) consiste de um líquido incolor e hidrofóbico. Antes era bastante usado no setor industrial, como solvente químico, pesticida e alvejante. Porém, após a constatação dos malefícios desse composto à saúde humana e ao ambiente, seu uso foi drasticamente reduzido (ATSDR, 2003). Dentre os efeitos deletérios causados pelo CCl_4, alterações hepáticas foram relatadas, tais como esteatose, necrose hepatocelular, fibrose e cirrose. Esses relatos conduziram o uso do CCl_4 para fins experimentais (ATSDR, 2003).

Durante o processo de metabolização, CCl_4 é convertido em radicais livres, tais como o triclorometil (Cl_3^-), através de uma reação de redução catalisada pelo citocromo p450 2E1 (CYP2E1) nos hepatócitos (MEDERACKE, 2013). Este radical é altamente reativo e sua produção resulta na auto-oxidação dos ácidos graxos insaturados presentes nos fosfolipídios (peroxidação lipídica) e consequente lesão de membrana, que culmina em necrose centro-lobular severa (WEBER; BOLL; STAMPFL, 2003). Outra característica evidente na ação hepática do CCl_4 é o acúmulo de moléculas lipídicas no citoplasma de hepatócitos (esteatose), resultante da inibição na produção de apoproteínas (proteínas carreadoras de lipídios). Esta toxina também provoca danos ao retículo endoplasmático, o que compromete diretamente a produção de proteínas. As lesões na membrana plasmática provocadas pelos radicais livres Cl_3^- resultam em influxo de íons cálcio para o interior das células hepáticas e conseqüente tumefação celular, o que pode levar o hepatócito à morte (Figura 3) (STARKEL; LECLERCQ, 2011).

Figura 3- Mecanismo de ação do CCl_4 no fígado.



Fonte: Adaptado de Oliveira (2007).
Nota: A metabolização do CCl_4 produz radicais livres, tais como o Cl_3^-, que induzem a peroxidação lipídica, o que resulta em lesão na membrana de hepatócitos. Este dano tecidual estimula uma resposta de reparo, com recrutamento de células inflamatórias e produção de MEC, que se acumula no parênquima hepático mediante estímulo contínuo.
Legenda: CCl_4 - tetracloreto de carbono; Cl_3^-triclorometil; MEC-matriz extracelular

O CCl_4 é um agente tóxico de ação rápida, provocando alterações morfológicas aparentes em 15 minutos. Uma única dose de CCl_4 leva a uma necrose centrizonal aguda reversível. A fibrose hepática desenvolve-se progressivamente, durante administrações repetitivas de CCl_4, iniciando em áreas pericentrais, seguido pela fibrose severa (DOMENICALI et al., 2009). O grau de lesões pode variar dependendo da espécie, cepa, dose, via e frequência de administração (LIU et al., 2012). Esse modelo tem sido usado em estudos de mecanismos que regulam a reversibilidade da fibrose hepática (KHAN et al., 2015; TROEGER et al., 2012).

Para o estabelecimento de um modelo de lesão hepática, o CCl_4 tem sido administrado isolado ou combinado a outras substâncias hepatotóxicas, como o etanol por exemplo, e por vias de administração distintas (oral, inalatória, intraperitoneal, subcutânea) (CONSTANDINOU; HENDERSON; IREDALE, 2005).

2.2.2 Modelo parasitário induzido pela esquistossomose mansônica

A esquistossomose mansônica é uma doença parasitária tropical e constitui um grande problema de saúde pública no Brasil (SOUZA et al., 2011). Trata-se de uma enfermidade causada por parasitos da espécie *Schistosoma mansoni*, no qual a infecção ocorre pela penetração percutânea de cercarias (LICHTENBERGOVA et al., 2008; MILAN; KEIM, 2007). Quando o parasito evolui para sua forma adulta, no sistema vascular do plexo venoso mesentérico, passa a liberar grande quantidade de ovos, muitos dos quais passam para o intestino e são liberados nas fezes (MELO; COELHO, 2011). Contudo, 30% dos ovos ficam presos nos sinusóides hepáticos e no intestino, causando morbidade e mortalidade associada à esquistossomose (HAMS; AVIELLO; FALLON, 2013; WYNN et al., 2004).

Durante o curso da esquistossomose mansônica, duas fases patológicas são descritas: I) A fase aguda, que ocorre entre a primeira e a quarta semana de infecção, pode apresentar sintomatologia variada, desde febre, fadiga, mialgia e eosinofilia até perda de peso e diarréia nos casos mais graves (GRYSEELS, 2012); II) A fase crônica, considerada a condição patológica mais relevante na esquistossomose mansônica e se reflete na resposta imunológica do hospedeiro aos ovos de *S. mansoni*, com a formação do granuloma hepático e da fibrose hepática periportal (GRYSEELS et al., 2006). Em virtude do contínuo estímulo antigênico dos ovos, células do sistema imunológico (células mononucleares, eosinófilos e neutrófilos) são recrutadas para os sítios de lesão, levando à formação de granulomas periovulares e eventualmente fibrose crônica em alguns indivíduos infectados (CARVALHO; MARTINS-FILHO; OLIVEIRA, 2008).

No granuloma maduro, proteínas da MEC e CEHs ativadas tornam-se elementos predominantes, resultando na fibrose. Esta fibrose é mediada pela citocina IL-13, que está envolvida na transdiferenciação das CEHs, responsáveis pela produção de colágeno e fibrogênese nas áreas de granuloma (DE WALICK; TIELENS; HELLEMOND, 2012). Além disso, a deposição maciça de fibras colágenas difusas nos espaços periportais (Fibrose de Symmer’s ou pipestem) também caracteriza a fase crônica da esquistossomose (LAMBERTUCCI; SILVA; VOIETA, 2005).

O uso de modelos animais tem trazido grande contribuição ao conhecimento da imunopatologia da esquistossomose mansônica, nas suas fases aguda e crônica (CHEEVER et al., 2002), bem como ao desenvolvimento de estratégias terapêuticas mais eficazes. No estudo da esquistossomose mansônica, os animais classicamente utilizados têm sido os camundongos (HAMS; AVIELLO; FALLON, 2013). Essa espécie atende ao pré-requisito de um bom modelo, por permitir a reprodução da infecção de forma regular e característica (MACHADO E SILVA et al., 1991). No modelo murino, a esquistossomose mansônica de fase crônica é estabelecida num curto espaço de tempo (cerca de dezesseis semanas), apresentando dois quadros histopatológicos distintos: formação de granulomas isolados e fibrose periportal, uma fibrose semelhante à fibrose de Symmers, que ocorre no homem (ANDRADE, 1987; ANDRADE; CHEEVER, 1993).

Camundongos infectados com *S. mansoni* desenvolvem granulomas no fígado decorrente de uma resposta imunológica contra os ovos do parasita que são depositados no trato portal (BARROS et al., 2014; BOROS, 1989). A patologia clínica em hospedeiros murinos compreende hepato-esplenomegalia, fibrose hepática e formação de ascite que se assemelha ao quadro observado na esquistossomose humana (ABDUL-GHANI; HASSAN, 2010). A esquistossomose murina é geralmente usada para o estudo da imunopatologia da esquistossomose humana e da fibrose hepática associada (WILSON et al., 2007).

2.3 Terapia Celular para as Doenças Hepáticas

Diante da diversidade de estímulos agressores ao fígado, diversas alternativas que possam ter atuação anti-fibrogênica estão sendo avaliadas nos últimos anos (MATA-SANTOS et al., 2014). Tendo em vista a necessidade de novas estratégias terapêuticas mais eficazes e menos invasivas, a medicina regenerativa, através da terapia celular, tem aberto novas possibilidades para o tratamento de hepatopatias crônicas. A terapia celular pode ser definida como a atividade que aplica os princípios da engenharia e das ciências da saúde para a obtenção de populações celulares que mantenham, melhorem ou restaurem as funções de órgãos e tecidos do corpo humano (CARVALHO et al., 2010).

Nesse sentido, a terapia celular consiste em um procedimento de intervenção que introduz células, com potencial terapêutico conhecido nos tecidos danificados, e que tem ajudado no tratamento de muitas doenças (POURNASR et al., 2011). O transplante celular pode se tornar uma alternativa minimamente invasiva com poucas complicações. Estudos experimentais e clínicos têm comprovado a eficiência da terapia celular no tratamento de

cânceres (MIMEAULT; HAUKE; BATRA, 2007), diabetes mellitus, doenças neuro-degenerativas, doenças cardiovasculares, e também tem apresentado resultados promissores frente às enfermidades hepáticas (VOSOUGH et al., 2011).

Nas últimas duas décadas, diversos estudos em terapia celular têm sido conduzidos. Essas pesquisas têm como objetivos identificar as populações celulares mais indicadas para cada doença, o modelo experimental mais apropriado, o número de células a serem transplantadas, a melhor via para a infusão celular e o estado da doença, aos quais será possível obter benefícios para os pacientes hepatopatas (HOULIHAN; NEWSOME, 2008). Na terapia celular, procura-se utilizar células com capacidade de auto-renovação, proliferação e que contribuam para a recuperação do tecido danificado (MURACA, 2011). Diferentes populações celulares têm sido investigadas nos últimos anos.

Para que um tratamento seja considerado eficaz contra as lesões crônicas do fígado, ele deve proporcionar redução nos níveis de fibrose, reestabelecimento do parênquima hepático e consequente recuperação da função hepática. Na busca por populações celulares que apresentem potencial terapêutico frente às lesões do fígado, as células de medula óssea (CMO) tem se mostrado bastante promissoras, sendo a fonte celular mais explorada dos últimos anos (BALDO et al., 2010; TERAI; SAKAIDA, 2011). Estas células são de fácil obtenção e manipulação, além de apresentar baixo risco de rejeição, favorecendo, desta forma, o seu uso no tratamento de doenças do fígado (LODI; IANNITTI; PALMIERI, 2011).

A medula óssea é um órgão heterogêneo e tem sido considerada pela medicina regenerativa uma rica fonte de células com potencial terapêutico (CHO et al., 2012). Ela é composta por vários tipos de células, incluindo células progenitoras adultas, células-tronco mesenquimais, células progenitoras endoteliais e células-tronco hematopoiéticas. Trabalhos têm descrito a importante contribuição dessas populações celulares na regeneração hepática, através de processos de transdiferenciação, efeitos parácrinos e atividades anti-fibróticas (ALISON; ISLAM; LIM, 2009; HOULIHAN; NEWSOME, 2008).

Estudos experimentais têm demonstrado os efeitos do transplante de CMO frente à lesão hepática crônica. Em ensaios *in vitro*, foi possível observar que CMO podem se diferenciar em células semelhantes à hepatócitos, em termos funcionais, quando expostas ao microambiente adequado (CHEN et al., 2007; SCHWARTZ et al., 2002). Esses estudos também evidenciaram a presença de marcadores específicos de hepatócitos nas CMO induzidas à diferenciação (POURNASR et al., 2011).

Em estudos *in vivo* foi possível observar, em modelos murinos de lesão hepática, diversos efeitos benéficos do transplante de CMO. Essas pesquisas têm sugerido que as

células transplantadas migram para as áreas de lesão e contribuem para o aumento dos níveis de albumina sérica (ALI; MASOUD, 2012) e da sobrevida dos animais submetidos ao transplante (BELARDINELLI et al., 2008; DE FREITAS SOUZA et al., 2012; SHIZHU et al., 2012), além de induzir a formação de novos hepatócitos no fígado de camundongos com lesão hepática (SHIZHU et al., 2012). Esses novos hepatócitos podem ter se originado de alguma célula precursora medular (PAREKADDAN et al., 2007) ou a partir da diferenciação de células ovais, uma população de células indiferenciadas presentes no fígado e que estão envolvidas na regeneração hepática (SAKAIDA, 2008).

Com base nos estudos experimentais, novas propostas foram feitas a respeito do papel terapêutico das CMO nas hepatopatias crônicas. Um estudo desenvolvido por Oliveira et al. (2012) indicou a contribuição das CMO transplantadas na redução nos níveis de fibrose hepática, em modelo murino de cirrose hepática induzida pelo CCl_4. A melhora hepática foi associada à diminuição dos níveis de galectina-3 no tecido hepático, uma proteína diretamente envolvida na indução da resposta pró-fibrogênica (OLIVEIRA et al., 2012).

A diminuição do tecido fibroso observada em animais com lesão hepática crônica submetidos à terapia com CMO foi associada a um menor número de CEHs ativadas observada pela redução da expressão de α-SMA, sugerindo que a população celular infundida tenha participação na indução da apoptose ou da inativação das CEHs (TANIMOTO et al., 2013). Em outras investigações, nos quais foram utilizadas CMO obtidas de camundongos transgênicos GFP positivos cultivadas para expansão, a diminuição na deposição de colágeno foi relacionada ao aumento na expressão de MMP-9 em camundongos cirróticos submetidos ao transplante celular (IWAMOTO et al., 2013) e em camundongos BALB/c-Abcb4$^{-/-}$, modelo transgênico de fibrose hepática (RODERFELD et al., 2013).

Alterações nos níveis de diversos sinalizadores fibrogênicos também são observadas em estudos de terapia com CMO em modelo experimental de lesão hepática. Alguns resultados demonstram que, a diminuição da fibrose está associada à diminuição de TGF-ß1 em modelo murino de esquistossomose (OLIVEIRA et al, 2008) e de lesão hepática crônica induzida por CCl_4 em ratos (SUN et al., 2011). Tanimoto et al. (2013) também observaram diminuição nos níveis de TGF-ß1 e do Fator de necrose tumoral – alfa (TNF-α), após o transplante de CMO humanas em camundongos cirróticos.

Estudos de fase clínica têm sido conduzidos, com resultados promissores. Salama e colaboradores (2010) desenvolveram uma pesquisa, no qual 48 pacientes em estágio final de lesão hepática crônica foram submetidos ao transplante autólogo de células da medula óssea. A terapia promoveu melhora da função hepática, com diminuição dos sinais clínicos

(SALAMA et al., 2010). Em outra pesquisa, pacientes com cirrose hepática foram submetidos ao transplante autólogo de CMO via artéria hepática, e apresentaram aumento nos níveis de albumina sérica e diminuição nos níveis de bilirrubina, o que indica melhora na função do fígado, sem constatação de efeitos colaterais (LYRA et al., 2007). Pacientes diagnosticados com cirrose alcoólica submetidos ao transplante celular com CMO tiveram níveis de transaminases (ALT e AST) e de bilirrubina diminuídos, o que indica diminuição de lesão hepática (PAI et al., 2008). A ativação de células progenitoras do fígado também foi relatada em pacientes com cirrose submetidos ao transplante autólogo de CMO (KIM et al., 2010).

Esses resultados citados acima demonstram que as CMO são uma ferramenta promissora no tratamento das hepatopatias. Porém os mecanismos envolvidos na melhora hepática após o transplante celular não estão totalmente esclarecidos. A utilização de populações celulares heterogêneas (medula óssea) dificulta a compreensão dos fenômenos pós-terapia (HOULIHAN; NEWSOME, 2008). A possibilidade de identificar uma população celular que esteja diretamente envolvida na melhora tecidual hepática é fundamental para o aprimoramento desta modalidade terapêutica.

2.4 Monócitos/macrófagos na terapia celular

A terapia com CMO tem mostrado bons resultados frente às lesões do fígado. Os benefícios pós-terapia podem estar relacionados à presença de monócitos na camada mononuclear da medula óssea. Os monócitos representam 5-10% dos leucócitos do sangue periférico e se originam a partir de precursores mieloides da medula óssea (TACKE, 2012). São definidos como células circulantes precursoras de macrófagos e de células dendríticas, e estão presentes no sangue, na medula óssea e no baço (JUNQUEIRA; CARNEIRO, 2008).

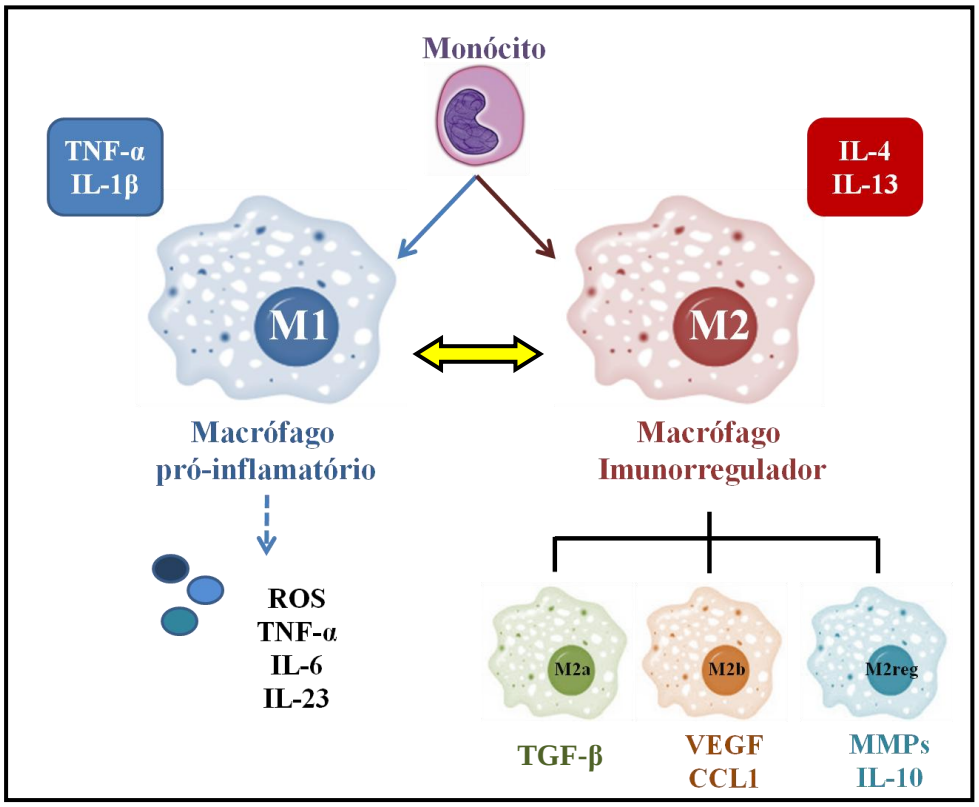
Os macrófagos, a forma madura dos monócitos, têm papel fundamental na resposta imune inata, e distribuem-se amplamente nos tecidos (osteoclastos – macrófagos do tecido ósseo; células de Kupffer – macrófagos do fígado; micróglias - macrófagos do tecido nervoso) (GORDON, 1995). A participação dos macrófagos em diversos processos biológicos e patológicos tem sido tema de estudo de muitos grupos de pesquisa nos ultimos anos. Essa plasticidade funcional dos macrófagos é dirigida pelo microambiente imunológico, podendo ser ativados por diferentes vias (MORI et al., 2009). Estudos intensivos com modelos experimentais de lesão hepática e com pacientes hepatopatas têm buscado elucidar e estabelecer dicotomias que descrevem a complexa heterogeneidade de subconjuntos de monócitos e macrófagos no fígado (JU; TACKE 2016). Monócitos e macrófagos podem ser

distinguidos no fígado com base em sua origem, diferenciação/polarização, pela expressão de marcadores específicos e pelas suas funções durante condições fisiológicas e patológicas (JU; TACKE, 2016).

A heterogeneidade dos macrófagos é dirigida por uma grande diversidade de citocinas liberadas, marcadores da superfície celular e perfis transcricionais (TACKE, 2012). Os macrófagos ativados diferenciam-se em vários subtipos diferentes, que têm funções distintas desde a inflamação e a fibrogênese até à resolução da fibrose (LICHTNEKERT et al., 2013). Em resposta à vários sinais, os macrófagos podem sofrer polarização pela via clássica M1 ou via alternativa M2 (Figura 4). O fenótipo M1, também conhecido como macrófago pró-inflamatório, é induzido por citocinas de perfil Th1, caracterizado pela elevada expressão de mediadores pró-inflamatórios (TNF-α, IL-1 β, IL-12 e IL-23) e alta produção de espécies reativas de oxigênio (ROs), com forte atividade microbicida e tumoricida; O fenótipo M2 ou imunorregulador está envolvido no remodelamento tecidual, na resposta a infecções parasitárias e alergias, através da secreção de fatores imuno-modulatórios (BRAGA; AGUDELO; CAMARA, 2015; GORDON; MARTINEZ, 2010). Investigações mais recentes afirmam que os macrófagos também podem sofrer alterações em seus fenótipos, passando de M1 para M2 e vice-versa (SICA; MANTOVANI, 2012).

Com base em estudos *in vitro*, investigações recentes têm classificado os macrófagos M2 em três subgrupos: M2a, ou macrófago pró-fibrótico, induzido por IL4/IL-13; M2b, ou angiogênico; M2c/M2reg, induzidos por IL-10, envolvidos na inativação de miofibroblastos e na promoção de efeitos anti-inflamatórios (DUFFIELD et al., 2013; LEE et al., 2014; LICHTNEKERT et al., 2013). Esses subtipos de macrófagos estão associados à regulação imune, tolerância e reparo tecidual (TRABER; ZOME, 2013).

Figura 4- Perfis de ativação dos monócitos/macrófagos.



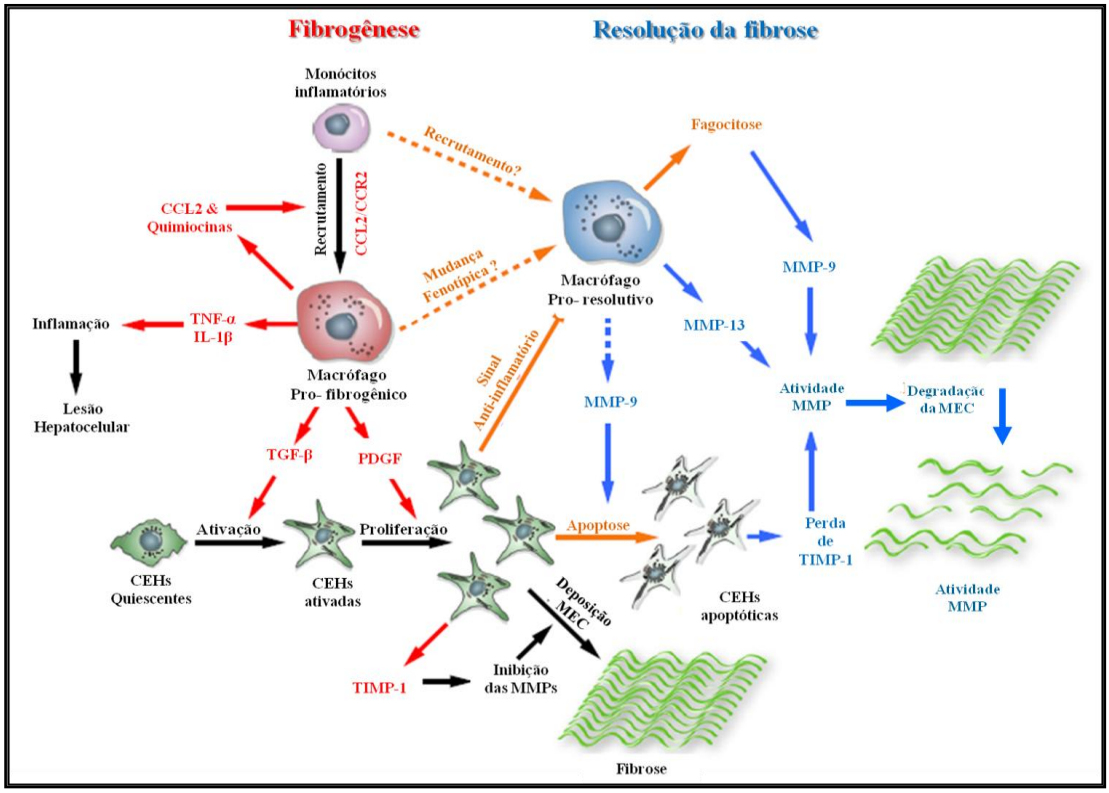
Fonte: Autora
Nota: Perfil de ativação clássica (M1), envolvido na resposta inflamatória, através da expressão de TNF-α, IL-1β, IL-12 e IL-23, e alta produção de ROs; Perfil de ativação alternativa (M2), envolvido no reparo tecidual, infecções parasitárias e alergias, através da secreção de TGF-β, VEGF, CCL1, MMPs e IL-10.
Legenda: TNF-α- Fator de necrose tumoral-alfa; IL-1β-Interleucina-1beta; IL-12- interleucina-12; IL-23-interleucina-23; ROs- espécies reativas de oxigênio; TGF-β-fator de cerscimento e transformação-beta; VEGF-fator de crescimento vascular endotelial; CCL1-quimiocina ligante-1; MMPs-metaloproteinases de matriz; IL-10-interleucina-10.

Em relação às doenças do fígado, subtipos funcionais de macrófagos também são designados, sendo classificados em: macrófagos inflamatórios, que promovem inflamação e fibrose hepática, ativação via receptores Tool-like (TLR), com liberação de citocinas inflamatórias (TNF, IL-1β), pró-fibrogênicas (TGF-β) e quimiocinas (MCP-1, CCL5); e macrófagos restauradores, envolvidos na resolução da inflamação (citocinas anti-inflamatórias) e da fibrose hepática (MMP-9, MMP-12, MMP-13), com baixa expressão da molécula de superfície antígeno de linfócito 6C (Ly6C), em camundongos (JU; TACKE, 2016; RAMACHANDRAN et al., 2012).

A compreensão do diferentes fenótipos de macrófagos, bem como de suas respectivas vias de ativação pode contribuir para o desenvolvimento de novas abordagens terapêuticas para várias doenças, inclusive às enfermidades hepáticas (Figura 5). Alguns estudos demonstram que populações de macrófagos podem desempenhar função protetora, participando ativamente do reparo hepático (WYNN; BARRON, 2010). Outros trabalhos têm

sugerido que subpopulações de macrófagos hepáticos assumem um papel anti-fibrótico em modelo experimental de lesão hepática crônica, expressam várias MMPs (MMP-9, MMP-12 e MMP-13) que estão envolvidas na degradação da MEC, favorecendo assim a resolução da fibrose hepática (FALLOWFIELD et al., 2007; PELLICORO et al., 2012). Além disso, os macrófagos podem participar na indução da apoptose das CEHs (RAMACHANDRAN; IREDALE, 2012).

Figura 5- Participação dos macrófagos na fibrogênese e na resolução da fibrose hepática.



Fonte: Adaptado de Ramachandran e Iredale (2012).
Nota: Subtipos de macrófagos podem desempenhar papel fibrogênico, participando da ativação das CEHs, ou assumindo papel anti-fibrótico, pela expressão de várias MMPs.
Legenda: CCL2-quimiocina ligante-2; TNF-α- Fator de necrose tumoral-alfa; IL-1β-Interleucina-1beta; TGF-β-fator de cerscimento e transformação-beta; PDGF-Fator de crescimento derivado de plaquetas; CEH-células estreladas hepáticas; MMPs-metaloproteinase; MMPs-metaloproteinases de matriz; TIMP-1: inibidor tecidual de metaloproteinase-1; MEC-matriz extracelular.

Algumas evidências têm reforçado o papel fundamental dos macrófagos na resolução da fibrose hepática. Em modelo murino de lesão hepática induzida pelo CCl_4, a depleção de macrófagos durante as fases de recuperação leva ao impedimento da degradação de fibras colágenas da MEC (DUFFIELD et al., 2005). O estudo conduzido por Yang e colaboradores (2014) mostrou que macrófagos derivados de monócitos circulantes são recrutados para o fígado durante a fase inflamatória da lesão e representam uma importante fonte de MMPs. Em

modelo murino de fibrose hepática induzida por ligação do ducto biliar, foi observado que durante a inflamação, os macrófagos atuam na fagocitose dos detritos celulares, removendo assim potencias sinais pró-inflamatórios, o que altera consequentemente seu fenótipo, e dessa forma induz o aumento a expressão de MMPs e a degradação da MEC (POPOV et al., 2010). Em modelo parasitário também foi possível avaliar a importante atuação dos macrófagos na resolução da fibrose hepática. Foi observado que macrófagos localizados na periferia dos granulomas de fígados de animais com esquistossomose apresentam propriedades resolutivas em relação ao tecido fibroso, com participação efetiva no remodelamento da cicatriz (CHUAH et al., 2013).

Diante do conhecimento da importante participação de subpopulações de macrófagos no reparo tecidual, pode-se considerar que as células da linhagem monócito/macrófago possam ser fortes candidatas à terapia celular para as doenças do fígado. Além disso, a possibilidade de obtenção deste tipo celular de uma fonte facilmente acessível reforça sua possível utilização para transplantes autólogos (ZHAO; GLESNE; HUBERMAN, 2003). Ensaios *in vitro* demonstraram que monócitos cultivados em meio de cultura condicionado com fator de crescimento hepático (HGF) originaram células funcionalmente semelhantes à hepatócitos, com capacidade bioquímica e metabólica equivalentes às células hepáticas originais (EHNERT et al., 2011; RUHNKE et al., 2005a; RUHNKE et al., 2005b; ZHAO; GLESNE; HUBERMAN, 2003). Em outra pesquisa, monócitos purificados de sangue periférico de pacientes portadores do vírus da hepatite B e diagnosticados com insuficiência hepática foram mantidos em cultura associados a fatores específicos. Nesse experimento, foi possível observar a habilidade de diferenciação dessas células em hepatócitos funcionalmente ativos (YAN et al., 2007).

Um estudo experimental realizado por Thomas e colaboradores (2011) demonstrou que, camundongos com lesão hepática crônica induzida pelo CCl_4 submetidos ao transplante de macrófagos obtidos através de cultura condicionada de monócitos apresentaram nítida melhora nos aspectos morfológicos, com diminuição nos níveis de fibrose, aumento da regeneração hepática e da apoptose das CEHs. No entanto, algumas questões do estudo permanecem inconclusivas, como por exemplo, qual fenótipo de macrófagos é mais predominante na melhora hepática.

Os estudos em terapia com monócitos/macrófagos vêm se destacando frente a diversas doenças crônico-inflamatórias, o que leva à necessidade de novas abordagens sobre o real potencial terapêutico dessa população celular, principalmente para o tratamento de enfermidades para as quais as opções terapêuticas existentes são limitados e/ou ineficazes.

3 JUSTIFICATIVA

As doenças crônicas do fígado são responsáveis por um considerável número de atendimentos e internações hospitalares com índice crescente de risco de morte pelos pacientes. Os tratamentos existentes são limitados e diferenciados, a depender da causa da doença e/ou persistência do estímulo. Quando as terapias vigentes não são eficientes, os pacientes podem evoluir para a insuficiência hepática, sendo o transplante hepático o tratamento mais indicado. A baixa disponibilidade de doadores de órgãos, o uso de drogas imunossupressoras e existência de co-morbidades podem contra indicar o transplante justificando a importância de novas estratégias terapêuticas que melhorem a função hepática dos pacientes.

Um possível caminho é a utilização da terapia celular através da medicina regenerativa. Dentro deste contexto, nosso grupo vem realizando estudos com células obtidas da medula óssea, demonstrando, através de ensaios pré-clínicos, uma melhora tecidual e dos parâmetros bioquímicos nos animais, após terapia celular. Tais achados incentivaram a condução de estudos clínicos com pacientes hepatopatas, Os resultados obtidos foram satisfatórios, no qual o procedimento de infusão de células-tronco mononucleares de medula óssea mostrou-se seguro e exeqüível, sendo possível avaliar também melhora das funções hepáticas desses pacientes após terapia.

Novos estudos, capazes de identificar quais populações dentre as células presentes na camada mononuclear da medula óssea estão diretamente envolvidas na regeneração hepática, se fazem necessários. Pretendeu-se nesse trabalho avaliar os macrófagos, devido ao potencial de diferenciação dessas células e sua capacidade de sintetizar e secretar mediadores químicos capazes de contribuir para melhora morfofisiológica do tecido hepático. Os macrófagos constituem uma população de células bastante heterogênea que podem ser classificadas em diferentes fenótipos que se relacionam antagonicamente com diversos processos patológicos. Desta forma, buscamos avanços tecnológicos que melhorem a qualidade de vida dos pacientes com hepatopatias agudas ou crônicas.

4 HIPÓTESE

Os monócitos da medula óssea assumem um perfil anti-fibrogênico e apresentam potencial terapêutico frente às lesões crônicas do fígado, contribuindo para a diminuição nos níveis de fibrose hepática.

5 OBJETIVOS

5.1 Objetivo Geral

Avaliar o potencial terapêutico de monócitos obtidos de medula óssea e as vias de ativação dos macrófagos no reparo hepático em modelos murinos de hepatopatias crônicas.

5.2 Objetivos Específicos

a) Avaliar os efeitos do transplante de monócitos sobre a fibrose em modelos experimentais de lesão hepática crônica;
b) Avaliar as células estreladas ativadas no tecido hepático dos animais com lesão crônica de fígado após terapia;
c) Avaliar vias de imuno-modulação do reparo hepático após terapia com monócitos de medula óssea;
d) Quantificar reguladores das vias fibrogênicas e das vias de ativação de macrófagos após o transplante de células monocíticas;
e) Avaliar os níveis de expressão de marcadores da fibrogênese e de perfis de macrófagos no fígado dos animais submetidos à terapia.

6 MATERIAIS E MÉTODOS

6.1 Animais

Cento e vinte Camundongos isogênicos C57BL/6, de ambos os sexos, pesando entre 20 e 23g, foram obtidos do Centro de Criação de Animais de Laboratório (CECAL- Fundação Oswaldo Cruz, Rio de Janeiro, Brasil). Os animais foram mantidos no Biotério Experimental do IAM/FIOCRUZ-Recife, e o protocolo de experimentação animal foi desenhado pra minimizar o desconforto aos animais, que foram mantidos em condições ideais de temperatura (± 23ºC) e luminosidade (ciclos de 12h claro/escuro), recebendo água e dieta *ad libitum*.

6.2 Indução de lesão hepática crônica por intoxicação pelo CCl_4

O modelo experimental de lesão hepática crônica foi induzido em camundongos C57BL/6 isogênicos pela administração, por via orogástrica, de CCl_4 a 20%, diluído em azeite de oliva, duas vezes por semana (OLIVEIRA et al., 2012). Os animais receberam ainda etanol (EtOH 5%) diluído na água de beber *ad libitum*. O estímulo foi mantido por um período de seis meses.

6.3 Infecção pelo *Schistosoma mansoni*

A infecção foi realizada em camundongos C57BL/6 isogênicos, por via percutânea, com cerca de 40 cercárias de *S. mansoni* obtidas da cepa LE (Belo Horizonte). Após 45 dias, foi realizado o exame parasitológico das fezes para confirmação da infecção. Um período de 16 semanas foi necessário para o estabelecimento da fase crônica infecção esquistossomótica. Os animais infectados foram então submetidos ao tratamento quimioterápico com Praziquantel (dose única, via oral – 400mg/Kg), para a eliminação dos vermes adultos.

6.4 Isolamento de monócitos de medula óssea

A medula óssea foi obtida de fêmures e tíbias de camundongos doadores (n=50), da linhagem C57BL/6, e a fração enriquecida com células mononucleares de medula óssea (CMMO) foi obtida por centrifugação em gradiente de Ficoll (Histopaque 1119 e 1077, Sigma Aldrich, St Louis, MO, USA), a 1000xg, por 15 minutos, a 25°C. Uma parte da

suspensão de CMMO foi utilizada para o isolamento de monócitos através de um sistema de separação imunomagnética. Para isso, as CMMO (aproximadamente 10^7 células/mL) foram incubadas com anticorpos anti-CD11b conjugados a microesferas magnéticas (MACS units, Milteny Biotec™) durante 15 minutos. Em seguida, as células foram lavadas e passadas por uma coluna magnética (MACS, Milteny Biotec™), onde os monócitos $CD11b^+$ foram retidos e então recuperados em tampão (PBS/BSA 0,5% + 2 EDTA mM, pH = 7,2). Por fim, os monócitos $CD11b^+$ foram lavados e ressuspendidos em salina estéril 0,9%, e posteriormente infundidos nos animais.

6.5 Caracterização da população de monócitos por citometria de fluxo

As CMMO e os monócitos obtidos por separação imunomagnética foram incubados com os seguintes anticorpos: anti-CD11b (PE rat anti-mouse CD11b, M1/70 clone, BD Pharmingen™), anti-CD14 (FITC rat anti-mouse CD14, rmC5-3 clone, BD Pharmingen™), anti-CD45 (APC rat anti-mouse CD45, 30-F11 clone, BD Pharmingen™), anti- CD34 (PE rat anti-mouse CD34, RAM34 clone, BD Pharmingen™), anti-Ly6A (FITC rat anti-mouse Ly-6A/E, D7 clone, BD Pharmingen™). Após 30 minutos de incubação, as células foram lavadas com 2 mL PBS wash (PBS BSA 0,5% azida sódica 0,1%, pH 7,2), centrifugadas a 400xg por 5 minutos e então ressuspendidas em 300µl de PBS wash. As amostras foram então caracterizadas fenotipicamente por citometria de fluxo, (FACS Calibur, BD Biosciences, San Jose, CA). O mínimo de 10.000 eventos/amostra foi coletado

6.6 Grupos experimentais

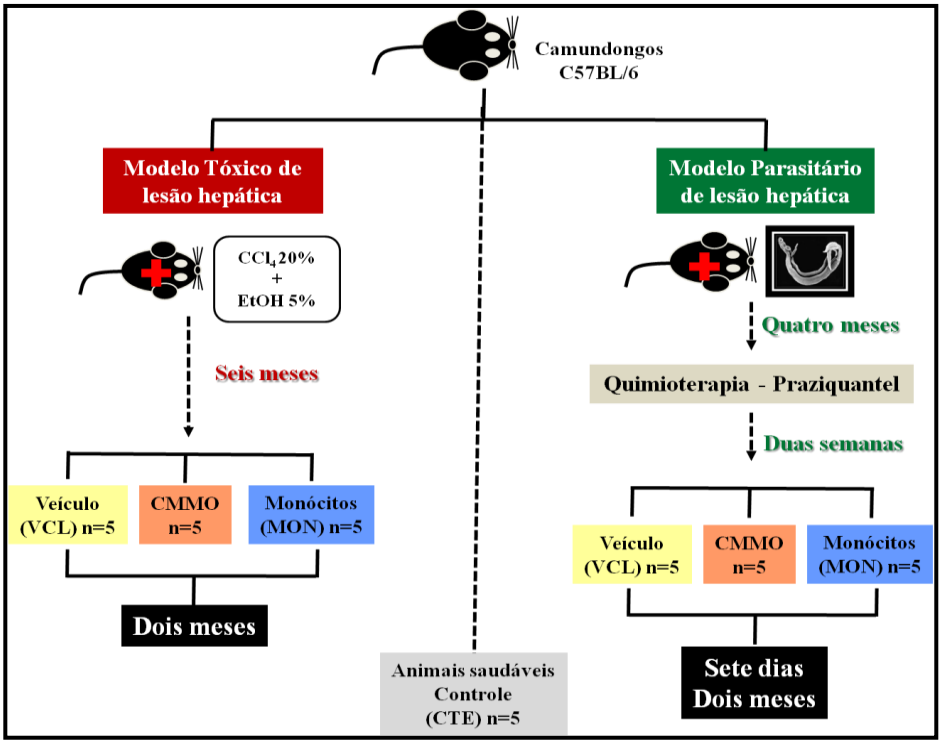
Após o estabelecimento dos modelos de lesão hepática (tóxico e parasitário), os animais foram randomicamente distribuídos nos seguintes grupos (figura 6):

a) Modelo tóxico (CCl_4): Animais sem tratamento, que receberam salina estéril (veículo - VCL); animais tratados com CMMO; e animais tratados com monócitos $CD11b^+$ $CD14^+$ (MON);

b) Modelo parasitário (esquistossomos mansônica): Animais sem tratamento, que receberam salina estéril (Veículo -VCL); animais tratados com CMMO; animais tratados com monócitos $CD11b^+$ $CD14^+$ (MON).

Um grupo de animais sadios também foi formado no desenho experimental (grupo controle - CTL).

Figura 6- Fluxograma do desenho experimental.



Fonte: autora
Nota: Camundongos C57BL/6 foram utilizados para o estabelecimento dos modelos tóxico e parasitário de lesão hepática crônica. Esses animais foram distribuídos randomicamente em grupos experimentais, submetidos àadiferentes modaliddes de terapia celular: Veículo, CMMO e monócitos. Em modelo tóxico, a avaliação foi realizada dois meses após o transplante celular. No modelo parasitário, os efeitos pós-terapia foram obsevados sete dias e dois meses após a infusão celular. Um grupo controle com animais saudáveis foi mantido durante todo o experimento.
Legenda: CCl_4-tetracloreto de carbono; EtOH-etanol; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos.

6.7 Infusão dos monócitos nos animais com lesão hepática crônica

Os grupos experimentais foram tratados com CMMO e monócitos $CD11b^+$ $CD14^+$ por via endovenosa (10^6 células/animal, diluídas em 200 µL de salina estéril). Os animais receberam tratamento uma vez por semana, durante três semanas consecutivas. Os grupos de animais com lesão hepática crônica induzida pelo CCl_4 foram submetidos à eutanásia dois meses após a terapia. Os camundongos submetidos à infecção esquistossomótica crônica foram eutanasiados sete dias e dois meses após a terapia. Durante a eutanásia, o fígado foi coletado.

6.8 Análise morfológica e morfometria

Fragmentos de fígados dos animais tratados e não tratados foram fixados em formol 10% por 24 horas, embebidos em parafina, seccionados (5 µm) e corados pelo Sírius-

vermelho e Fast green para estudo do colágeno (JUNQUEIRA; CARNEIRO, 2008). As imagens de secções histológicas coradas em Sirius Vermelho foram obtidas em microscópio óptico (DM LB 2, Leica Microsystems) equipado com uma câmera digital LEICA JVC TK (modelo C 1380, Pine Brook, NJ, EUA) e analisadas utilizando o sistema de processamento e análise de imagens LEICA QWIN, versão 2.6 MC (Leica, Cambridge, England). Dez campos microscópicos (aumento 100X) contendo áreas de tecido fibroso foram selecionadas para quantificação. A partir das secções histológicas coradas em Sirius-vermelho foram calculados alguns parâmetros de avaliação dos granulomas hepáticos: densidade numérica (número de granulomas/unidade de volume) e volume dos granulomas. As análises foram feitas em microscópio óptico (DM LB 2, Leica Microsystems).

6.9 Dosagem de hidroxiprolina

Amostras de fígado, obtidas do lobo maior, pesando entre 100 e 200 mg, foram usadas para determinação de hidroxiprolina constituinte do colágeno. As amostras foram processadas e analisadas segundo a metodologia de Bergman e Loxley (1963), lidas em espectrofotômetro automático (Pharmacia, modelo Ultrospec 3000), em densidade óptica de 558 nm, para a obtenção dos valores de hidroxiprolina de cada amostra (nmol).

6.10 Ensaios imunológicos

Fragmentos hepáticos congelados em nitrogênio líquido imediatamente após a coleta (~50mg) foram homogeneizados em um tampão de lise (Tris-HCl 50 mM, NaCl 300 mM, EDTA 5 mM, Triton X-100 1%, Azida sódica 0,02%, pH 7,2) contendo um coquetel de inibidores de protease (Roche, Mannheim, Germany). Os lisados foram centrifugados a 16000xg à 4ºC por 15 minutos, e então os sobrenadantes foram usados para quantificar, através do ELISA sanduíche, os níveis de TNF-α, IL-1β, IL-6 (BD OptEIA set mouse, San Diego, CA, USA), IL-13, IL-17, IL-23, MMP-9, TIMP-1 (R&D Systems, Minneapolis, MN, USA), TGF-β1 e IL-10 (e-Bioscience, San Diego, CA, USA). As amostras foram lidas em um comprimento de onda de 450 nm através de um leitor de microplacas (modelo 3550, Thermo Scientific).

6.11 Avaliação do estresse oxidativo

Para avaliar o estresse oxidativo, o conteúdo de glutationa (GSH) foi quantificado a partir de fragmentos de fígado de camundongos utilizados no estudo (RAHMAN; KODE; BISWAS, 2006). As amostras de tecido hepático foram pesadas, maceradas em solução de ácido metafosfórico 5% e centrifugadas a 12000xg, a 4°C, por 10 minutos. GSH foi detectada utilizando o Glutathione Assay Kit (Sigma Aldrich, St Louis, MO, EUA), e mensurada em um leitor de microplacas (BioRad - 415 nm).

6.12 RT-qPCR

A extração do RNA total de fragmentos hepáticos foi feita por Trizol (Invitrogen). A concentração de RNA total foi quantificada utilizando o espectrofotômetro NanoDrop D-1000, e 5 µg de RNA foi usado para a síntese de cDNA utilizando o GoScript Transcription System (Promega). A PCR quantitativa em tempo real (qPCR) foi feita no ABI Prism 7500 (Applied Biosystems) usando SYBR® Green PCR Master Mix (Applied Biosystems). A sequência dos pares de primes para os genes CCL5, arginase-1 (arg-1), proteína 3 semelhante a chitinase-3 (YM-1), receptor de manose 1 tipo-C (CD206), α-SMA, TGF-β1, Gal-3 e molécula alfa 1 semelhante a resistina (Fizz1) foram desenhados com o auxílio da base de dados NCBI. O desenho dos pares de primers usados para a amplificação dos genes IL-12β (MANUELPILLAI et al., 2012) e CCR2/Ly6C (RAMACHANDRAN et al., 2012) foram obtidos de literatura. A amplificação de β-actina foi usada como controle endógeno. A expressão relativa foi calculada utilizando como referência os valores de CT das amostras de animais sadios e não tratados, através do método ΔΔCT. A sequência dos primers utilizados está listada no quadro 1.

Quadro 1 - Primers utilizados para a RT-qPCR.

Gene	Forward 5' .....> 3'	Reverse 5'.....> 3'
CCL5	CCAGAGAAGAAGTGGGTTCAAG	AGCAATGACAGGGAAGCTATAC
IL-12β	GTAACCAGAAAGGTGCGTTCC	GAACACATGCCCACTTGCTG
Arg-1	CCAGGGACTGACTACCTTAAAC	GAAGGCGTTTGCTTAGTTCTG
YM-1	ACCATGGCCAAGCTCATT	GTCCTTAGCCCAACTGGTATAG
CD206	GTTCACCTGGAGTGATGGTTCTC	GACATGCCAGGGTCACCTTT
Fizz1	ACTTGCAACTGCCTGTGCTTAC	TCAAAGCTGGGTTCTCCACCTC
CCR2	CAAATCAAAGGAAATGGAAGACAAT	GCCCCTTCATCAAGCTCTTG
α-SMA	TCAGGGAGTAATGGTTGGAATG	GGTGATGATGCCGTGTTCTA
TGF-β1	GGTGGTATACTGAGACACCTTG	CCCAAGGAAAGGTAGGTGATAG
Gal-3	CTGAGAGATACCCATCGCTTTG	GTAGCTCAGTGAGAGAACACTT
B-actina	CCGTAAAGACCTCTATGCCAAC	AGGAGCCAGAGCAGTAATCT

Fonte: Autora

6.13 Imunomarcação

A imuno-histoquímica foi conduzida para avaliar as CEHs α-SMA^{+} e OPN. Secções hepaticas (5 μm) foram inicialmente desparafinizadas com xilol; desidratatadas em crescentes concentrações de etanol. Para α-SMA, as secções foram incubadas com anticorpo biotinilado anti-α-SMA (Santa Cruz Biotechnology, Santa Cruz, California, EUA), overnight. Em seguida foi adicionado o conjugado estreptavidina-peroxidase por 10 minutos. Para marcação da OPN, as amostras foram incubadas com o anticorpo primário anti-OPN (AF808, R&D Systems, Minneapolis, MN, USA), como previamente descrito (PEREIRA et al., 2015). O anticorpo secundário foi ligado a um polímero sintético conjugado com peroxidase (HRP, horseradish peroxidase). O substrato 3,3'diaminobenzidina (DAB) foi usado para revelar a marcação. As secções foram contra-coradas com hematoxilina de Harris. A imunomarcação foi mensurada em dez campos por secção (aumento 200X), utilizando um Sistema de Processamento de Análise de Imagem LEICA QWIN, version 2.6 MC (Leica Cambridge, Cambridge – Inglaterra).

6.14 Análise estatística

Os dados foram expressos em valores médios (média ± EP). Inicialmente os dados quantitativos foram submetidos a um teste de normalidade (Shapiro-Wilk), e baseados no valor de P, os dados foram submetidos a um teste paramétrico (ANOVA), ou não-paramétrico (Kruskal-Wallis, com pós-hoc de Dunn). As análises estatísticas foram realizadas utilizando o programa Graphpad Prism (versão 5.0, San Diego, CA, USA). O valor de $P < 0.05$ foi considerado estatisticamente significativo.

7 CONSIDERAÇÕES ÉTICAS

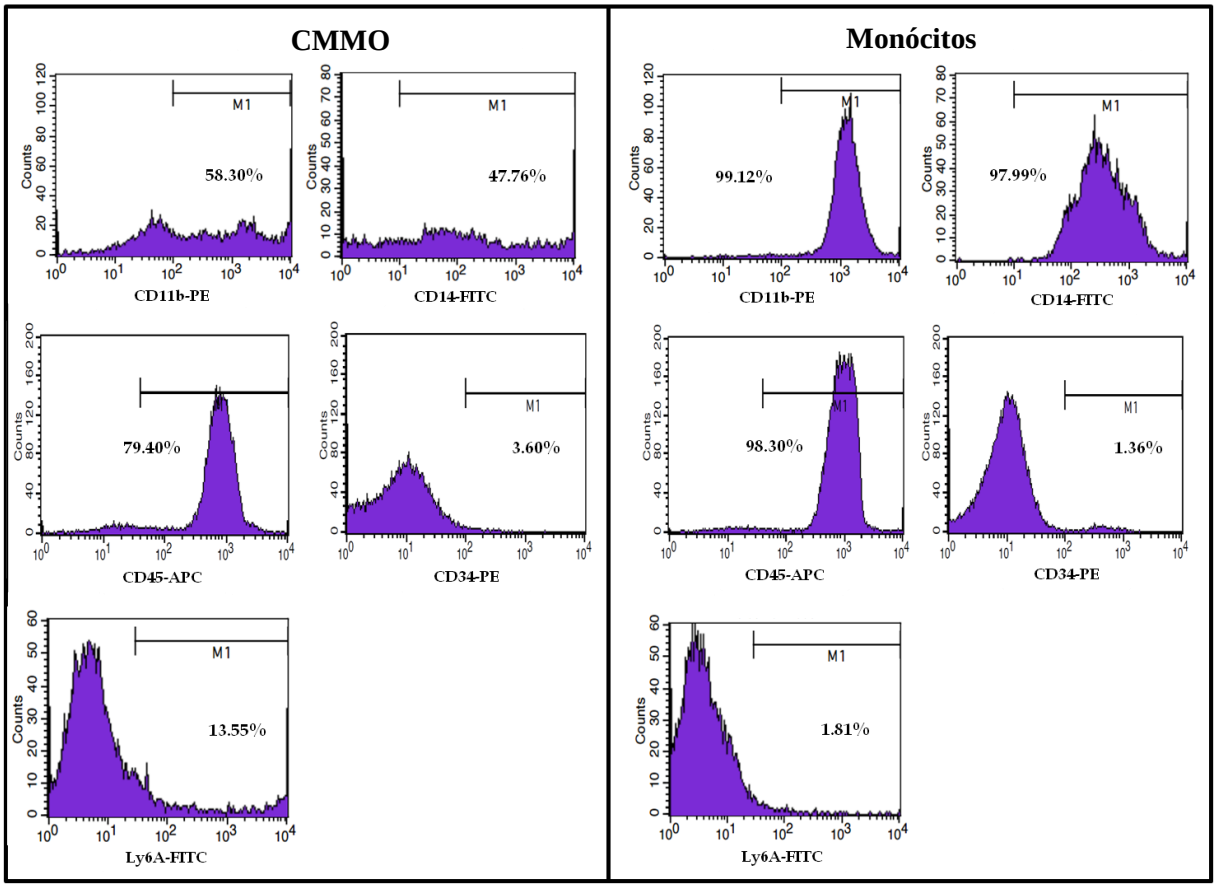
Os procedimentos experimentais realizados no presente estudo estão de acordo com os padrões éticos da Fundação Oswaldo Cruz e foram aprovados pela Comissão de Ética para o Uso dos Animais (CEUA - CPqAM 15/2011 – ANEXOS A e B).

8 RESULTADOS

8.1 Caracterização celular

As CMMO foram caracterizadas como: 58.3% $CD11b^+$; 47.46% $CD14^+$; 79.4% $CD45^+$; 3.6% $CD34^+$ e 13.55% $Ly6A^+$. A população celular obtida por separação Imunomagnética apresentou a seguinte distribuição fenotípica: 99.12% $CD11b^+$; 97.99% $CD14^+$; 98.3% $CD45^+$; 1.36% $CD34^+$ e 1.81% $Ly6A^+$, demonstrando uma enriquecida população homogênea de monócitos na nossa preparação celular. A figura 7 mostra histogramas FACS representativos de CMMO e de monócitos $CD11b^+$ isolados por separação imunomagnética.

Figura 7- Histogramas FACS representativos de CMMO e monócitos $CD11b^+$ isolados por separação imunomagnética.



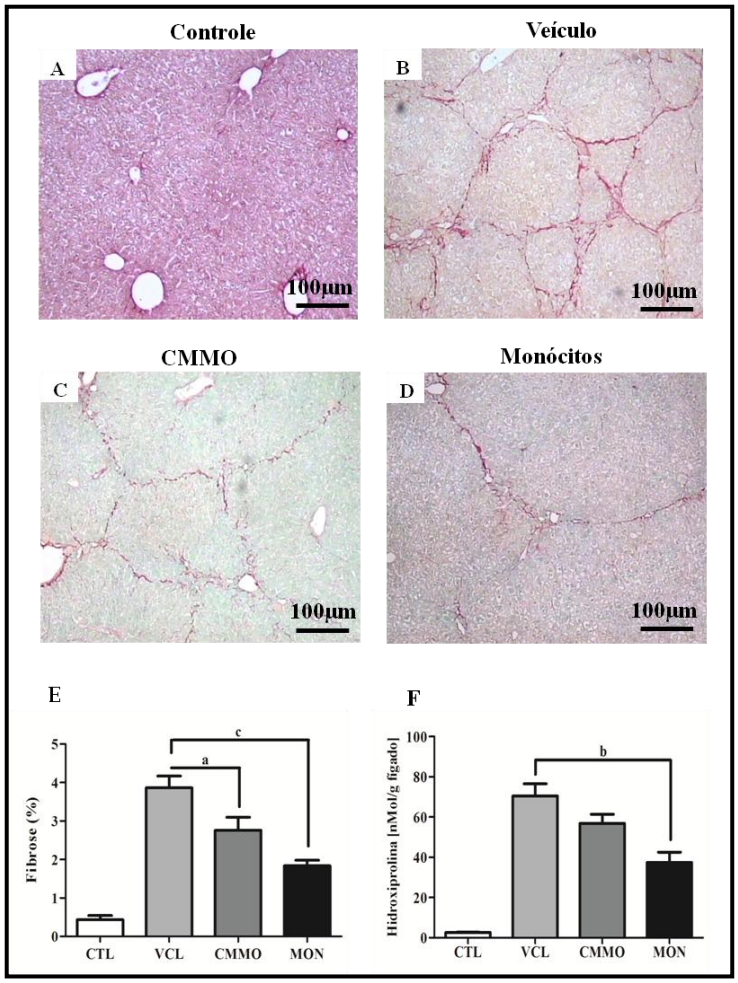
Fonte: autora
Legenda: CMMO-células mononucleares de medula óssea

8.2 Modelo tóxico de lesão hepática crônica

8.2.1 Avaliação da fibrose hepática

A análise morfométrica demonstrou que, dois meses após o transplante celular, houve redução significativa das áreas de fibrose hepática (Figuras 8A-8E) em animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$ comparados aos animais tratados com salina. Essa diminuição também foi observada no grupo de animais tratados com CMMO. Uma significativa redução no conteúdo de hidroxiprolina foi encontrada no grupo submetido à terapia com monócitos (Figura 8F).

Figura 8- Avaliação da fibrose hepática após terapia celular, em modelo tóxico de lesão hepática crônica.

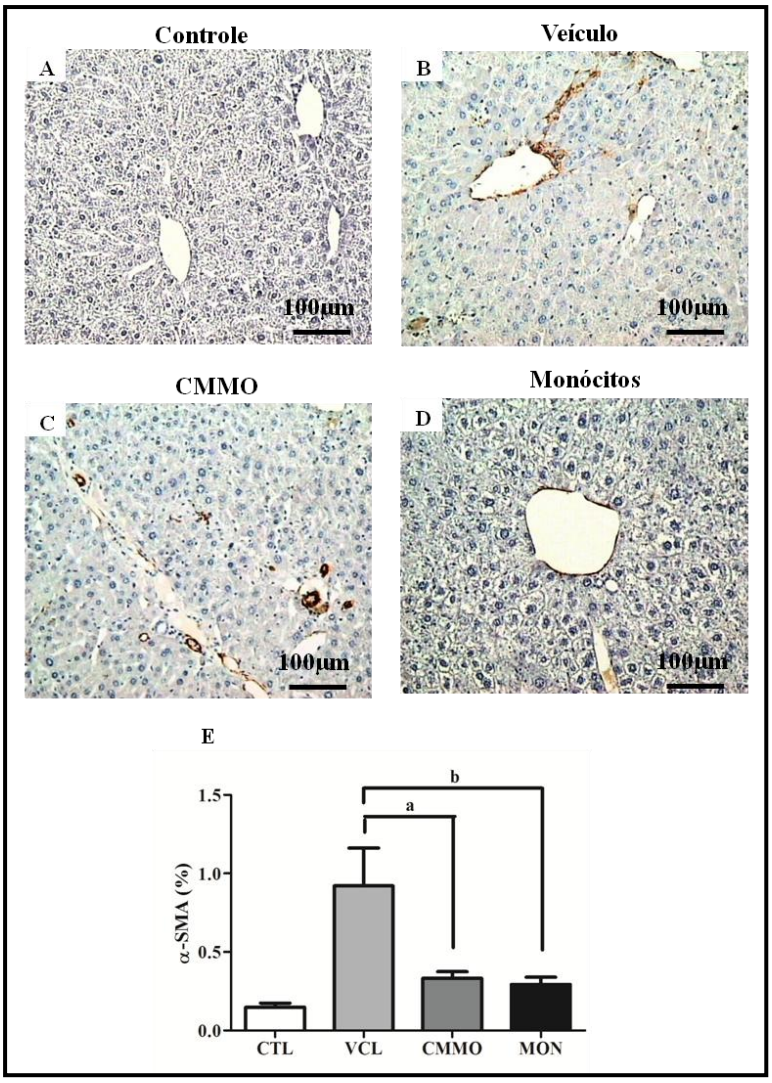


Fonte: Autora
Nota: (A-D) Fotomicrografias de secções histológicas de fígado coradas com picro-sirius, de animais do grupo (A) CTL, e dois meses após a administração de (B) VCL, (C) CMMO e (D) monócitos (100X). (E) Análise morfométrica do colágeo hepático. (F) Concentração de hidroxiprolina em fragmentos hepáticos de animais submetidos ao transplante de monócitos (a = $P < 0.05$; b = $P < 0.01$, c = $P < 0.001$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos.

8.2.2 Imuno-histoquímica

Como demonstrado na figura 9, o percentual de células positivas para α-SMA no parênquima hepático foi reduzido nos camundongos que receberam monócitos $CD11b^+$ $CD14^+$, bem como no grupo tratado com CMMO, comparados ao grupo de animais tratados com salina.

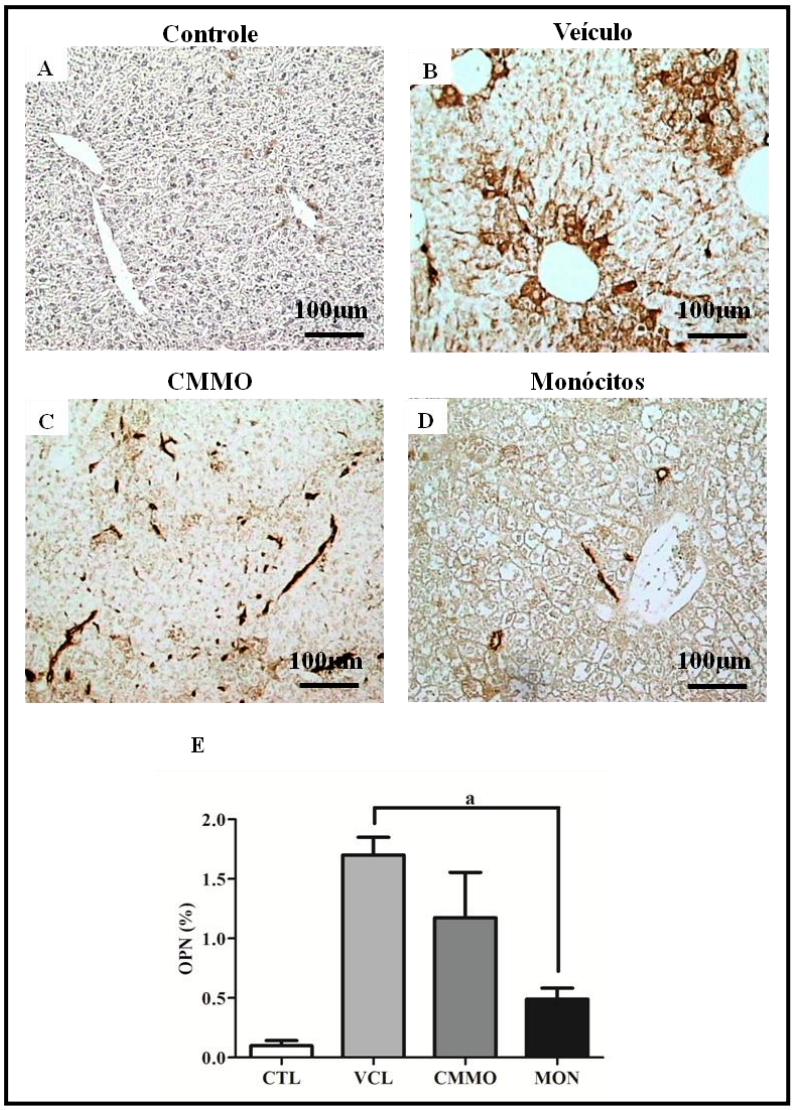
Figura 9- Imuno-histoquímica para a detecção de CEHs α-SMA^+.



Fonte: Autora
Nota: Secções histológicas de animais (A) do grupo CTL, tratados com (B) veículo, (C) CMMO e (D) monócitos (200x). (E) Mensuração de CEHs α-SMA^+ (a = P < 0.05; b = P < 0.01; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; CEH-célula estrelada hepática; α-SMA^+: alfa-actina de músculo liso.

No presente estudo foi possível observar redução na marcação para OPN após terapia com monócitos $CD11b^{+}$ $CD14^{+}$ (Figura10).

Figura 10- Imuno-histoquímica para a detecção de osteopontina.



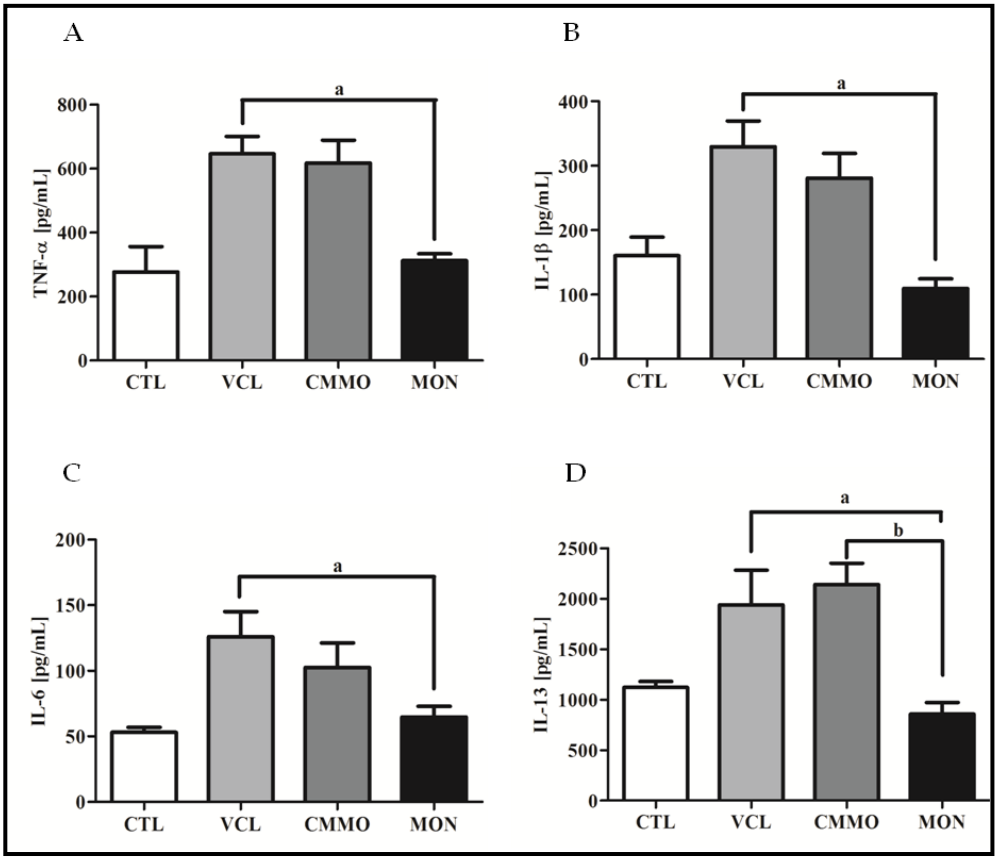
Fonte: Autora
Nota: Secções histológicas de fígado de (A) animais saudáveis, tratados com (B) veículo, (C) CMMO e (D) monócitos (200x). (E) Quantificação da imunomarcação para OPN hepático (a = $P < 0.05$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; OPN-osteopontina.

8.2.3 Avaliação do perfil de citocinas

Para investigar os mecanismos envolvidos na melhora da fibrose hepática após a terapia com monócitos $CD11b^{+}$ $CD14^{+}$, os níveis de mediadores inflamatórios e pró-fibrogênicos foram quantificados. As concentrações de TNF-α (Figura 11A), IL-1β (Figura 11B), IL-6

(Figura 11C) e IL-13 (Figura 11D) em lisados hepáticos foram significativamente reduzidas no grupo de animais tratados com monócitos também apresentaram-se em níveis significativamente menores em animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$, comparados aos camundongos tratados com salina.

Figura 11- Efeitos da terapia com monócitos de medula óssea no perfil hepático de citocinas pró-inflamatórias.

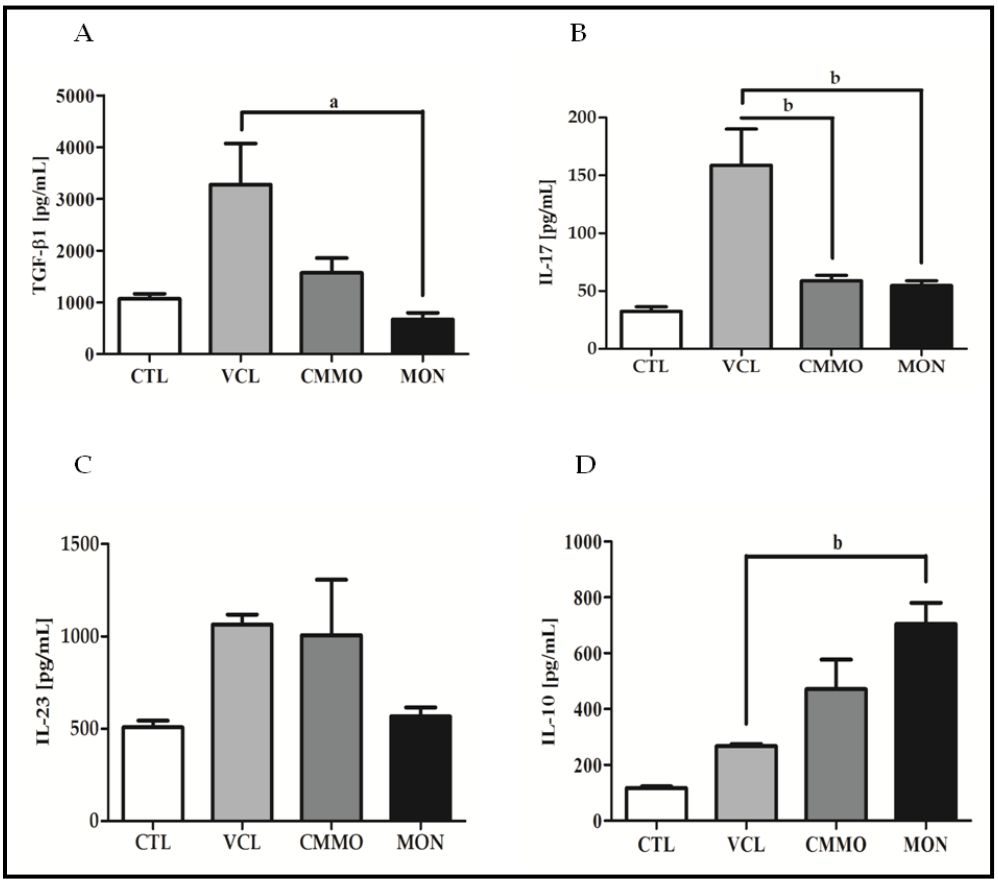


Fonte: Autora
Nota: Níveis hepáticos de (A) TNF-α, (B) IL-1β, (C) IL-6 e (D) IL-13, mensuradas por ELISA sanduíche, em camundongos com lesão hepática crônica induzida por CCl_4. (a = P < 0.05; b = P < 0.01; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; TNF-α- fator de necrose tumoral-alfa; IL-1β-interleucina-1beta; IL-6-interleucina-6; IL-13- interleucina-13.

O mediador pró-fibrogênico TGF-β1 também se apresentou em níveis significativamente menores em animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$, comparados aos camundongos tratados com salina (Figura 12A). A terapia com monócitos $CD11b^{+}$ $CD14^{+}$ também promoveu diminuição significativa nos níveis de IL-17 (Figura 12B) e uma tendência a redução nos níveis hepáticos de IL-23, em relação ao grupo tratado com salina (Figura 12C). Os animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$ também demonstraram aumento

significativo nos níveis de IL-10, em comparação com os animais tratados com salina (Figura 12D).

Figura 12 - Efeitos da terapia com monócitos em camundongos com lesão hepatica crônica nos níveis hepáticos de mediadores da fibrose hepática.



Fonte: Autora
Nota: Níveis hepáticos de (A) TGF-β1, (B) IL-17, (C) IL-23 e (D) IL-10 (a = P < 0.05; b = P < 0.01; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; TGF-β1-fator de crescimento e dtransformação-beta; IL-17-Interleucina-17; IL-23-interleucina-23; IL-10-interleucina-10.

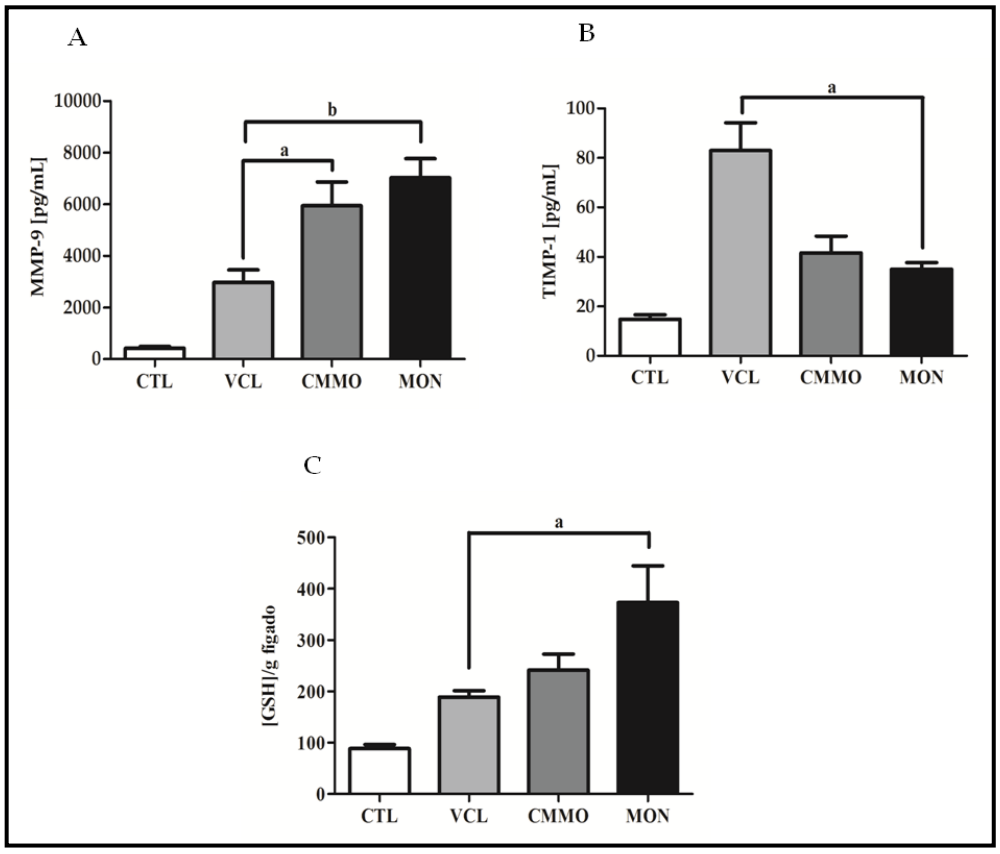
8.2.4 Avaliação de reguladores da fibrogênese hepática

Os níveis de MMP-9 e TIMP-1, dois importantes fatores associados à fibrogênese hepática, foram avaliados. Um aumento significativo na concentração de MMP-9 foi observado nos lisados hepáticos de animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$ e com CMMO (Figura 13A). Os níveis hepáticos de TIMP-1 foram significativamente reduzidos no grupo tratado com monócitos $CD11b^{+}$ $CD14^{+}$ (Figura 13B).

8.2.5 Avaliação do Estresse Oxidativo

Para avaliar a influência da terapia com monócitos $CD11b^+$ $CD14^+$ no estresse oxidativo envolvido na patogênese do modelo tóxico de lesão hepática crônica, os níveis de GSH foram determinados. Os animais com fibrose hepática tratados com monócitos tiveram níveis significativamente maiores desta molécula antioxidante quando comparados aos animais tratados com salina (Figura 13C).

Figura 13- Efeitos da terapia com monócitos de medula óssea nos níveis hepáticos de reguladores da fibrose hepática e do estresse oxidativo.



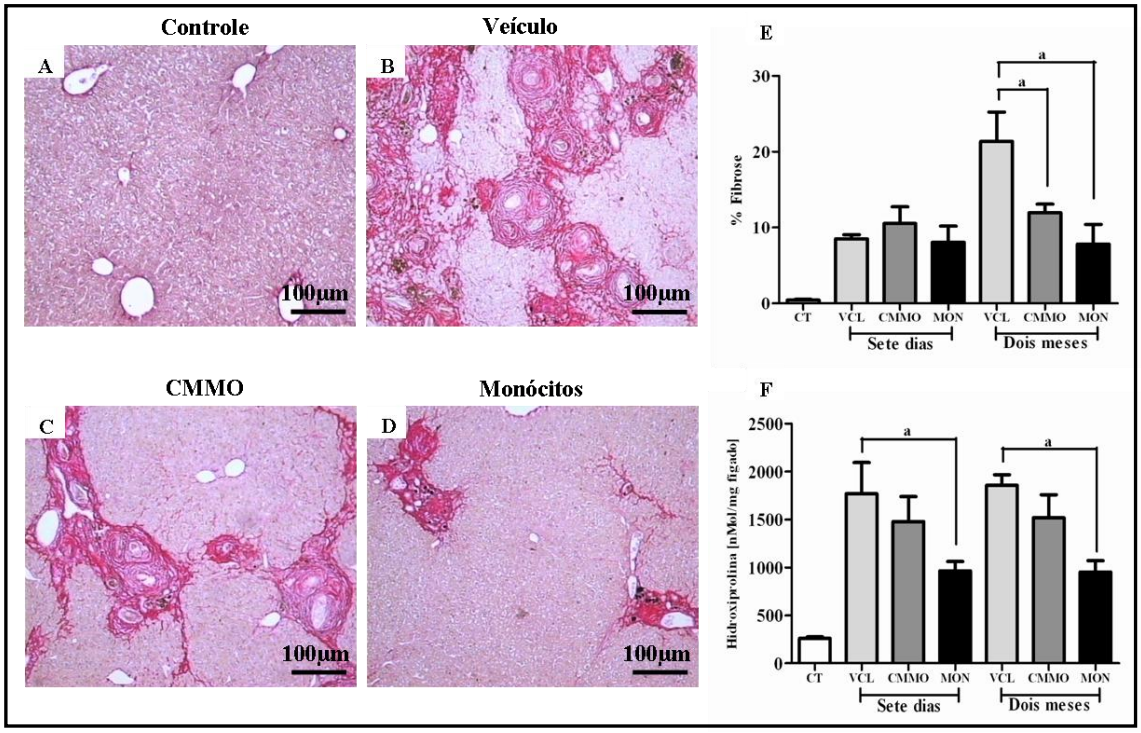
Fonte: Autora
Nota: níveis hepáticos de (A) MMP-9, (B) TIMP-1 e (C) GSH, em camundongos com lesão hepática crônica induzida por CCl_4 (a = $P < 0.05$; b = $P < 0.01$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; MMP-9-metaloproteinase-9; TIMP-1-inibidores teciduais de metaloproteinase-1; GSH-glutationa.

8.3 Modelo parasitário de lesão hepática crônica

8.3.1 Avaliação da fibrose hepática

Em modelo murino de esquitossomose, a quantificação dos níveis de fibrose hepática através da análise morfométrica demonstrou que, sete dias após a terapia celular, não houve diferença no percentual de tecido fibroso entre os grupos experimentais (Figura 14A). Porém, dois meses após terapia, o grupo tratado com monócitos $CD11b^{+}$ $CD14^{+}$ apresentou níveis diminuídos de fibrose hepática em comparação aos animais não tratados (Figuras 14A-14E). A quantificação bioquímica de hidroxiprolina em tecido hepático mostrou diferenças significativas entre os grupos tratados com monócitos e o grupo veículo, nos dois tempos avaliados (Figura 14F).

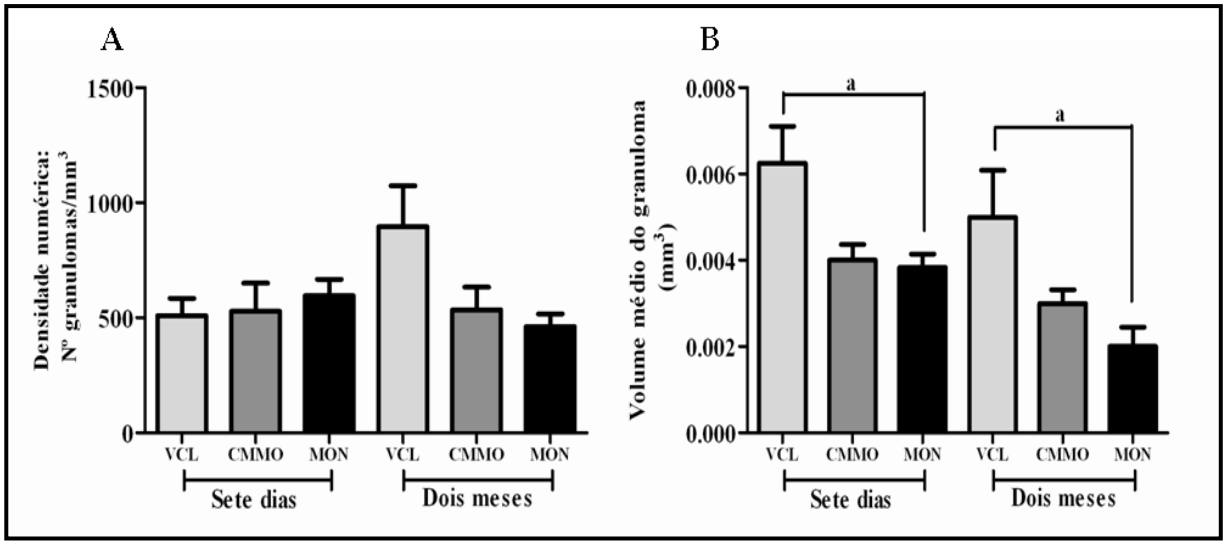
Figura 14- Avaliação da fibrose hepática após terapia celular, em modelo parasitário de lesão hepática crônica.



Fonte: Autora
Nota: (A-D) Fotomicrografias de secções histológicas de fígado**,** coradas com picro-sirius, em animais dos grupos (A) CTL, (B) VCL, (C) CMMO e (D) monócitos (100X), dois meses após o transplante celular. (E) Quantificação de tecido fibroso por morfometria em modelo parasitário de lesão hepática crônica, sete dias e dois meses pós-terapia. (F) Quantificação dos níveis de hidroxiprolina em modelo parasitário de lesão hepática crônica, sete dias e dois meses pós-terapia. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = $P < 0.05$; b = $P < 0.01$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos.

No presente estudo, a mensuração da densidade numérica dos granulomas do tecido hepático não apresentou diferenças significativas entre os grupos avaliados (Figura 15A). No entanto, a morfometria do volume dos granulomas demonstrou redução significativa sete dias e dois meses após o transplante de monócitos $CD11b^{+}$ $CD14^{+}$ (Figura 15B).

Figura 15- Avaliação dos granulomas hepáticos após terapia celular.

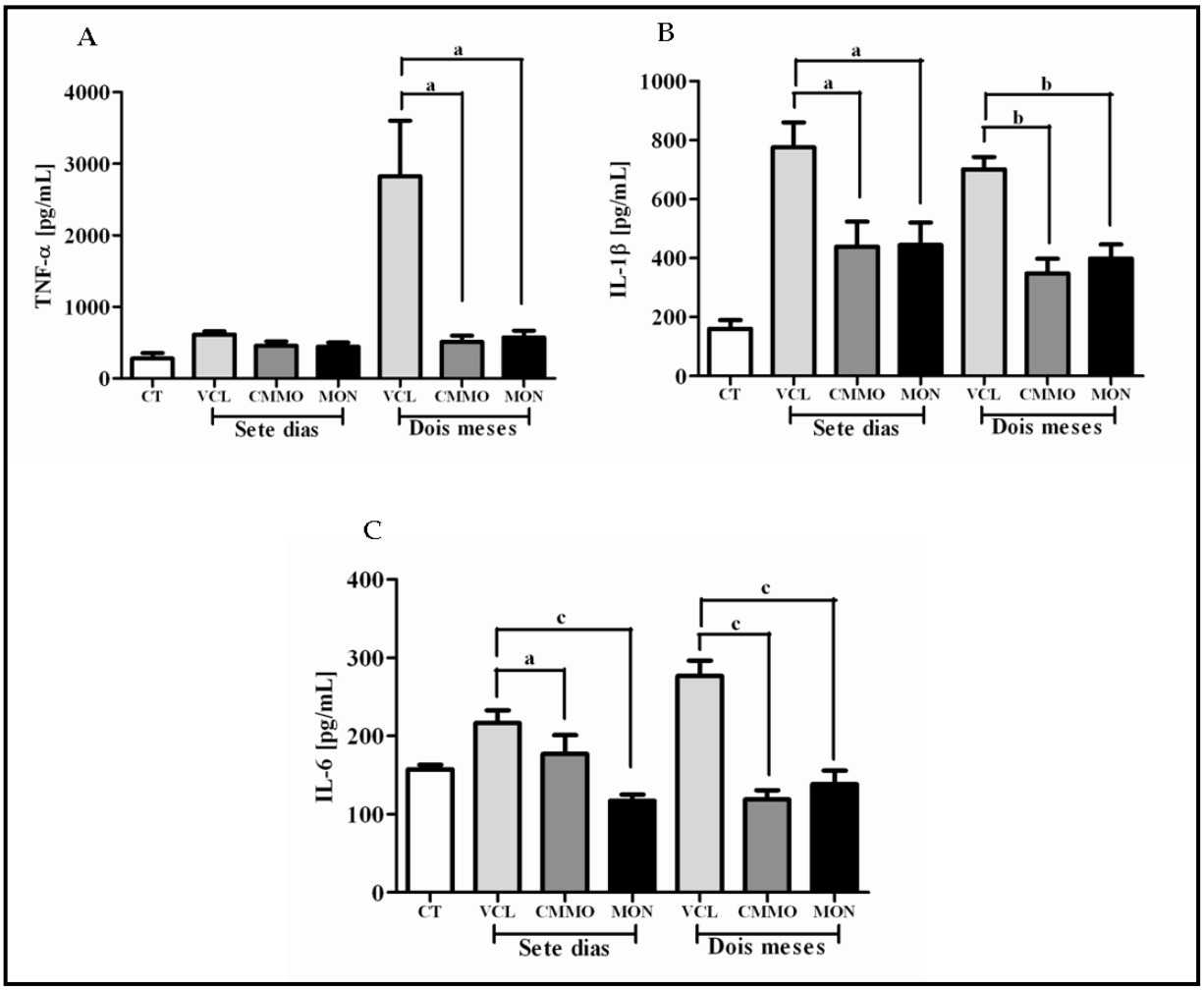


Fonte: autora
Nota: (A) Mensuração da densidade numérica de granulomas hepáticos por morfometria em modelo parasitário de lesão hepática crônica, sete dias e dois meses pós-terapia celular. (B) Mensuração do volume dos granulomas hepáticos em modelo parasitário de lesão hepática crônica, sete dias e dois meses pós-terapia celular. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = $P < 0.05$; b = $P < 0.01$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos.

8.3.2 Análises imunológicas

A quantificação de mediadores inflamatórios em modelo crônico de esquistossomose mostrou que as concentrações da citocina TNF-α foram significativamente diminuídas, dois meses após terapia celular (Figura 16A). Os níveis hepáticos de IL-1β e IL-6 foram significativamente reduzidas no grupo de animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$, bem como em animais submetidos ao transplante de CMMO, nos dois tempos avaliados (Figuras 16B e 16C).

Figura 16- Efeitos da terapia celular no perfil hepático das citocinas pró-inflamatórias.

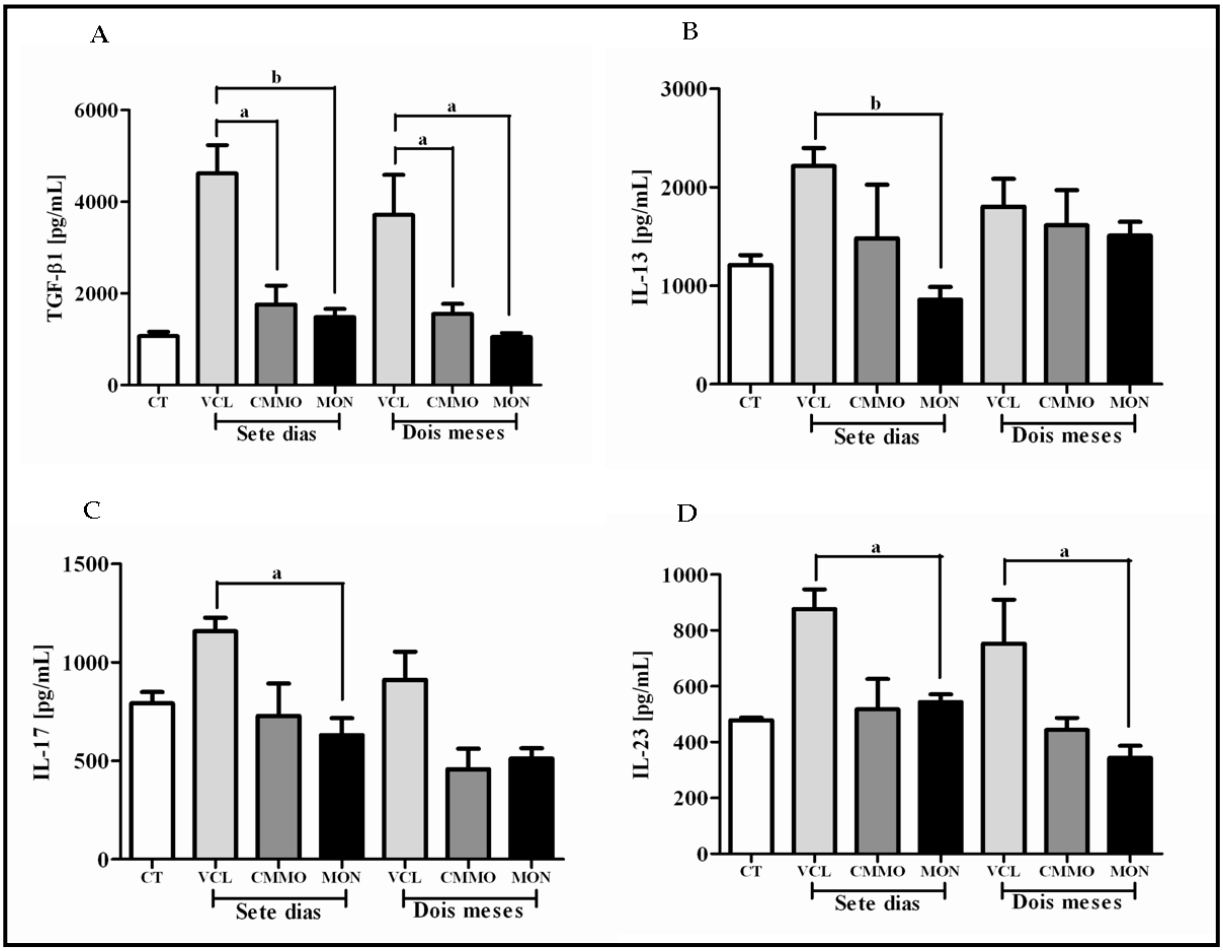


Fonte: autora
Nota: Níveis hepáticos de (A) TNF-α, (B) IL-1β e (C) IL-6, em modelo crônico de esquistossomose, mensurados por ELISA sanduíche. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = P < 0.05; b = P < 0.01; c = P < 0.001; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; TNF-α- fator de necrose tumoral-alfa; IL-1β-interleucina-1beta; IL-6-interleucina-6.
.

Em modelo crônico de esquistossomose também foram observadas alterações nos níveis hepáticos de mediadores pró-fibrogênicos, após terapia celular com monócitos $CD11b^{+}$ $CD14^{+}$. Uma redução significativa nos níveis de TGF-β1 foi observada nos dois tempos avaliados (Figura 17A), enquanto que a redução dos níveis hepáticos de IL-13 ocorreu somente nos animais avaliados sete dias após a terapia celular (Figura 17B).

O estudo também demonstrou redução significativa dos níveis hepáticos da citocina IL-17, somente sete dias após a infusão dos monócitos $CD11b^{+}$ $CD14^{+}$ (figura 17C), e da citocina IL-23, nos dois tempos estudados (figura 17D).

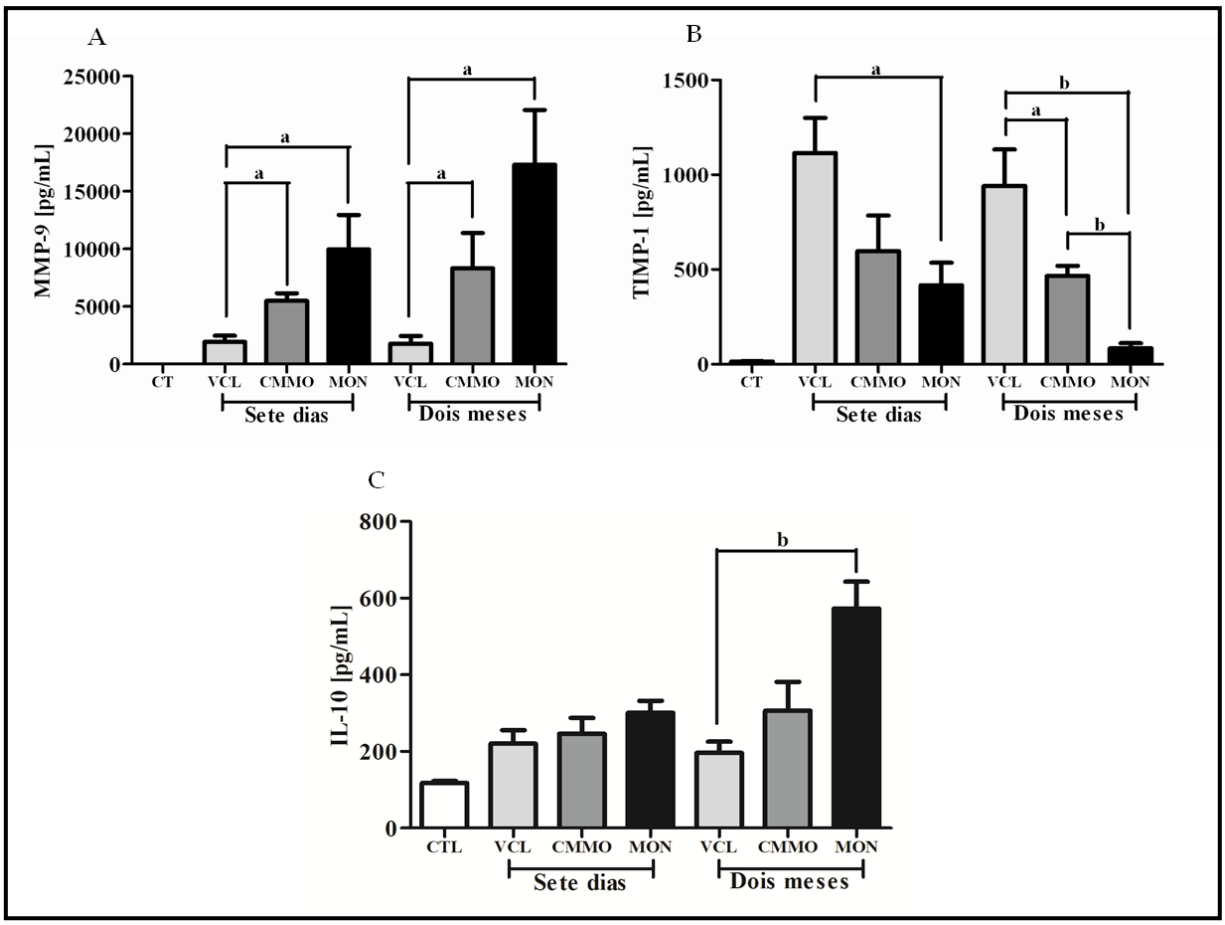
Figura 17- Efeitos da terapia celular no perfil hepático dos mediadores da fibrogênese hepática.



Fonte: Autora
Nota: Níveis hepáticos de (A) TGF-β1, (B) IL-13, (C) IL-17 e (D) IL-23, em modelo crônico de esquistossomose, mensurados por ELISA sanduíche. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = $P < 0.05$; b= $P < 0.01$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; TGF-β1-fator de crescimento e dtransformação-beta; IL-13-interleucina-13; IL-17-Interleucina-17; IL-23-interleucina-23.

Nossos resultados demonstraram um aumento significativo nos níveis de MMP-9 após o transplante celular (Figura 18A). Consistente com esses resultados, uma redução significativa na concentração de TIMP-1 hepático também foi observada (Figura 18B). Os animais tratados com monócitos $CD11b^{+}$ $CD14^{+}$ também apresentaram aumento significativo nos níveis de IL-10, em comparação com os animais tratados com salina, dois meses após a terapia (Figura 18C).

Figura 18- Efeitos da terapia celular no perfil hepático de fatores reguladores da fibrose hepática.

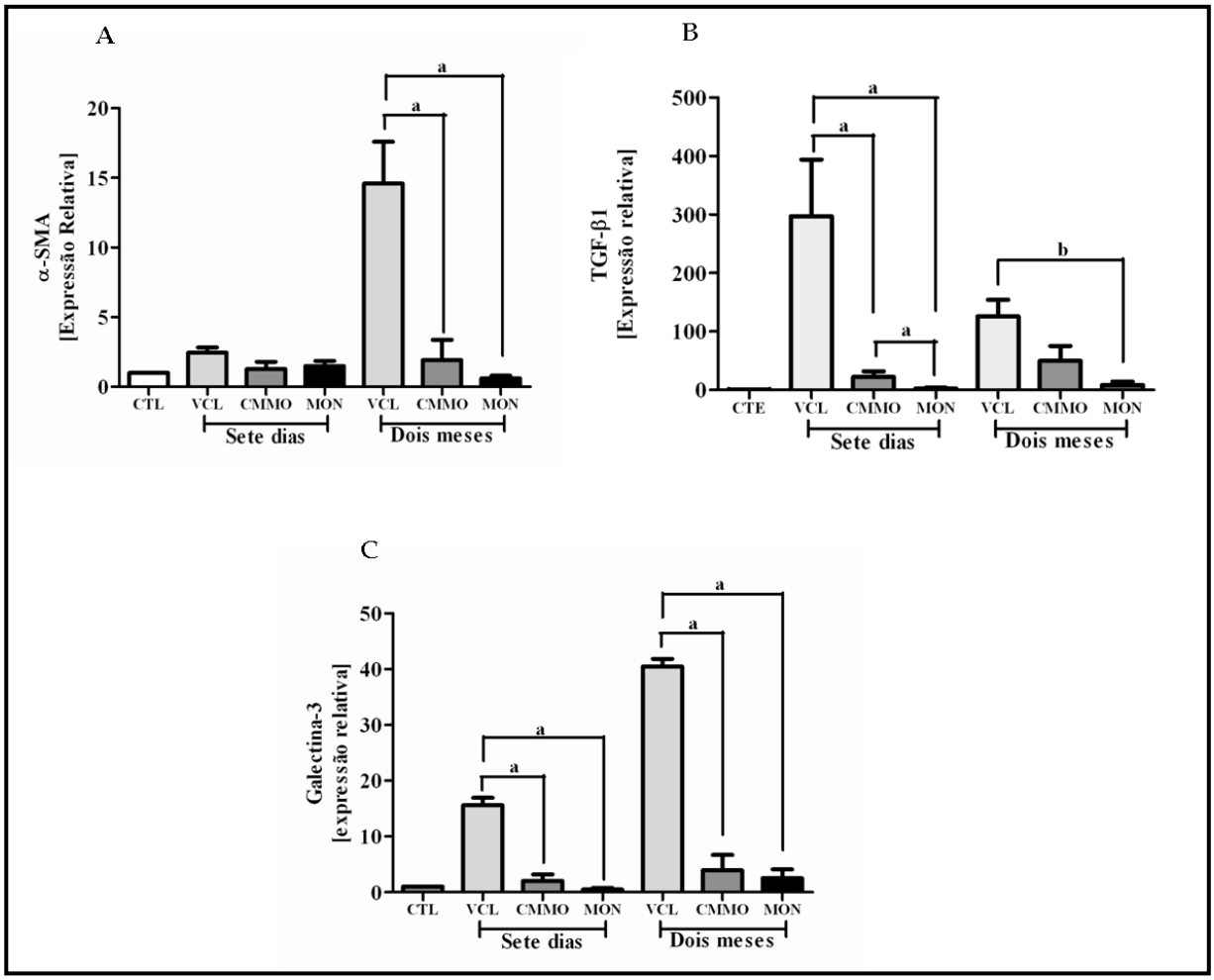


Fonte: Autora
Nota: Níveis hepáticos de (A) MMP-9, (B) TIMP-1 e (C) IL-10, em modelo crônico de esquistossomose, mensurados por ELISA sanduíche. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = $P < 0.05$; b = $P < 0.01$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; MMP-9-metaloproteinase-9; TIMP-1-inibidores de metaloproteinase-1; IL-10-interleucina-10.

8.3.3 Avaliação dos níveis de expressão gênica

O presente estudo identificou, através da RTqPCR, uma redução significativa na expressão do marcador de CEHs, o α-SMA, dois meses após o transplante celular (Figura 19A). A expressão dos mediadores TGF-β1 e gal-3, mostraram-se significativamente diminuídos, no fígado de animais submetidos ao transplante celular, nos dois tempos avaliados (Figuras 19B e 19C).

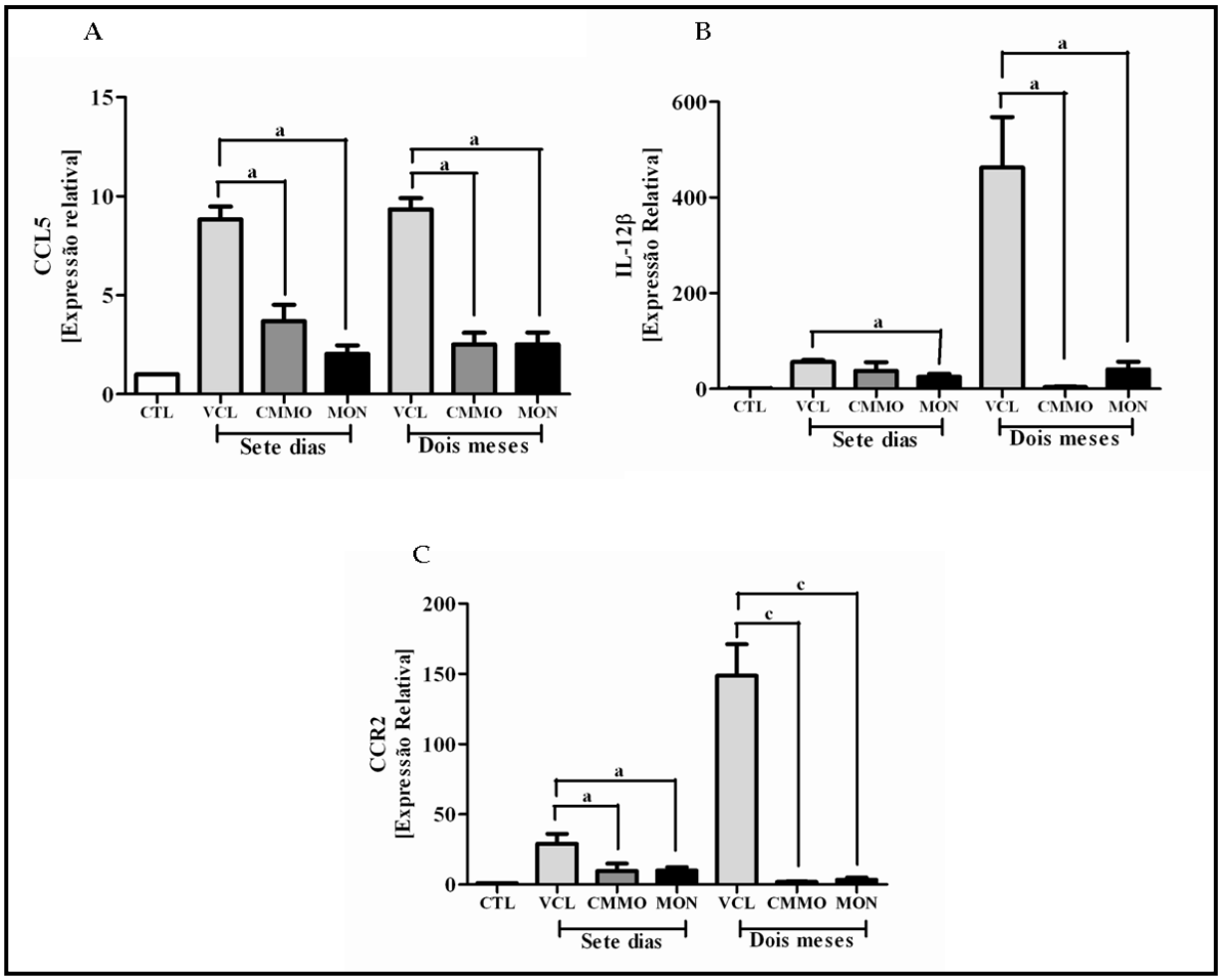
Figura 19- Efeitos da terapia celular nos níveis de expressão de marcadores da fibrose hepática.



Fonte: autora
Nota: Quantificação dos níveis de expressão hepática, por qPCR, dos alvos (A) α-SMA, (B) TGF-β1 e (C) Galectina-3, em modelo crônico de esquistossomose, sete dias e dois meses após terapia celular. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = $P < 0.05$; b = $P < 0.01$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; α-SMA-alfa-actina de músculo liso; TGF-β1-fator de crescimento e dtransformação-beta.

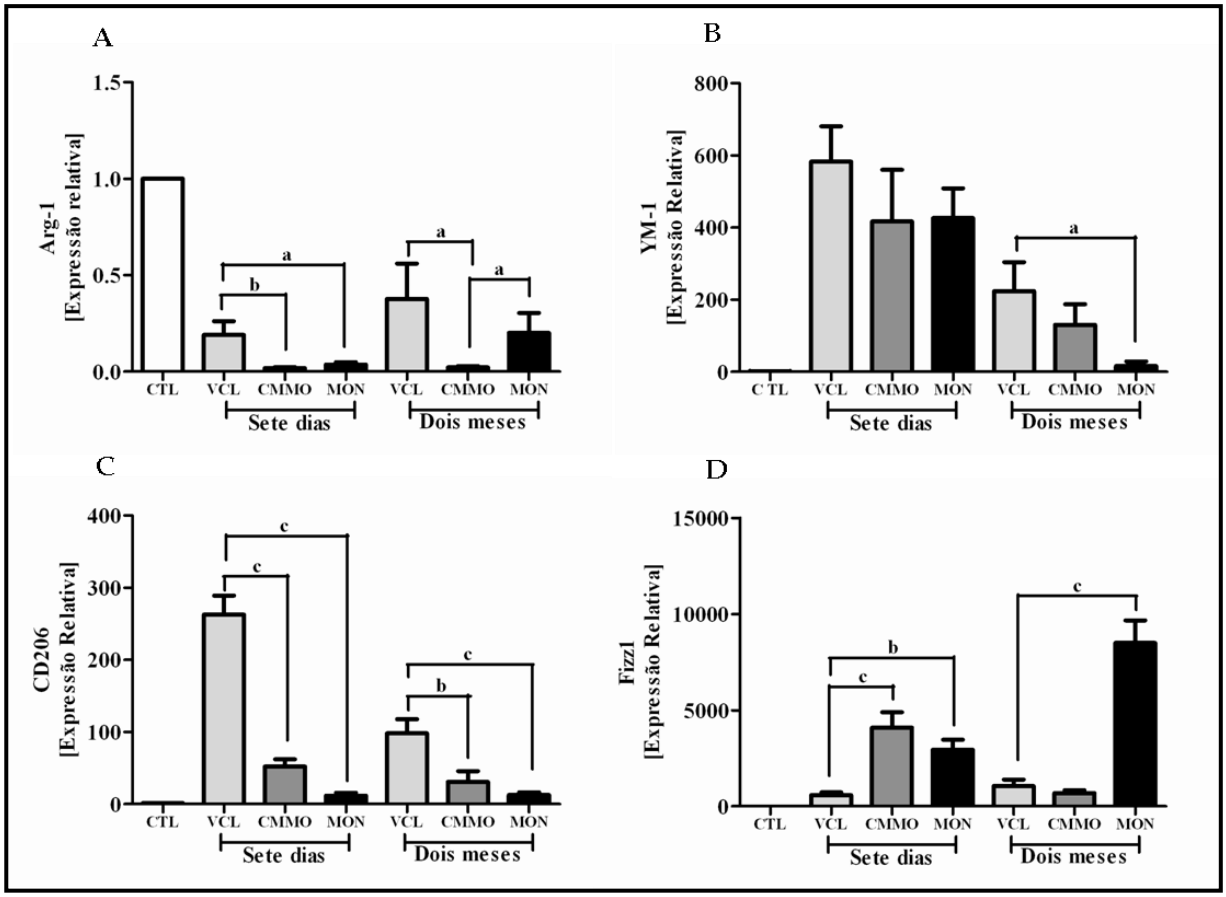
A terapia com monócitos $CD11b^{+}$ $CD14^{+}$ induziu, em modelo parasitário de fibrose hepática, redução na expressão hepática de marcadores associados ao perfil de ativação de macrófagos M1, CCL5 (figura 20A), IL-12β (figura 20B) e CCR2/Ly-6C (Figura 20C), sete dias e dois meses após terapia celular. Interessantemente, o estudo molecular também mostrou redução significativa na expressão dos marcadores do perfil M2 Arg-1 (figura 21A), YM-1 (figura 21B) e CD206 (figura 21C). No entanto, o marcador Fizz1 mostrou-se aumentado significativamente após o transplante celular (figura 21D).

Figura 20- Efeitos da terapia celular na expressão hepática de marcadores de macrófagos M1.



Fonte: autora
Nota: Quantificação dos níveis de expressão hepática, por qPCR, dos alvos (A) CCL5, (B) IL-12β e (C) CCR2. Os alvos foram quantificados por qPCR, em modelo crônico de esquistossomose. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = $P < 0.05$; c = $P < 0.001$; n = 5 animais/grupo).
Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; CCL5-C-C motif chemokine ligand 5; IL-12β-interleunina-12beta; CCR2-C-C motif chemokine receptor 2.

Figura 21- Efeitos da terapia celular na expressão hepática de marcadores de macrófagos M2.



Fonte: autora

Nota: Quantificação dos níveis de expressão hepática, por qPCR, dos alvos (A) Arginase-1, (B) YM-1, (C) CD206 e (D) Fizz1. Os alvos foram quantificados por qPCR, em modelo crônico de esquistossomose. Análise estatística pelo teste de Kruskal-Wallis, com pós-hoc de Dunn (a = P < 0.05; b = P < 0.01; c = P < 0.001; n = 5 animais/grupo).

Legenda: CTL-controle; VCL-veículo; CMMO-células mononucleares de medula óssea; MON-monócitos; Arg-1-arginase-1; YM-1- proteína 3 semelhante a chitinase-3; CD206- receptor de manose 1 tipo-C; Fizz1- molécula alfa 1 semelhante a resistina.

9 DISCUSSÃO

O presente estudo reforça a importância dos monócitos/macrófagos no reparo hepático, mostrando, através de avaliações morfológicas, bioquímicas, imunológicas e moleculares, a atuação desta população celular na regulação de vias fibrogênicas relevantes, nos dois modelos de lesão hepática crônica avaliados (tóxico e parasitário). Investigações recentes têm atribuído uma grande plasticidade às células da linhagem monócito/macrófago, e que a depender do microambiente tecidual, tais células podem ser conduzidas a assumir um perfil pro-resolutivo, contribuindo assim para regressão da fibrose hepática experimental (WYNN; VANNELLA, 2016).

Nossos resultados demonstraram que o transplante de monócitos $CD11b^{+}$ $CD14^{+}$ derivados de CMMO promoveu efeitos benéficos nas lesões hepáticas, causando assim significativa redução na fibrose, como demonstrado através da análise morfométrica do tecido fibroso e da avaliação dos níveis de hidroxiprolina, além da mensuração do volume dos granulomas hepáticos em modelo de esquistossomose crônica. Os benefícios observados pós-transplante celular podem ser o resultado da regulação de importantes citocinas envolvidas nos processos de reparo tecidual. Trabalhos anteriores, conduzidos por nosso grupo, têm mostrado diminuição dos níveis de colágeno hepático em animais submetidos à terapia com CMMO (OLIVEIRA et al., 2008, 2012). No entanto, os resultados obtidos no presente estudo mostraram uma melhora acentuada nesses parâmetros após a infusão de monócitos $CD11b^{+}$ $CD14^{+}$, usando os mesmos modelos experimentais.

Os macrófagos, importantes elementos na mediação da resposta inflamatória, apresentam uma dicotomia quando ativados, assumindo diferentes fenótipos a depender dos estímulos do microambiente tecidual (MAHBUB; DEBURGHGRAEVE; KOVACS, 2012). Com base nos resultados obtidos na presente investigação e nos dados encontrados na literatura (JU; TACKE, 2016), pode-se sugerir que subtipos de macrófagos restauradores possam estar envolvidos no reparo tecidual através da inibição da produção de citocinas inflamatórias (TNF-α, IL-1β and IL-6), e aumento da produção da citocina anti-inflamatória IL-10. Estudos prévios têm reportado o papel dos macrófagos na mediação da fibrogênese hepática, e tem proposto a participação de subpopulações de monócitos durante a lesão e reparo hepático (DUFFIELD et al., 2005; RAMACHANDRAN; IREDALE, 2012). Terapias realizadas em modelos experimentais têm indicado que a infusão de macrófagos obtidos de cultura de monócitos de medula óssea pode promover diminuição da fibrose hepática e potencializar os processos de regeneração do fígado (MOORE et al., 2015; SUH et al., 2012;

THOMAS et al., 2011). Nosso estudo reforça o potencial anti-fibrogênico desta população celular frente a modelos murinos de lesão hepática crônica.

A diminuição dos níveis de tecido fibroso hepático observada em nosso estudo pode estar correlacionada com um baixo número de CEHs ativadas. O papel pró-fibrogênico deste tipo celular tem sido reportado na literatura, que descreve uma relação direta entre a fibrose hepatica murina e o número de CEHs ativadas (JIANG; TOROK, 2013; TROEGER et al., 2012). Nesse sentido, estudos têm evidenciado que a terapia com CMMO promoveram diminuição no número de células α-SMA$^+$ em modelo murino de lesão hepática (TANIMOTO et al., 2013). Em nossa investigação, a diminuição de CEHs ativadas após o transplante de monócitos CD11b$^+$ CD14$^+$ foi identificada através da imuno-histoquímica para α-SMA, em modelo tóxico de lesão hepática, bem como pela redução da expressão hepática de α-SMA avaliada por RTqPCR, em modelo parasitário de fibrose hepática. Considerando que a ativação das CEHs é mediada por sinalização parácrina e autócrina (JANG et al., 2015), nós hipotetizamos que monócitos CD11b$^+$ CD14$^+$ transplantados modulam a atividade das CEHs através da regulação de citocinas e fatores de crescimento, incluindo TGF-β1, TNF-α, além de ROs, produzidos por hepatócitos danificados.

A produção das citocinas pró-inflamatórias TNF-α, IL-1β e IL-6 foram inibidas nos animais submetidos à terapia com monócitos CD11b$^+$ CD14$^+$, nos dois modelos estudados. Várias citocinas inflamatórias parecem participar na patogênese da fibrose através da promoção da diferenciação de CEHS quiescentes em miofibroblastos (DUFFIELD et al., 2013). TNF-α e IL-1β são importantes mediadores da fibrose: TNF-α tem um papel crítico na fibrose hepática induzida por CCl_4 (TOMITA et al., 2006); IL-1β é um potente mediador inflamatório que promove a ativação de miofibroblastos através de mecanismos dependentes de TGF-β (FAN et al., 2001). Ambas as citocinas também induzem a produção de IL-6, que exibe atividade autócrina em fibroblastos, além de estar envolvida na indução da fibrose hepática (DUFFIELD et al., 2013).

Além disso, foi evidenciado um aumento na produção da citocina IL-10 após terapia com monócitos, nos dois modelos avaliados. Esta citocina anti-inflamatória tem um papel central na regulação da resposta imune, atenuando a inflamação e assim, prevenindo lesões ao hospedeiro, e tem sido fortemente associada ao perfil M2reg (LICHTNEKERT et al., 2013; YAO et al., 2015). No fígado, IL-10 tem função protetora durante o estabelecimento de lesões crônicas (HAMMERICH; TACKE, 2014). Além disso, tem sido relatada como uma citocina supressora da atividade pró-fibrogênica das CEHs (SUH et al., 2012), e efetora na inibição da produção de colágeno e na secreção de TGF-β (HAMMERICH; TACKE, 2014). Os

resultados obtidos mostram a influência da infusão de monócitos na expressão hepática dos referidos mediadores, permitindo inferir que as células utilizadas na terapia possam ter atuado na imunorregulação das vias inflamatórias e fibrogênicas.

A modulação da inflamação durante os processos de reparo tecidual através da elevada expressão de IL-10 e inibição da produção de TNF-α, IL-1β and IL-6 estão bem descritas na literatura (MA et al., 2016). Devido ao seu papel na ativação e proliferação das CEHs, essas citocinas têm sido implicadas na patogênese da inflamação hepática crônica, principalmente pelo aumento da produção de colágeno, e na regulação de MMPs e TIMPs na lesão de fígado (HUNG et al., 2005; ROBERT et al., 2016). Estudos em terapia gênica têm mostrado que um aumento na expressão de IL-10 reduz a expressão de moléculas pró-fibróticas, tais como TGF-β1 e TNF-α (HUNG et al., 2005), inibindo assim a resposta inflamatória e a ativação das CEHs, com consequente restabelecimento da função hepática (HUNG et al., 2005; ROBERT et al., 2016).

Em nossa investigação foi encontrado, tanto por ensaios imunológicos como por avaliações moleculares, uma redução significativa nos níveis hepáticos de TGF-β1. Esses resultados corroboram outros achados do presente estudo, indicando assim que os monócitos transplantados desempenham uma importante atividade anti-fibrogênica. TGF-β1 está diretamente envolvido na ativação das CEHs e na síntese de componentes da MEC, principalmente colágeno tipo I (JIANG; TOROK, 2013). TGF-β1 também desempenha um importante papel na inibição da degradação da MEC, bloqueando a síntese de MMPs e induzindo um aumento na produção de TIMPs, o que leva a deposição excessiva de colágeno e ao estabelecimento da fibrose hepática (ZHOU; ZHANG; QIAO, 2014). Estudos prévios têm associado à melhora no quadro de fibrose hepática experimental após terapia com CMMO com a redução nos níveis de TGF-β1 (OLIVEIRA et al., 2008; THOMAS et al., 2011). Nossos dados sugerem que a terapia com monócitos atue nesta via fibrogênica, contribuindo assim para a redução da fibrose hepática murina.

A presente investigação mostrou que o transplante celular causou, nos dois modelos estudados, uma diminuição significativa nos níveis de IL-17, uma citocina pró-inflamatória efetora, produzida por células T CD4. (WEAVER et al., 2007). Em condições de lesão hepática, este mediador induz o recrutamento de células inflamatórias para o fígado, bem como ativa diretamente células da imunidade inata, tais como neutrófilos e células dendríticas, para a liberação de citocinas que perpetuam a inflamação crônica (YASUMI et al., 2007). Achados anteriores tem relatado que células T helper (Th) 17 são hábeis em participar da patogênese de lesões hepáticas associadas com a infecção pelo vírus da hepatite

B (WANG; CHEN; XU, 2011), bem como da imunomodulação do granuloma hepático decorrente da esquistossomose mansônica (RUTITZKY; STADECKER, 2011). Recentemente, evidencias indicaram que a IL- 17 pode estar implicada na indução da fibrose hepática, contribuindo para a ativação de CEHs *in vitro* (WANG; CHEN; XU, 2011).

Adicionalmente, o presente estudo identificou, através de ensaios imunológicos, redução nos níveis hepáticos da citocina IL-23, após terapia com monócitos $CD11b^{+}$ $CD14^{+}$ em modelo parasitário de fibrose hepática. Esses achados corroboram estudos anteriores que identificaram a IL-23 como uma importante citocina pró-inflamatória indutora da resposta Th17 fibrogênica (DUFFIELD et al., 2013; GASSE et al., 2011; LARKIN et al., 2012), além de sua conhecida participação na reposta produzida por macrófagos M1 (BRAGA; AGUDELO; CAMARA, 2015).

Nossos resultados mostraram que a terapia com monócitos $CD11b^{+}$ $CD14^{+}$ promoveu uma redução significativa na expressão de Osteopontina (OPN) em modelo tóxico de lesão hepática crônica. OPN é uma glicoproteína expressa numa variedade de tecidos, encontrada principalmente na MEC e em sítios de reparo tecidual (SUBRAMAN et al., 2015). Estudos têm mostrado que essa proteína é altamente expressa no tecido fibroso hepático e influencia a função de células progenitoras do fígado (LANCHA et al., 2014). Nessas condições, níveis aumentados de TGF-β e a ativação das CEHs podem também ser observados (LANCHA et al., 2014; SETH et al., 2014). Nesse contexto, pode-se supor que a supressão da OPN após o transplante de monócitos de medula óssea poderia levar à atenuação da fibrose hepática (COOMBES t al., 2016; WANG et al., 2016).

A glutationa (GSH) é uma conhecida molécula anti-oxidante, que atua como um modulador da sinalização redox, da proliferação celular, da apoptose, da resposta imune e da fibrogênese (LIU; GASTON PRAVIA, 2010; LU, 2013). Foi demonstrado que níveis reduzidos desta molécula foram encontrados em modelos pré-clínicos de fibrose e em doenças fibróticas humanas (LIU; GASTON PRAVIA, 2010). Estudos prévios têm mostrado que uma maior produção de GSH inibe a atividade fibrogênica do mediador TGF-β1(LIU; GASTON PRAVIA, 2010). Foi observado, em modelo tóxico de lesão hepática crônica, um aumento de GSH após o transplante de monócitos $CD11b^{+}$ $CD14^{+}$, sugerindo uma associação entre os efeitos anti-fibróticos observados nos animais tratados com monócitos e a atividade anti-oxidante desta população celular.

Alterações nos níveis de algumas moléculas envolvidas na fibrogênese, bem como no remodelamento do tecido fibroso foram avaliadas nesse estudo. A terapia com monócitos $CD11b^{+}$ $CD14^{+}$ em camundongos com lesão hepática crônica, induzida pela intoxicação por

CCl_4 ou pela infecção por *S. mansoni*, provocou um aumento significativo nos níveis hepáticos de MMP-9, reforçando mais uma vez o potencial anti-fibrogênico que a terapia com monócitos de medula óssea tem apresentado no presente trabalho. Estudos anteriores associam a redução da fibrose hepática com a degradação do tecido fibroso (TROEGER et al., 2012). MMP-9 desempenha um importante papel na resolução da fibrose hepática, e tem sido considerado um potente alvo terapêutico (PHAM VAN et al., 2008). Yang et al. (2014) sugeriram que no microambiente hepático, subpopulações de macrófagos exercem um papel anti-fibrótico, uma vez que expressam várias MMPs, incluindo a MMP-9, esta diretamente envolvida na degradação da MEC, favorecendo assim a resolução da fibrose hepática. Em outra investigação foi identificado que uma população de macrófagos $Ly6C^{low}$ tem um papel anti-fibrótico no fígado, uma vez que secretam MMPs (RAMACHANDRAN et al., 2012).

A presente investigação também mostrou, em modelo murino de esquistossomose crônica, que a terapia com monócitos $CD11b^+$ $CD14^+$ promoveu redução significativa nos níveis de expressão gênica da Gal-3. A galectina-3 consiste de uma proteína pleiotrópica, que desempenha um papel importante na proliferação celular, adesão, diferenciação, angiogênese e apoptose, e mais recentemente tem sido associada à patogênese da fibrose hepática (LI; LI; GAO, 2014). Durante a infecção helmíntica (i.e *S. mansoni*), a Gal-3 modula a resposta inflamatória, recrutamento e migração das células do sistema imune, bem como a liberação de citocinas (BREUILH et al., 2007). Embora se saiba que a gal-3 está envolvida na ativação das CEHs, ainda é incerto se os efeitos observados estão relacionados a uma ação direta da Gal-3 sobre as CEHs, ou se ocorre um efeito secundário responsável pela alteração na concentração hepática de citocinas que promovam o estabelecimento da fibrose hepática (HENDERSON et al., 2006; JIANG et al., 2012). Estudos pré-clínicos têm relatado que a utilização de inibidores de Gal-3 promove resolução da fibrose hepática, em diferentes modelos experimentais (TRABER; ZOME, 2013; TRABER et al., 2013). Oliveira et al. (2012) demonstraram que a terapia com CMMO foi capaz de promover redução da fibrose hepática associada à diminuição na expressão hepática de Gal-3 em modelo murino de cirrose induzida por CCl_4. Baseado nesses achados pode-se dizer que a terapia com monócitos de medula óssea atuou, mais uma vez, em uma importante via fibrogênica.

O transplante de monócitos $CD11b^+$ $CD14^+$ induziu redução nos níveis hepáticos de TIMP-1 e IL-13, dois importantes mediadores pró-fibrogênicos. A presença de grandes quantidades de TIMP-1 durante os eventos de lesão crônica do fígado pode contribuir para o estabelecimento da fibrose hepática, e tem sido considerado um promissor alvo terapêutico no tratamento da fibrose hepática (GAO; BATALLER, 2011). IL-13 é uma citocina associada

com as formas severas de fibrose hepática associada à esquistossomose mansônica, bem como em doenças hepáticas não esquistossomóticas (LIU et al., 2012). IL-13 é considerada um dos mediadores centrais da patogênese hepática, e está diretamente associada à produção de TGF-β1 pelas células hepáticas, além de induzir células progenitoras à transdiferenciação em miofibroblastos produtores de colágeno (PELLICORO et al., 2014). Nossos dados reforçam o papel protetor de monócitos/macrófagos nos processos de reparo tecidual, com atuação na vias fibrogênicas.

Várias investigações têm buscado identificar e correlacionar subgrupos funcionais distintos de macrófagos nos processos de reparo tecidual (RAMACHANDRA; IREDALE, 2012; SUH et al., 2012; TACKE; ZIMMERMANN, 2014). No presente estudo, foram avaliados marcadores moleculares associados aos perfis de macrófagos M1 e M2, após terapia com monócitos $CD11b^{+}$ $CD14^{+}$, em modelo parasitário de lesão hepática crônica. Em nossos achados, observou-se uma redução significativa na expressão de CCl5 e IL-12β, marcadores associados ao perfil clássico de ativação de macrófagos (M1). Tais resultados corroboram os achados das análises imunológicas, nos quais foram observadas reduções significativas nos níveis de mediadores inflamatórios associados ao perfil M1, após o transplante de monócitos. Wynn e Ramalingam (2012) descrevem que macrófagos M1 estão associados com funções pró-inflamatórias, que promovem a iniciação de processos pró-fibróticos através da ativação de miofibroblastos.

O estudo molecular também indicou que o transplante celular promoveu redução dos marcadores Arg-1, YM-1 e CD206, associados ao perfil M2, mais especificamente ao perfil M2a ou M2 fibrogênico (BRAGA; AGUDELO; CAMARA, 2015; LECH; ANDERS, 2013; LEE et al., 2014; LICHTNEKERT et al, 2013). A arginase-1 (arg-1) é considerada um protótipo marcador de macrófagos M2, e trata-se de uma enzima que utiliza o aminoácido L-arginina como substrato para a produção de L-ornitina. Esta serve de matéria-prima para a biossíntese de colágeno, e por isso, vários estudos têm sugerido que arg-1 esteja envolvida nos processos de fibrogênese (MUNDER, 2009), e que macrófagos M2a $arg\text{-}1^{+}$ estão diretamente associados ao quadro de fibrose (GIBBONS et al., 2011; WANG et al., 2015). Na esquistossomose, um grande número de macrófagos $arg\text{-}1^{+}$ estão localizados ao redor dos granulomas hepáticos (PESCE et al., 2006). Ym1, é uma lectina amplamente considerada como marcador de macrófagos M2 em camundongos, e sua expressão é altamente regulada por IL-4, IL-13 e TGF-β (RÕSZER, 2015); CD206 é um marcador bem conhecido tanto para macrófagos M2 de camundongos quanto para macrófagos M2 de humanos (BRAGA;

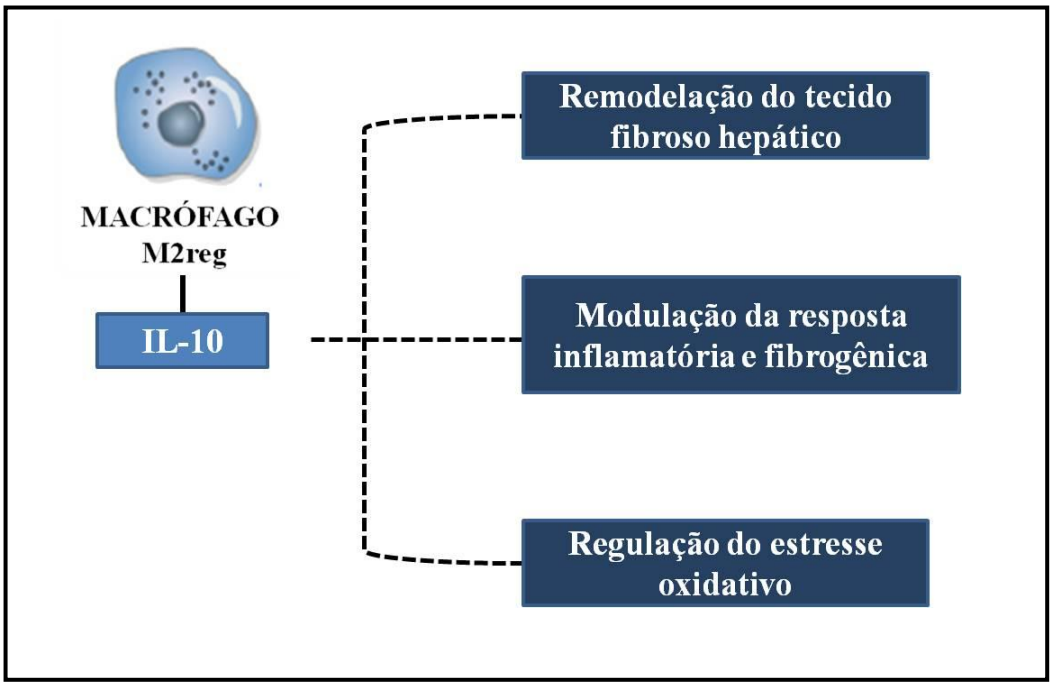
AGUDELO; CAMARA, 2015). Beljaars et al. (2014) associaram elevada expressão de CD206 e de YM-1 às áreas de fibrose hepática induzida por CCl_4.

Interessantemente, a avaliação dos níveis de expressão, em modelo murino de esquistossomose crônica, mostrou aumento significativo na expressão gênica hepática de Fizz1, após terapia com monócitos de medula óssea. O papel de Fizz1 na resposta imune Th2, bem como na fibrogênese ainda não está totalmente elucidado. Não há um consenso a respeito da relação deste marcador com um subtipo de macrófago M2. Algumas publicações associam a alta expressão de Fizz1 a macrófagos M2 pró-fibrogênicos (WILSON et al., 2007). Murthy et al. (2017) afirmaram que macrófagos com fenótipo pró-fibrótico, positivos para arg-1 apresentaram níveis de expressão reduzidos de Fizz1.

O presente estudo também evidenciou uma redução significativa na expressão hepática de CCR2, após terapia com monócitos de medula óssea. O receptor de quimiocina CCR2 está envolvido no recrutamento de monócitos para as áreas de lesão, e é altamente expresso em monócitos/macrófagos $Ly6C^{hi}$, induzidos durante a progressão da fibrose (KARLMARK et al., 2009; MITCHELL et al., 2009). Um estudo conduzido por Mitchell e cols (2009) demonstrou que camundongos knockouts para CCR2 apresentaram níveis significativamente mais baixos de fibrose hepática induzida por CCl_4, em comparação com animais selvagens. Recentemente, foi identificado um novo subtipo de macrófago, que tem sido considerado um elemento crítico para a resolução da fibrose hepática. Ramachandran e colaboradores (2012) encontraram em suas investigações que macrófagos $Ly6C^{low}$ secretam grandes quantidades de MMPs fibrolíticas, tais como MMP-9 e MMP-13, bem como IL-10. O aumento nos níveis de MMP-9 e IL-10 também foram observados em nosso estudo, sugerindo que a terapia com monócitos de medula óssea influenciou na regulação das vias de ativação dos macrófagos envolvidos na resposta inflamatória crônica.

Baseado nos resultados obtidos em nosso estudo, aliados aos achados encontrados na literatura, podemos inferir que a terapia celular com monócitos derivados da medula óssea contribui para a melhora hepática em modelos pré-clínicos de lesão crônica de fígado. Tais achados nos levam a supor que a modalidade terapêutica proposta no presente trabalho, pode conduzir ao predomínio de macrófagos M2reg, produtores de citocinas imunossupressoras (IL-10), e estes podem estar envolvidos na remodelação das proteínas fibrolíticas, na modulação da resposta inflamatória e da fibrogênese, bem como na regulação do estresse oxidativo decorrente da lesão tecidual hepática (figura 22).

Figura 22- Mecanismos envolvidos na participação de macrófagos M2 regulatórios no reparo hepático.



Fonte: autora
Nota: Eventos de remodelação do tecido fibroso hepático, modulação da resposta inflamatória e fibrogênica, além de regulação do estresse oxiadtivo foram observados após terapia com onócitos de meula óssea em modelos experimentais de hepatopatias.
Legenda: M2reg-macrófagos M2 regulatórios; IL-10: interleucina-10.

10 CONCLUSÃO

A terapia com monócitos $CD11b^{+}$ $CD14^{+}$ em modelos murinos de lesão hepática crônica parece contribuir para a regressão da fibrose hepática, independente do estímulo agressor, através de uma combinação de mecanismos, que envolve a modulação da resposta inflamatória e remodelação da MEC constituinte do tecido fibroso, através da expressão de MMPs, da supressão do microambiente hepático pró-fibrogênico e pela indução da inibição das CEHs ativadas.

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APÊNDICE A - Manuscrito I: Bone Marrow-derived Monocyte Infusion Improves Hepatic Fibrosis by decreasing Osteopontin, TGF-β1, Interleukin-13 and Oxidative Stress.

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Bone Marrow-derived Monocyte Infusion Improves Hepatic Fibrosis by decreasing Osteopontin, TGF-β1, Interleukin-13 and Oxidative Stress.

Souza VCA *et al*. Monocyte Therapy Improves Hepatic Fibrosis

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Abstract

AIMS: To evaluate the therapeutic effects of bone marrow-derived $CD11b^+$ $CD14^+$ monocytes in a murine model of chronic liver damage.

METHODS: Chronic liver damage was induced in C57BL/6 mice by administration of carbon tetrachloride (CCl_4) and ethanol for six months. Bone marrow-derived monocytes isolated by immunomagnetic separation were used for therapy. The cell transplantation effects were evaluated by morphometry, biochemical assessment, immunohistochemistry and enzyme-linked immunosorbent assay (ELISA).

RESULTS: $CD11b^+$ $CD14^+$ Monocyte therapy significantly reduced liver fibrosis and increased hepatic glutathione (GSH) levels. Levels of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and IL-1β, in addition to pro-fibrotic factors, such as interleukin (IL)-13, transforming growth factor-beta (TGF-β1) and tissue Inhibitor of Metalloproteinase-1 (TIMP-1) also decreased, while IL-10 and matrix metalloproteinase (MMP)-9 increased in the monocyte-treated group. $CD11b^+$ $CD14^+$ Monocyte transplantation caused significant changes in the hepatic expression of alpha-Smooth Muscle Actin (α-SMA) and osteopontin (OPN).

CONCLUSION: The results show that monocyte therapy is capable of bringing about improvement of liver fibrosis by reducing oxidative stress and inflammation, as well as increasing anti-fibrogenic factors.

Key words: Monocytes, Bone Marrow Mononuclear Cells, Cell Therapy, Liver Fibrosis, Macrophages, Glutathione.

Core Tip: Chronic inflammation is now recognized as a central player in the development of liver fibrosis. Studies have shown that activated macrophages establish a link between chronic inflammation and fibrosis in various organs. The present study evaluated the therapeutic effects of bone marrow-derived $CD11b^+$ $CD14^+$ monocytes in a murine model of liver damage. The results show that mice with transplants showed improvement of liver fibrosis by way of a reduction in oxidative stress and inflammation and an increase in anti-fibrogenic factors. The study demonstrates the beneficial effects of cellular therapy in liver fibrosis and also reports on the important modulatory mechanisms involved.

Souza VCA, Pereira TA, Teixeira VW, Carvalho H, de Castro MCAB, D'assunção CG, Barros AF, Carvalho CL, Lorena VMB, Costa VMA, Teixeira AAC, Figueiredo RCBQ, Oliveira SA. Bone Marrow-derived Monocyte Infusion Improves Hepatic Fibrosis by decreasing Osteopontin, TGF-β1, Interleukin-13 and Oxidative Stress.

INTRODUCTION

The abuse of alcohol, infections caused by hepatitis viruses B and C, and nonalcoholic steatohepatitis (NASH) are the main causes of liver tissue damage[1]. These risk factors can lead to focal or diffuse hepatocellular degeneration and necrosis. Persistent inflammatory stimulus in the liver can induce the formation of fibrous tissue, and ultimately lead to the development of liver cirrhosis[2]. Hepatic stellate cells (HSCs) play an important role in liver fibrogenesis because they are the main source of secreted extracellular matrix components (ECM)[3]. When severe liver damage occurs, HSCs are activated, mainly by the action of transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α) and reactive oxygen species (ROS), produced by damaged hepatocytes or liver-resident macrophages[4].

The ECM contain various types of proteins, including osteopontin (OPN)[5], a proinflammatory cytokine that modulates the pro-fibrogenic phenotype of HSCs and is involved in many physiological and pathological processes, including inflammation, fibrosis and angiogenesis[5,6]. OPN has also been described as a mediator induced by the Hedgehog pathway (Hh) and plays an important role in the repair of acute and chronic liver damage, both in humans and experimental models[7,8].

The remodeling of fibrous tissue is a complex mechanism by which multiple cell types, producing molecules such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), play an important role in the synthesis and degradation of the ECM[9]. In chronic liver damage, the establishment of hepatic fibrosis is directly related to MMP/TIMP imbalance [10], thereby showing that MMPs and TIMPs may be potent therapeutic targets[11].

Although important advances in the knowledge of chronic liver diseases have been made, the existing treatments are still limited. New, more effective and less invasive therapeutic strategies are therefore needed. In this context, several studies of regenerative medicine have demonstrated the potential of cell therapy as a promising emerging treatment for liver diseases[12] and various cell populations have been

investigated to this end[12,13]. Bone marrow mononuclear cells (BMMCs) have shown promising results in both experimental[14] and clinical[15,16] studies. Previous studies of experimental models of liver injury have demonstrated that cell therapy is able to decrease mortality[17] and levels of hepatic fibrosis[14]; improve biochemical parameters[18], increase MMP-9 expression[19]; and reduce levels of TGF-β1 [20] and galectin-3 expression[14].

Identifying which components of the BMMC population are responsible for the beneficial effects of cell therapy is extremely important for clinical application. Recent studies have reported that monocytes may have important therapeutic potential in chronic liver diseases[21,22]. These cells are the precursors of the heterogeneous macrophage population involved in liver repair responses. In the liver, macrophages perform various functions, such as phagocytosis and cytokine production, which are important in the inflammatory response to damage, liver fibrosis and degradation of ECM[23,24]. *In vitro* assays have shown that monocytes maintained in culture supplemented with hepatocyte growth factor (HGF) exhibited similar behavior to those hepatic cells obtained from the liver culture[21]. One preclinical study has shown that cellular therapy with cultured macrophages decreases murine liver fibrosis and this is followed by changes in the levels of some mediators involved in liver repair[22].

Although these findings are of great importance, information about the functions of monocyte/macrophage cell lineages in cell therapy for liver diseases is still limited. The present study evaluated the therapeutic potential of bone marrow-derived monocytes in a murine model of chronic liver damage induced by carbon tetrachloride (CCl_4) and ethanol (EtOH).

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (4-6 weeks of age), weighing 20-23 g were obtained from the Animal Breeding Center Laboratory (CECAL) Fundação Oswaldo Cruz (FIOCRUZ,

Rio de Janeiro, Rio de Janeiro, Brazil), and housed in the animal research facility in the Aggeu Magalhães Research Center (CPqAM), (FIOCRUZ, Recife, Pernambuco, Brazil). The animal protocol was designed to minimize pain or discomfort to the animals, which were maintained in rooms with a controlled temperature (22± 2ºC) and humidity (55 ±10%) environment under continuous air renovation conditions. Animals were housed in a 12-h light/12-h dark cycle and free access to food (Nuvilab, Curitiba, Paraná, Brazil) and water. Experimental procedures were in accordance with the ethical standards of the Oswaldo Cruz Foundation and approved by the Ethics Committee for the Certified Use of Animals (CEUA-CPqAM 15/2011).

Chronic liver damage and experimental design

Chronic liver damage in the mice was induced by orogastric administration of 200 µL of 20% carbon tetrachloride (CCl_4) solution diluted in olive oil in twice weekly doses[14]. The mice also received a 5% ethanol solution in water *ad libitum*. CCl_4 treatment was carried out for six months. The mice were randomly divided into four experimental groups with chronic hepatic damage: Group I: Control mice (normal mice) (n = 5); Group II: Saline-treated mice (n = 5); Group III: Mice treated with BMMCs (n = 5); Group IV: Mice treated with BMMC-derived monocytes (n = 5).

Isolation of BMMCs and Monocytes

Bone marrow was harvested from the femurs and tibiae of donor C57BL/6 mice (n = 15) and BMMCs were purified by centrifugation in a Ficoll gradient (Histopaque 1119 and 1077, Sigma Aldrich, St Louis, MO, USA) at 1000 g, for 15 minutes. This protocol facilitates the rapid recovery of viable BMMCs using two ready-to-use separation mediums in conjunction. The BMMC preparation was used to isolate monocytes by way of the immunomagnetic cell separation system. For this, the BMMCs (approximately 10^7 cells/mL) were incubated with anti-CD11b antibodies conjugated to magnetic microbeads (MACS units, Milteny Biotec™), washed and passed through a magnetic column (MACS, Milteny Biotec™), where $CD11b^+$ monocytes were retained and recovered in a buffer (0.5% PBS/BSA 0.5% bovine

serum albumin-BSA 2 mM EDTA+ 2 mM EDTA). Finally, the cells were washed and re-suspended in 0.9% sterile saline, which was later infused into the mice.

Cell characterization

The BMMCs and monocytes obtained by imunomagnetic separation were first incubated with Anti-CD11b (PE Rat Anti-Mouse CD11b, M1/70 clone, BD Pharmingen™), Anti-CD14 (FITC Rat Anti-Mouse CD14, rmC5-3 clone, BD Pharmingen™), Anti-CD45 (APC Rat Anti-Mouse CD45, 30-F11 clone, BD Pharmingen™), Anti- CD34 (PE Rat Anti-Mouse CD34, RAM34 clone, BD Pharmingen™) and Anti-Ly6A (FITC Rat Anti-Mouse Ly-6A/E, D& clone, BD Pharmingen™). After 30 minutes of incubation, cells were washed with 2mL of PBS wash (PBS 0.5% BSA 0.1% sodium azide), centrifuged at 400xg for 5 minutes and then resuspended in 300µl of PBS wash. The samples were then phenotypically characterized by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA). A minimum of 10.000 events/sample were collected. The cell population obtained by immunomagnetic separation presented the following phenotype distribution: 99.12% $CD11b^+$; 97.99% $CD14^+$; 98.3% $CD45^+$; 1.36% $CD34^+$ and 1.81% $Ly6A^+$ cells, differed from those of BMMC which were: 58.3% $CD11b^+$; 47.76% $CD14^+$; 79.4% $CD45^+$; 3.6% $CD34^+$ and 13.55% $Ly6A^+$ cells, demonstrating an enrichment of homogeneous monocytes population in our cell preparation. Figure 1 shows representative FACS histograms of BMMCs and $CD11b^+$ monocytes isolated by immunomagnetic separation.

Cell infusion in mice with chronic liver damage

Six months after treatment with CCl_4/ethanol, bone marrow-derived $CD11b^+$ $CD14^+$ monocytes and BMMCs were administered endovenously to the mice (10^6 cells/animal) for three consecutive weeks. Two months after transplantation, mice

were euthanized and the liver and the spleen were extracted for further analysis (Figure 2).

Morphometric Evaluation

In order to characterize and quantify liver fibrosis, treated and non-treated samples were fixed for 24 hours in 10% formalin, embedded in paraffin, sectioned (5 μm) and stained with picro-Sirius. The images were obtained using an optical microscope (DM LB 2, Leica Microsystems) equipped with LEICA JVC TK (model - C 1380, Pine Brook, NJ, USA) and analyzed using the Image Analysis Processing System LEICA QWIN, version 2.6 MC (Leica, Cambridge, UK). Ten microscopic fields (100 X magnification) containing fibrous tissue areas were chosen for quantification. To detect and quantify Kupffer cells, the histological sections were stained with haematoxylin and eosin (H&E) and observed under an optical microscope (DM LB 2, Leica Microsystems). The cell counts were performed in 10 fields/sections (400 X magnification).

Hydroxyproline Assay

Liver samples (approximately 200 mg) were immersed in 6N HCl at approximately 120^{o}C for 18 hours followed by filtration. The hydroxyproline (Hyp) concentration was determined by a colorimetric assay at 558 nm as previously described [25] and expressed as nMol/g liver.

Immunohistochemistry Analysis

Immunohistochemistry was carried out to evaluate the activated HSCs (alpha-smooth muscle actin, α-SMA) and OPN. To stain α-SMA, liver sections (5 μm) were initially deparaffinized with xylene; dehydrated in increased concentration of ethanol; incubated overnight with biotinylated antibody anti-α-SMA (Santa Cruz Biotechnology, Santa Cruz, California, USA); and then incubated with streptavidin-peroxidase for 10 minutes. For OPN staining, the samples were incubated overnight with primary anti-OPN antibodies (AF808, R&D Systems, Minneapolis, MN, USA), as previously described[5]. Thereafter, a secondary antibody bound to a synthetic

polymer conjugate with peroxidase (HRP, horseradish peroxidase). 3,3'diaminobenzidine (DAB) was used for staining. The sections were counter-stained with Harris hematoxilin. The staining was measured in ten-fields/sections (200 X magnification) using an Image Analysis Processing System LEICA QWIN, version 2.6 MC (Leica Cambridge, Cambridge - UK).

GSH measurement

To evaluate oxidative stress, the amount of Glutathione (GSH) was quantified using liver fragments from mice submitted to the cell therapy and those that were not. The liver fragments were weighed, macerated in 5% metaphosphoric acid solution and centrifuged at 12000 g at 4°C for 10 minutes. GSH was detected using the Glutathione Assay Kit (Sigma Aldrich, St Louis, MO, USA) and measured with a microplate reader (BioRad - 415 nm).

Enzyme-Linked Immunosorbent Assay (ELISA)

Frozen liver fragments (~100 mg) were homogenized in a lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.02% Sodium Azide) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were centrifuged at 16000 g for 15 minutes at 4ºC and supernatants were used to quantify the levels of TNF-α, IL-6, IL-1β, IL-13, IL-10**,** IL-17, IL-23, TGF-β1, MMP-9 and TIMP-1 by way of a sandwich ELISA assay following the manufacturers' instructions (IL-13, IL-17, IL-23, MMP-9 and TIMP-1: R&D Systems, Minneapolis, MN, USA; TGF-β1: Human/mouse TGF-beta1, e-Bioscience, San Diego, CA, USA; TNF-α, IL-1β, IL-6 and IL-10: BD OptEIA set mouse, San Diego, CA, USA). Samples were read at a 450 nm wavelength using a microplate reader (model 3550, Thermo Scientific). The concentration of TGF-β1 was also determined from supernatants of splenocyte culture obtained from mice used in the study, as previously described[26]. The cytokine concentration was expressed in pg/mL.

Statistical Analysis

Quantitative data were submitted to the normality test (Shapiro-Wilk). Differences were evaluated using the ANOVA test, for parametric analysis, and the Kruskal-Wallis test, with post-hoc Dunn, for non-parametric analysis. Statistical analyses were performed using Prism Software (version 5.0, GraphPad Software, San Diego, CA, USA) and Bioestat 5.3 (Mamirauá Institute, Manaus, AM, Brazil). A p value of < 0.05 was considered statistically significant. Data were expressed as mean values (mean ± SEM).

RESULTS

Monocyte Therapy alters Hepatic Fibrosis

Morphometric analysis, two months after therapy, showed a significant decrease in fibrotic areas in the liver from $CD11b^+$ $CD14^+$ in the monocytes-treated group compared to the saline-treated group ($P < 0.001$; Figures 3B, 3D and 3E). This decrease was also found in mice treated with BMMCs ($P < 0.05$; Figures 3B, 3C and 3E). A marked reduction in the amount of hydroxyproline was also observed in the group that received monocyte treatment ($P < 0.01$; Figure 3F). The number of Kupffer cells significantly increased in the monocyte-treated ($P < 0.001$) and BMMC-treated ($P < 0.01$) groups, when compared to the saline-treated group (Figure 3G).

To test whether $CD11b^+$ $CD14^+$ monocyte transplantation was able to alter the number of activated HSCs, α-SMA positive cells were assessed by immunohistochemistry. As shown in Figure 4, α-SMA-positive cells in the hepatic parenchyma decreased in the mice that received monocytes ($P < 0.01$, Figures 4D-E) as well as in the BMMC-treated group ($P < 0.05$, Figures 4C-E) compared with the group treated with saline (Figures 4B-E). Furthermore, OPN also decreased after $CD11b^+$ $CD14^+$ monocyte therapy (Figure 5).

Monocyte Transplantation reduces Hepatic Inflammatory and Pro-Fibrotic Cytokine Levels

To investigate the mechanisms involved in the improvement of hepatic fibrosis after $CD11b^+$ $CD14^+$ monocyte therapy, the levels of hepatic inflammatory and pro-fibrotic

cytokine were quantified. The levels of TNF-α, IL-1β and IL-6 in liver lysates were significantly lower in the $CD11b^+$ $CD14^+$ monocytes -treated group ($P < 0.05$, Figure 6A-6C). IL-13 (Figure 6D) and TGF-β1 (Figure 7A), important fibrogenic mediators, were significantly lower compared than in mice treated with saline ($P < 0.05$). In the supernatant splenocyte culture obtained from the monocyte- and BMMC-treated groups there was a significant decrease in TGF-β1 compared with the saline-treated mice ($P < 0.0$, figure 7B). IL-17 cytokine levels were also lower in animals undergoing cell transplantation ($P <0.01$, Figure 7C). A trend was also observed for decreased IL-23 cytokine levels (Figure 7D).

Monocyte Therapy altered MMP-9, TIMP-1 and IL-10 Hepatic Levels

The levels of MMP-9 and TIMP-1, two relevant factors associated with liver fibrosis, were evaluated. A significant increase in the production of MMP-9 was found in animal treated with $CD11b^+$ $CD14^+$ monocytes and BMMC ($P < 0.05$, Figure 8A). Interestingly, TIMP-1 levels were significantly lower in $CD11b^+$ $CD14^+$ monocyte-treated mice ($P < 0.05$; Figure 8B). The monocyte-treated group also showed significantly increased levels of IL-10 in comparison with the saline-treated group ($P < 0.05$, figure 8C).

Monocyte Therapy Increases GSH Levels

GSH levels were determined to evaluate the influence of $CD11b^+$ $CD14^+$ monocyte therapy on oxidative stress. Monocyte-treated mice with chronic liver damage had significantly higher levels of this antioxidant molecule than the saline-treated group ($P < 0.05$, Figure 8D).

DISCUSSION

The present study corroborates the importance of monocytes/macrophages in liver repair. These may act to regulate some significant fibrogenic pathways, in a murine model of chronic liver damage. Monocytes/Macrophages are cells with great

plasticity and, depending on the tissue microenvironment, may be caused to adopt a profile that contributes to resolution/regression of experimental hepatic fibrosis[24].

The results of the present study demonstrate that transplantation of BMMC-derived CD11b$^+$ CD14$^+$ monocytes had beneficial effects on liver lesions, thereby causing a significant reduction in fibrosis, mainly by regulating important cytokines involved in the liver repair process. Previous work carried out by our group has already shown a decrease in collagen levels in a liver undergoing BMMC therapy[14]. However, the results obtained in the present study demonstrated an improvement in these parameters on BMMC-derived CD11b$^+$ CD14$^+$ monocyte infusion, with an almost twofold decrease in the collagen levels using the same experimental model.

Macrophages, important mediators of inflammatory responses, have a dichotomous response when activated, assuming a classical (M1) or alternative (M2) pathways phenotype depending on the environmental stimulus[27]. The increase in the number of hepatic resident macrophages (Kupffer cells) after cell therapy observed in our study suggests that the subsets of restorative macrophages are involved in the tissue repair by inhibiting the production of proinflammatory cytokines (TNF-α, IL-1β and IL-6)[28]. Previous studies have reported the role of macrophages in mediating liver fibrogenesis, and have proposed using macrophage subpopulations during liver damage and repair[23, 29]. Treatment carried out in experimental models have shown that the infusion of bone marrow-derived macrophages decreases fibrous tissue, and enhances hepatic regeneration[22, 30,31].

The decrease in fibrous liver tissue observed in the present study may be associated with the lower number of activated HSCs found. The pro-fibrogenic role of this cell type has been already reported in the literature, indicating a direct relationship between murine liver fibrosis and the rise in the number of activated HSCs[3, 32]. In this regard, some studies have reported a decrease in the number of α-SMA$^+$ cells in murine models of liver damage treated with BMCs. This decrease is probably due to an alteration in the modulation of HSCs by specific cytokines and growth factors, including TGF-β1, TNF-α, and also ROS, produced by hepatocytes in

a damaged liver[33]. As activation of HSCs mediated by autocrine and paracrine signaling and these cells not only secrete cytokines, but also respond to them [33], it was hypothesized that BMMC-derived $CD11b^+$ $CD14^+$ monocytes modulate the activity of HSCs by regulating the secretion of cytokines and growth factors.

The production of the proinflammatory cytokine profiles of TNF-α, IL-1β and IL-6 were inhibited in mice submitted to liver damage and treated with $CD11b^+$ $CD14^+$ monocytes. Furthermore, there was an increase in the synthesis of IL-10 cytokine, which is known for its Th2 profile and anti-inflammatory activity[34]. These results show the influence of $CD11b^+$ $CD14^+$ monocyte infusion in the hepatic production of inflammation and fibrogenesis mediators. The modulation of inflammation during liver repair processes by way of increased expression of IL-10 and inhibition of the production of TNF-α, IL-1β and IL-6 is well described in the literature[35]. Because of their role in activating and proliferating HSCs, these cytokines have been implicated in the pathogenesis of chronic liver inflammation, mainly by increasing the production of collagen and regulating MMPs and TIMPs in liver damage[36, 37]. Gene therapy studies have shown that the overexpression of IL-10 reduces the expression of pro-fibrotic molecules such as TGF-β1 and TNF-α [37], thereby downregulating the inflammatory response and reducing activated HSCs, which ultimately leads to the reestablishment of liver function[36,37].

The present study found a significant decrease in TGF-β1 levels in both the extracts of liver protein and the supernatant of cultured splenocytes. These results corroborate other findings of the study, thereby indicating that transplanted monocytes play an important anti-fibrogenic role. TGF-β1 is a growth factor which plays a crucial role in initiating and maintaining liver fibrogenesis[4]. This factor is directly involved in activating HSCs and synthesizing ECM components, mostly in type I collagen [4]. It also plays an important role in inhibiting the degradation of ECM, stimulating the decrease of MMP synthesis and increasing the production of TIMPs, which leads to excessive deposition of collagen and the establishment of hepatic fibrosis[2]. Previous studies have associated the improvement of experimental liver fibrosis after BMMC-treatment with the reduction in TGF-β1 levels[20, 22]. The

results of the present study suggest that monocyte therapy acts through this fibrogenic pathway, thereby contributing to reducing liver fibrosis in mice.

The present investigation showed that cell transplantation caused a significant decrease in IL-17 levels, an effector proinflammatory cytokine, produced by $CD4^+$ T-cell[38]. This mediator induces the recruitment of inflammatory into liver cells and also directly activates natural hepatic immunity systems, such as neutrophils and dendritic cells, to release cytokines that perpetuate chronic inflammation[39]. Previous reports have reported that helper (Th) 17 T cells are able to participate in the pathogenesis of hepatic lesions associated with HBV[40]. Recently, emerging evidence has indicated that IL- 17 may be implicated in the induction of liver fibrosis, contributing to the activation of HSCs *in vitro*[40].

OPN is a glycoprotein expressed in a variety of tissues, mainly found in MEC and the sites of healing wounds[41]. Studies have shown that this protein is highly expressed in fibrotic liver tissue and influences the function of hepatic progenitors[42]. Under this condition, increases in the level of TGF-β and activation of HSCs could be also observed[6,42]. It thus seems reasonable to suppose that deactivation of OPN could lead to attenuation of liver fibrosis[1,8]. The results of the present study accordingly showed a significant decrease in the production of OPN and in the number of activated HSCs

GSH is an important antioxidant molecule that acts as a modulator of redox signaling, cell proliferation, apoptosis, immune responses and fibrogenesis[43,44]. Reduced levels of this molecule have been found in preclinical fibrosis models and in human fibrotic diseases[43]. A previous study has shown that higher GSH production inhibits the fibrogenic activity of TGF-β1[43]. The present study also found an increase in this molecule after $CD11b^+$ $CD14^+$ monocyte transplantation, suggesting an association between the anti-fibrotic effects observed in the monocyte-treated group and increased antioxidant activity of this cell population.

Alterations in the quantities of some molecules involved in fibrogenesis, as well as fibrous tissue remodeling, were assayed in this study. The $CD11b^+$ $CD14^+$

monocyte therapy in mice with chronic liver damage caused an increase in MMP-9 hepatic levels. Previous studies have associated reduced liver fibrosis with fibrous tissue degradation[3]. MMP-9 plays an important role in resolving liver fibrosis and has been considered a potent therapeutic target[11]. Yang et al.[45] suggest that, in the hepatic microenvironment, macrophage subpopulations play an anti-fibrotic role, as they express several MMPs, including MMP-9, which are directly involved in degrading ECM, facilitating the resolution of hepatic fibrosis.

$CD11b^+$ $CD14^+$ monocyte transplantation gave rise to a reduction in hepatic TIMP-1 and IL-13, two important pro-fibrogenic mediators. TIMPs are involved in the regulation of fibrogenic response by inhibiting the enzymatic activity of MMPs, having an anti-apoptotic effect on HSCs[9]. The presence of high quantities of these inhibitors in chronically damaged hepatic tissue may contribute to the establishment of liver fibrosis[9]. IL-13 is a cytokine associated with severe forms of schistosomal liver fibrosis as well as non-schistosomiasis liver diseases[47]. IL-13 is considered one of the central mediators in liver pathogenesis and is involved in TGF-β1 production by liver cells, besides inducing progenitor cells to transdifferentiate into myofibroblasts, which produce collagen[48]. The data produced by the present study corroborates the protective role of monocytes/macrophages in tissue repair processes, by way of fibrogenic pathways.

Several studies have attempted to identify and to correlate different macrophage profiles to tissue repair processes[29,30, 49]. Ramachandran et al[29] found that $Ly6C^{low}$ macrophages secrete large amounts of fibrolytic MMPs such as MMP-9 and MMP-13, as well as IL-10. Therefore the increase in secretion of MMP-9 and IL-10 observed in this study suggests a downregulation of the activation pathways that lead to the chronic inflammatory response.

In conclusion, the present study shows the important contribution of bone marrow-derived monocyte/macrophage cell therapy to improving the state of liver fibrosis in a murine model of chronic liver damage. These cells act to modulate inflammation and fibrogenesis and regulate the oxidative stress caused by damaged

tissue. Further studies should be conducted to establish a promising therapeutic tool for treating chronic liver diseases.

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COMMENTS

Background

Chronic liver disease is characterized by alterations in the process of tissue repair, such as the excessive deposition of fibrous tissue and the inhibition of the dynamics of regeneration. The knowledge on bone marrow cell therapy has opened new perspectives towards treatment of hepatic diseases. However, the cell types are involved in liver recovery has not been fully elucidated. Monocytes have emerged as one set of potential candidates due to their plasticity and involvement in inflammation and tissue repair.

Research frontiers

Previous experiments have already showed that bone marrow cells transplantation promotes improvement in experimental model of liver fibrosis. Monocyte/macrophage lineage may have important therapeutic potential in chronic liver diseases.

Innovations and breakthroughs

This is an innovative study that evaluated the effects of monocyte transplantation isolated from bone marrow mononuclear cells, by morphological, biochemical and immunological assays.

Applications

Experimental hepatic fibrosis improvement after cell therapy reinforces the potential involvement of monocytes/macrophages in liver repair, being able to acquire pro-resolute profile, acting in the regulation of some relevant inflammatory and fibrogenic pathways.

Terminology

Bone marrow mononuclear cells (BMMCs) used to collectively denominate bone marrow cells whose nuclei are unilobulated and which lack granules in the cytoplasm. This cell population includes hematopoietic progenitor cells, lymphoid cells (lymphocytes, plasma cells) and monocytes.

Peer-review

The authors addressed an interesting, clinically relevant and important issue aiming to modify the state of chronic liver disease. To approach this goal the authors purified bone-marrow derived CD11bhigh monocytes, which were transfused to mice prior the administration of the provoking agents i.e. ethanol and CCl4. Using a C57BL/6 mice model system they showed that the transfusion of monocytes was more effective to decrease IL-13 levels in the liver as compared to the infusion of bone marrow derived mononuclear cells (BMMCs). The authors also demonstrated that monocyte transfusion could reduce the size of the fibrotic area, the amount of hydroxyproline and the concentration of pro-inflammatory cytokines, while the levels of IL-10 cytokine and the number of Kupffer cells in the liver were increased as compared to saline transfusion. Due to the limited information of cell based therapies in chronic inflammatory diseases, the identification of immunostimulatory and regulatory pathways in a preclinical setting and in the context of liver metabolism is of high importance.

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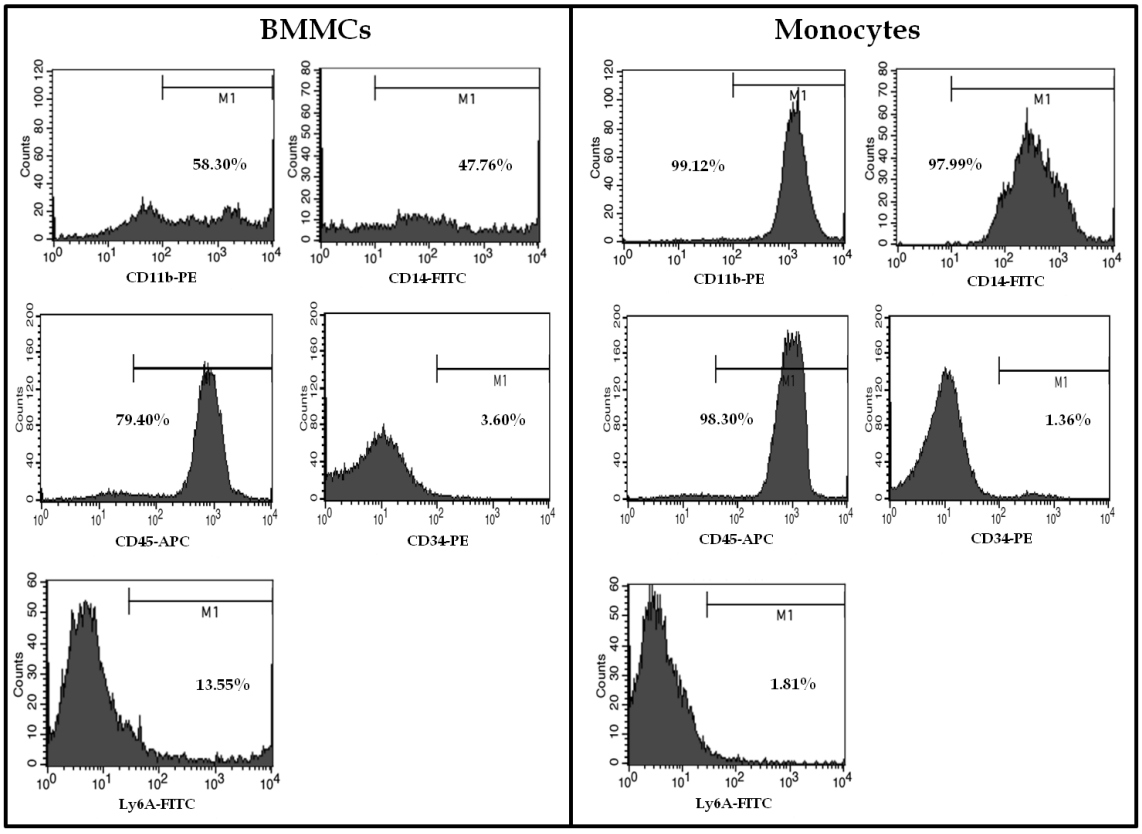


Figure 1: Representative FACS histograms of BMMCs and CD11b$^+$ monocytes isolated by immunomagnetic separation.

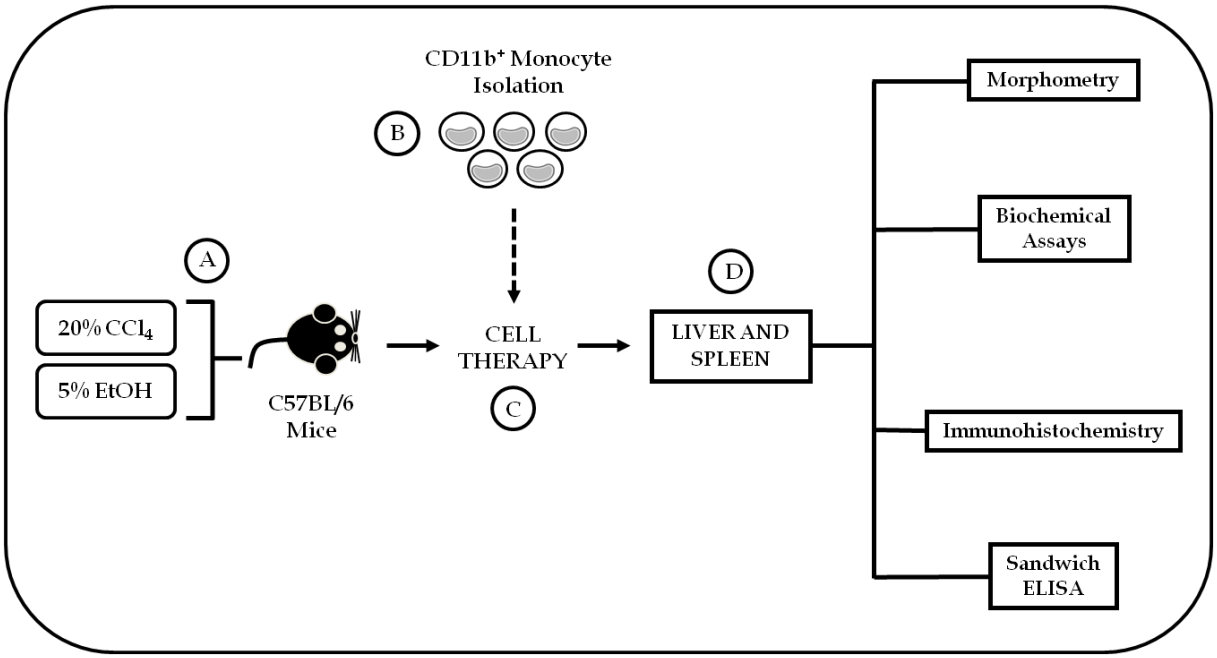


Figure 2. **Schematic flowchart of experimental design.** (A) Male C57BL/6 mice underwent chronic administration of CCl_4 and EtOH solutions for six months. (B) BMMCs were harvested from C57BL/6 donor mice for CD11b$^+$ monocyte isolation using immunomagnetic separation. (C) Chronically liver damaged mice underwent cell therapy, and after two months the effects of the treatment were evaluated (D) using morphometric, biochemical, immunohistochemistry and Sandwich ELISA analysis.

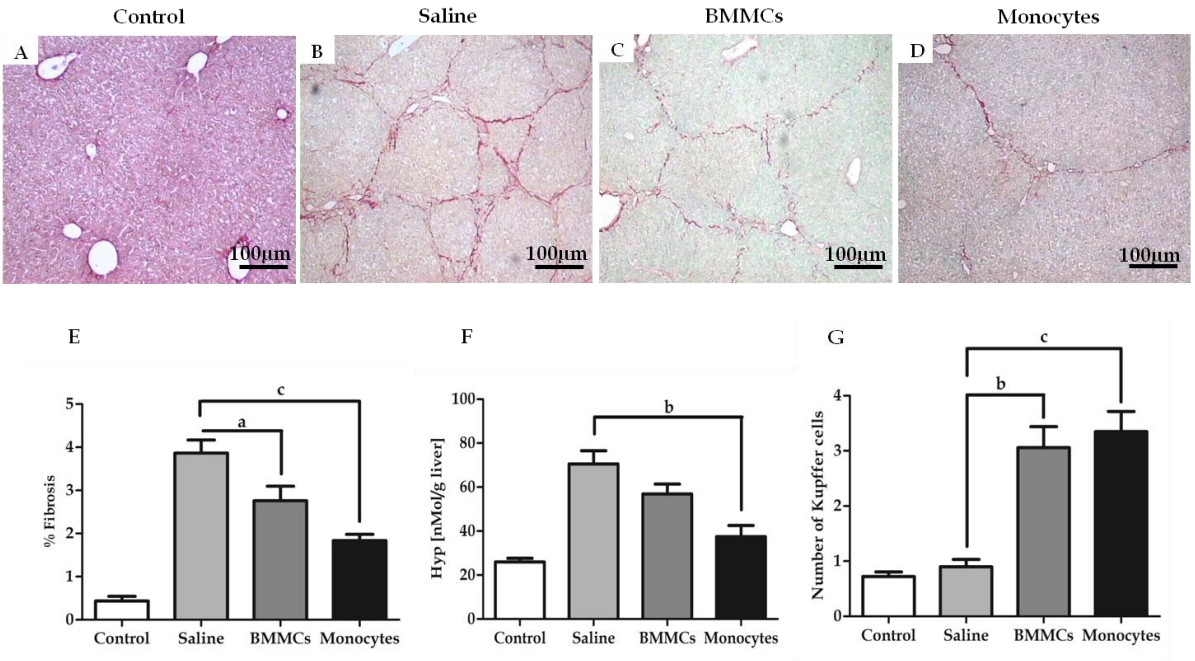


Figure 3. Photomicrographs of histological liver sections stained with picro-Sirius red showing hepatic collagens in (A) control mice, mice after CCl_4 administration and treated with (B) saline, (C) BMMCs, and (D) BMMC-derived monocytes (picro-Sirius red, 100X). (E) Morphometric evaluation of picro-Sirius Red-stained sections. (F) Hydroxyproline in liver fragments of mice undergoing cell transplantation. (G) Kupffer cell count in H&E-stained histological liver sections, in mice undergoing $CD11b^+$ $CD14^+$ monocyte therapy and BMMC-treated mice (a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$).

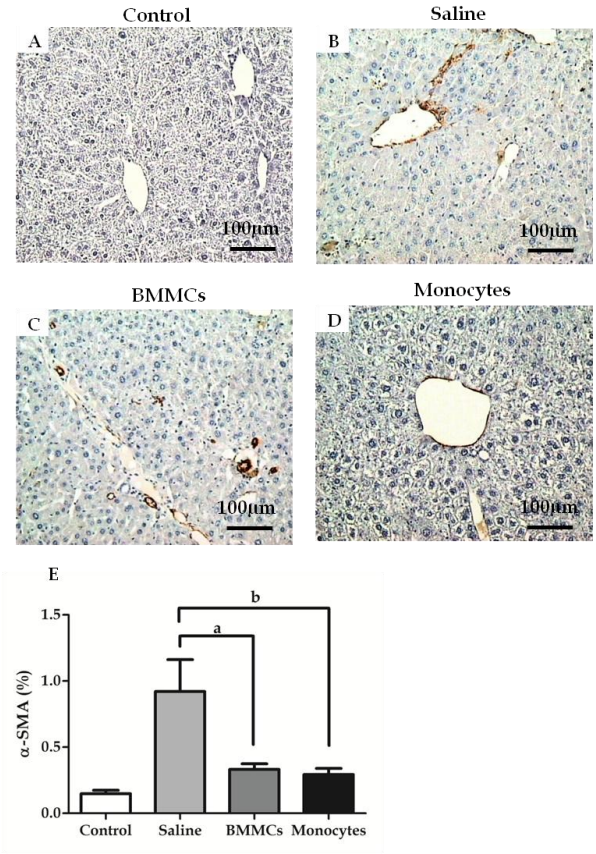
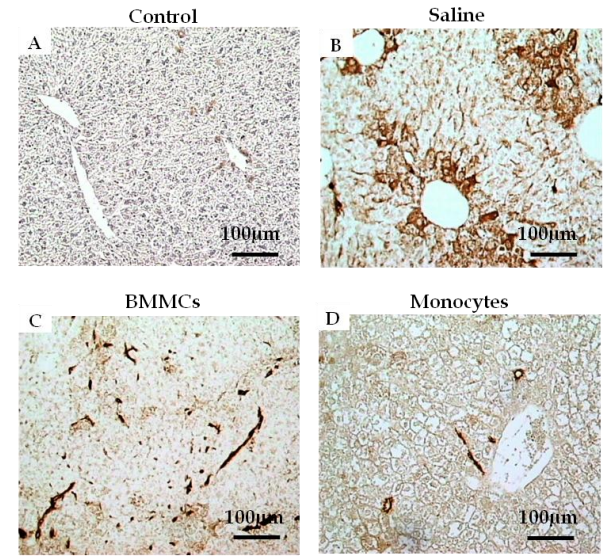


Figure 4. Immunohistochemistry for detection of α-SMA$^+$ HSCs in histological sections of (A) control mice, (B) saline-treated, (C) BMMCs-treated and (D) CD11b$^+$ CD14$^+$ monocyte-treated groups (200x). (E) Measuring of α-SMA$^+$ HSCs two months after treatment with CD11b$^+$ CD14$^+$ monocytes and BMMCs (a = $P < 0.05$; b = $P < 0.01$).



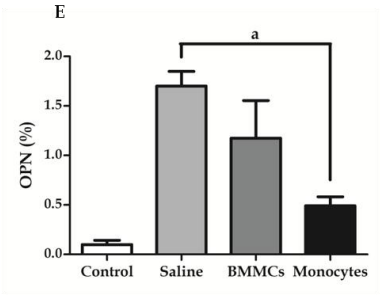


Figure 5. Immunohistochemistry for detection of OPN in histological sections from (A) control mice, (B) saline-treated, (C) BMMCs-treated and (D) $CD11b^+$ $CD14^+$ monocyte-treated groups (200x). (E) Levels of hepatic OPN two months after treatment with $CD11b^+$ $CD14^+$ monocytes (a = $P < 0.05$).

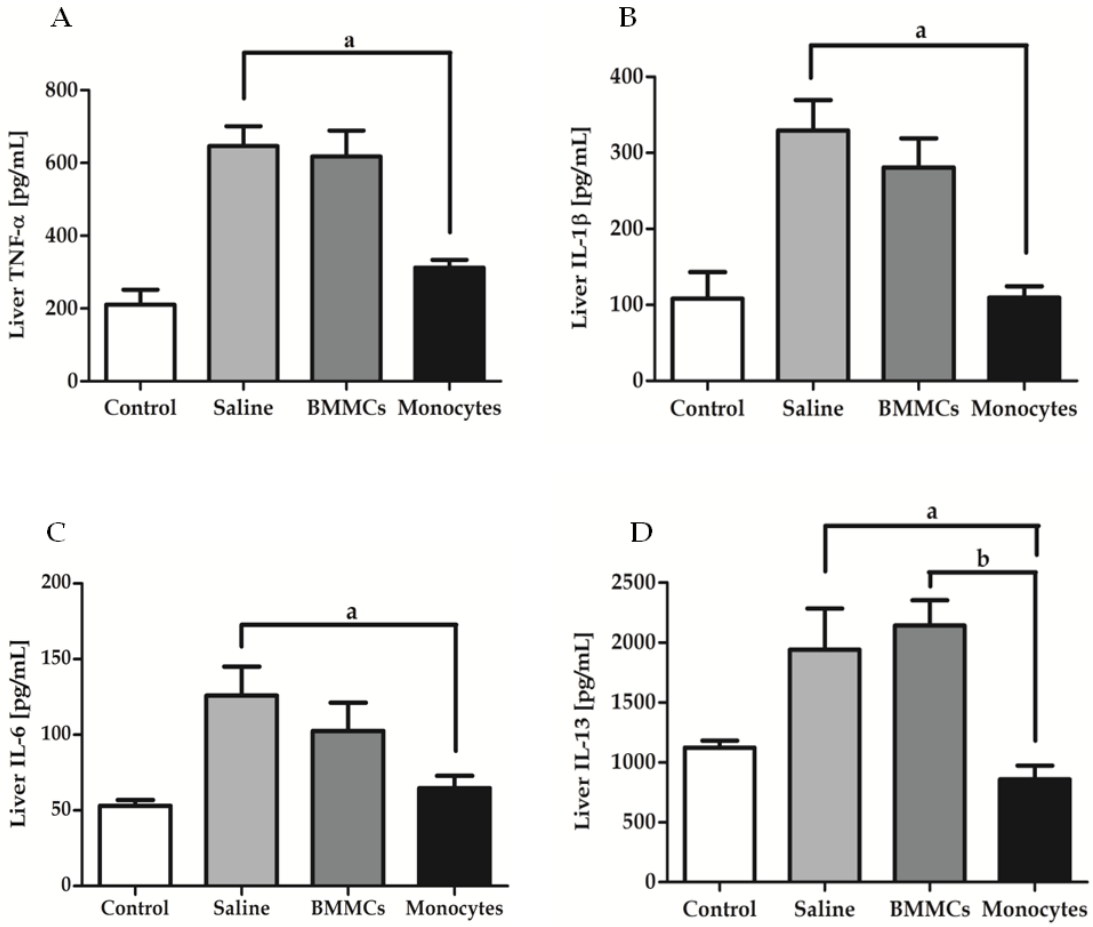


Figure 6. Effects of monocyte therapy on cytokine profile (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-13, as measured by ELISA. Data are represented graphically as the mean ± SEM of five mice/group (a = $P < 0.05$; b = $P < 0.01$).

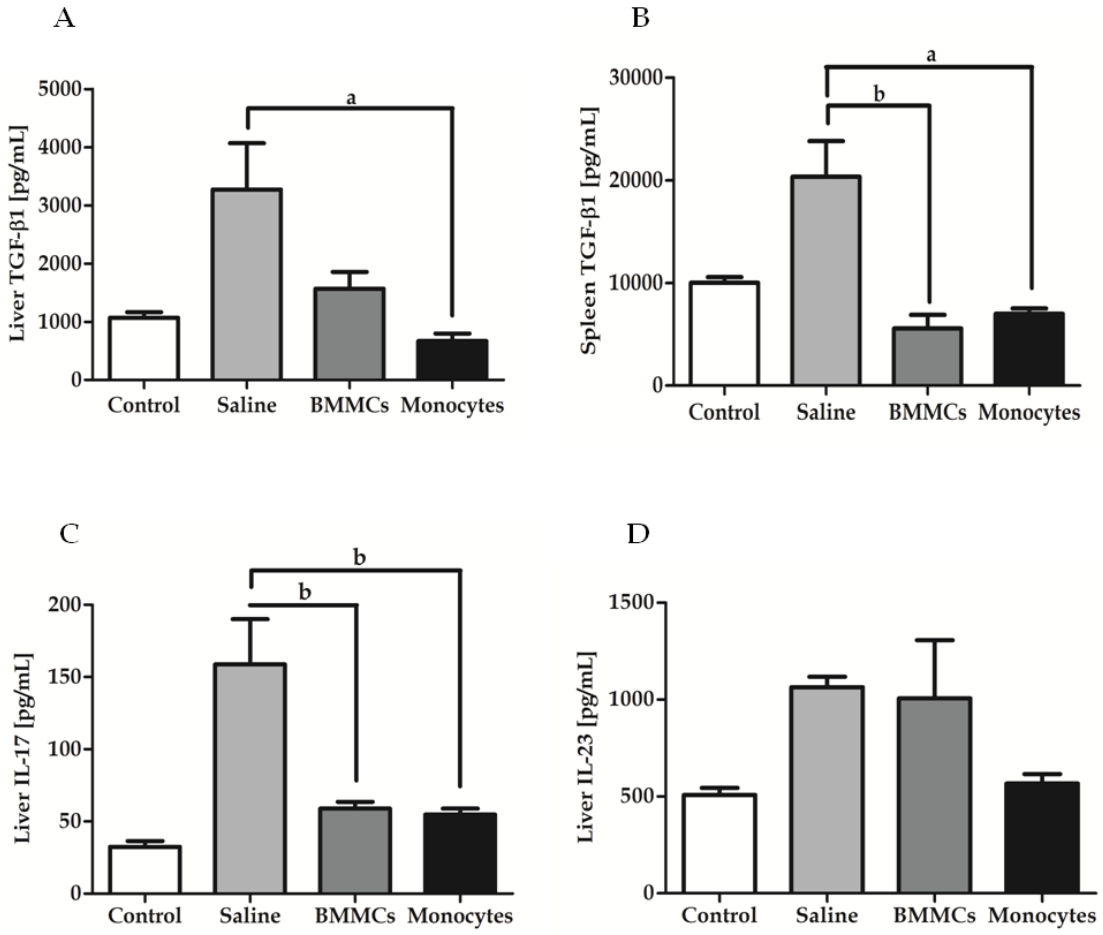


Figure 7. Effects of monocyte-based therapy on chronically liver damaged mice: (A) hepatic levels of TGF-β1; (B) splenic levels of TGF-β1; (C) hepatic levels of IL-17 and (D) IL-23 (a = $P < 0.05$; b = $P < 0.01$).

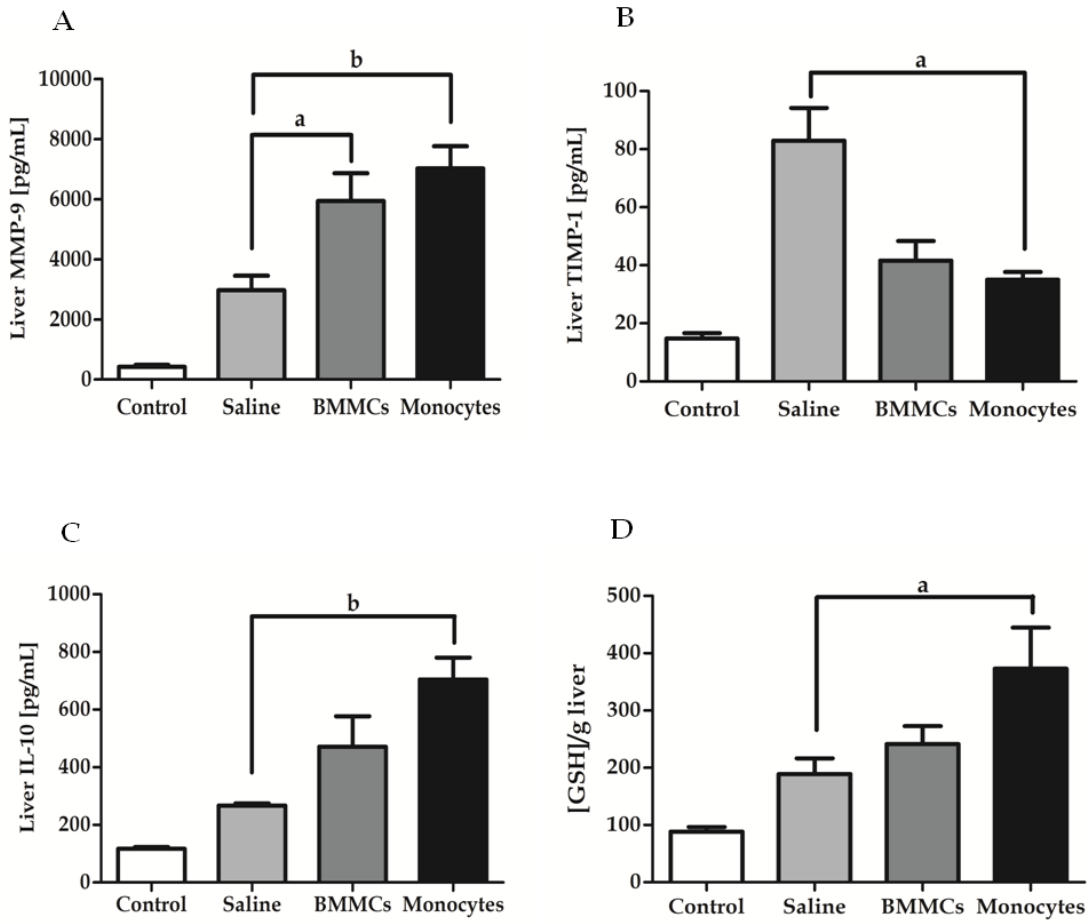


Figure 8. Effects of monocyte-based therapy on hepatic levels of (A) MPP-9, (B) TIMP, (C) IL-10 and (D) GSH, in chronically liver damaged mice (a = $P < 0.05$; b = $P < 0.01$).

APÊNDICE B - Manuscrito II: Bone marrow-derived monocytes combined with praziquantel ameliorate Schistosoma mansoni-induced liver fibrosis by increase of IL-10 and downregulation of CCR2

Título: **Bone marrow-derived monocytes combined with praziquantel ameliorate Schistosoma mansoni-induced liver fibrosis by increase of IL-10 and downregulation of CCR2.**

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Introduction

Liver diseases represent a serious public health problem in the world. Continuous hepatic injury may result in alterations in liver repair processes, which culminate in establishment of liver fibrosis (1,2). It is known that a variety of cell types and soluble mediators are involved in the course of chronic liver injury, integrating inflammatory processes, fibrosis and ECM remodeling (3). Hepatocellular necrosis resulting from tissue injury promotes inflammation, followed by a healing response, mediated in liver by of hepatic stellate cells (HSCs) activation, which orchestrate the excessive production of extracellular matrix components (ECM) (4).

Liver fibrosis is a pathological condition present in most chronic liver diseases, responsible for high morbidity and mortality rate (5). In this context, the development of therapies for liver diseases is of great importance. Currently, liver transplantation has been the only therapeutic strategy for patients with advanced liver diseases (6). However, it consists of a high risk procedure with a limited number of donors (6). In view of these aspects, it is important to develop new therapeutic strategies for chronic liver disease.

In the search for biological substitutes that maintain, improve or restore functions of organs and tissues of human body, cell therapy has been shown to be an attractive option for chronic liver diseases treatment (7). Investigations about the therapeutic potential of some cell populations indicate that bone marrow cells (BMC) may contribute to tissue repair process. Preclinical studies has been shown that BMCs are capable of differentiating into cells similar to hepatocytes (8). In addition, BMC transplantation was able to reduce mortality (9,10) and decrease liver fibrosis levels in murine models of liver injury (11, 12).

Recent studies have highlighted that monocyte/macrophage lineage cells present in bone marrow may present a relevant therapeutic potential for liver disease due to its plasticity

and its known participation in several processes, since inflammation and fibrogenesis to the resolution of fibrosis (13). Recent research has been investigated the behavior of monocytes/macrophages against chronic-degenerative diseases, in different preclinical models, as well as in studies with patients. Therefore, the results obtained allow consider these cells as potential therapeutic tools for the treatment of chronic liver diseases (14).

Involvement of macrophages in several biological and pathological processes has been the subject of study of many research groups in recent years. This functional plasticity of macrophages is directed by immunological microenvironment, and can be activated by different pathways (15). Intensive studies with liver injury experimental models and liver disease patients have sought to elucidate and establish dichotomies that describe the complex heterogeneity of monocyte and macrophago subsets in the liver (16).

In response to various signals, macrophages may undergo polarization by the M1 classical pathway or M2 alternatively pathway. The M1 phenotype is characterized by high expression of proinflammatory mediators (TNF-α, IL-1β, IL-12 and IL-23), with strong microbicidal and tumoricidal activity; The M2 or immunoregulatory phenotype is involved in tissue remodeling, response to parasitic infections and allergies, through secretion of immuno-modulatory factors (17,18). More recently, *in vitro* studies have designated M2 macrophages in subgroups, associated with immune regulation, tolerance and tissue repair (19).

Understanding the different macrophages phenotypes may contribute to the development of new therapeutic approaches for various diseases, including liver diseases. Some studies have shown that macrophage populations may play a protective role (14), actively participating in hepatic repair, by expressing MMPs (20) and inducing HSCs apoptosis (21). Studies on monocyte/macrophage therapy have been highlighting various chronic-inflammatory diseases, which leads to the need for new approaches on the real

therapeutic potential of this cell population, mainly for the treatment of diseases which the existing therapeutic options are ineffective.

Materials and methods

Animals. Sixty male C57BL/6 mice weighing 20-23 g were used in this study. Mice were Purchased from the Animal Breeding Center Laboratory (CECAL) Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Rio de Janeiro, Brazil), and housed in the animal research facility in the Aggeu Magalhães Research Center (CPqAM), (FIOCRUZ, Recife, Pernambuco, Brazil). They were acclimatized in a 12 h light/dark cycle. Animal had a free access to food and water. Animal care and the experiments were conducted in accordance with the approval of Ethics Committee for the Certified Use of Animals (CEUA-CPqAM 15/2011).

Experimental model of chronic schistosomiasis. Mice were submitted to infection by *S. mansoni*, subcutaneously, with 40 cercariae (LE strain). After 45 days, parasitological examination of feces was performed to confirm the infection. A period of 16 weeks was necessary for the establishment of the chronic phase of the schistosomiasis infection.

Chemotherapy. Before the cell therapy, infected animals were submitted to the chemotherapeutic treatment with Praziquantel, for elimination of adult worms. Administration was performed in a single dose orally at a concentration of 400 mg / kg body weight.

Isolation and characterization of bone marrow-derived monocytes: Bone marrow of femurs and tibia of donors C57BL/6 mice was extracted and used to obtain bone marrow mononuclear cells (BMMCs) by Ficoll gradient centrifugation (Histopaque 1119 and 1077 , Sigma Aldrich, St Louis, MO, USA) at 1000 g for 15 minutes. BMMCs were incubated with anti-CD11b antibodies conjugated to magnetic beads for monocyte isolation through immunomagnetic separation system as previously described (22). At the end of the procedure,

about 106 cells / mL were recovered. The monocytes obtained by immunomagnetic separation were characterized phenotypically following the previously described methodology (22), presenting as a homogeneous Cd11b $CD14^{+}$ monocytes population.

*Experimental design***.** After the establishment of chronic schistosomiasis model, liver fibrotic mice were randomly distributed into following groups with five mice in each group: Group I: Untreated animals, wich received saline; Group II: Animals BMMC-treated; Group III: Animals monocyte-treated. A group of healthy animals also added to study (Group IV-control).

Cell infusion. Mice were injected with three doses of 10^{6} BMMCs or monocytes per animal, suspended in 200 μL sterile saline, into intravenously. The procedure was performed once a week for three consecutive weeks. Two months after cell therapy, animals were submitted to euthanasia for liver collection.

Morphometry. Specimens of the liver tissues were fixed in 10% formalin and embedded in paraffin wax. Sections (5-μm tick) were stained by Sirius-red and Fast green for collagen study. Images of histological sections stained in Red Sirius were obtained by light microscope (DM LB 2, Leica Microsystems) equipped with a LEICA JVC TK digital camera (model C 1380, Pine Brook, NJ, USA) analyzed using the Image Analysis Processing System LEICA QWIN, version 2.6 MC (Leica, Cambridge, UK). Ten microscopic fields (100X magnification) containing fibrous tissue areas were selected for quantification. From the histological sections stained in Sirius-red, some parameters of hepatic granuloma evaluation were also calculated: numerical density (number of granulomas/unit volume) and granuloma volume. Analyzes were done under an optical microscope (DM LB 2, Leica Microsystems).

Hydroxyproline Mensuration. Liver samples (weighing 100-200 mg) were used to determine the hydroxyproline constituent of collagen. Samples were processed and analyzed according

to methodology previously described (23), read in automatic spectrophotometer (Pharmacia, model Ultrospec 3000), at 558 nm, to obtain the hydroxyproline values of each sample (nmol/g tissue).

Immunological assays. For cytokine and growth factors quantification in frozen livers were processed for obtation soluble proteins following a previously described protocol (22). Supernatants were used to quantifie TNF-α, IL-6, IL-1β (BD OptEIA set mouse, San Diego, CA, USA), IL-13, IL-17, IL-23, MMP-9, TIMP-1 (R & D Systems, Minneapolis, MN, USA), TGF-β1 and IL-10 (e-Bioscience, San Diego, CA, USA) levels. Samples were read at a 450 nm wavelength using a microplate reader (model 3550, Thermo Scientific).

Gene expression evaluation. Total RNA was extracted and purified from liver tissue using Trizol (Invitrogen). Complementary DNA was synthesized from total RNA by reverse transcription (RT) using GoScript Transcription System (Promega). Sequences of primers are listed in Table 1. Expression of mRNA was assessed by qPCR using SYBR® Green PCR Master Mix (Applied Biosystems) ABI PRISM 7500 sequence detector (Applied Biosystems, CA, USA). qPCR was performed for the following targets: CCl5, IL-12β, CCR2 / Ly6C, Arginase 1, chitinase-3-like protein 3 (YM-1) Mannose 1 receptor-type C (CD206), Fizz1 (alpha 1 molecule Similar to resistin), α-SMA, TGF-β1 and Gal-3. Quantification was performed using the comparative ΔΔCT method method by Applied Biosystems RQ Software, normalized with the reference gene, β-actin.

Statistical Analysis. Data were expressed as mean values (mean ± ED). Values were submitted to Kruskal-Wallis non-parametric test, with Dunn post hoc. Statistical analyzes were performed using Graphpad Prism program (version 5.0, San Diego, CA, USA). The P value <0.05 was considered statistically significant.

Results

Assessment of liver fibrosis. In a murine model of schistosomiasis, liver fibrosis quantification levels by morphometric analysis showed that seven days after cell therapy, there was no difference in percentage of fibrosis among the experimental groups. However, two months after therapy, monocyte-treated group had decreased levels of liver fibrosis compared to untreated animals (Figures 1B, 1D and 1E). Biochemical quantification of hydroxyproline in liver tissue showed significant differences between the monocyte-treated group and the saline-treated group, in the two evaluated times (Figure 1F). Numerical density of hepatic granulomas measurement did not present significant differences between the groups evaluated (Figure 2A). However, granuloma volume morphometry showed a significant reduction seven days and two months after monocyte transplantation (Figure 2B).

Immunological analyzes. Quantification of inflammatory mediators in chronic model of schistosomiasis showed that TNF-α concentration was significantly decreased, two months after cell therapy (Figure 3A). Hepatic levels of IL-1β and IL-6 were significantly reduced in monocyte-treated group, as well as in animals submitted to CMMO transplantation, in both evaluated times (Figures 3B and 3C). Changes in hepatic levels of pro-fibrogenic mediators following monocyte cell therapy. Significant reduction in TGF-β1 levels was observed in the two time points evaluated (Figure 4A), while the hepatic reduction in IL-13 levels occurred in the animals evaluated seven days after cell therapy (Figure 4B). The study also demonstrated significant reduction liver IL-17 levels seven days after infusion of monocytes (Figure 4C), and cytokine IL-23, in the two times studied (Figure 4D).

The present study also evidenced the significant increase in MMP-9 levels after cell transplantation (Figure 5A). Consistent with these results, significant reduction in hepatic TIMP-1 concentration was also observed (Figure 5B). Monocyte-treated animal also

demonstrated significant increase in IL-10 levels compared to saline-treated animals two months after therapy (Figure 5C).

Gene expression evaluation. The present study identified, by RTqPCR, significant reduction in expression of the marker of HSCs, α-SMA, two months after cell transplantation (Figure 6A). TGF-β1 and gal-3 expression were significantly decreased in liver of animals submitted to cell transplantation, in two evaluated times (Figures 6B and 6C). Monocyte therapy induced reduction in liver expression of markers associated with activation M1macrophages profile CCL5 (Figure 7A), IL-12β (Figure 7B) and CCR2 / Ly-6C (Figure 7C), seven days and Two months after cell therapy. Interestingly, the molecular study also showed a significant reduction in expression of markers M2 profile Arg-1 (Figure 8A), YM-1 (Figure 8B) and CD206 (Figure 8C). However, the Fizz1 marker was shown to be significantly increased after cell transplantation (Figure 8D).

Discussion

Our research has reinforced the use of monocyte therapy as promising therapeutic modality for chronic liver diseases, and has already been reported in previous studies developed by our group (22). Monocyte/macrophage cell lineage have been an interesting object of investigation in recent years, taking into account their role in inflammation and tissue repair (24). Inflammatory macrophages, which promote inflammation and liver fibrosis, are activated by use of TLR, with release of inflammatory (TNF, IL-1β) , pro-fibrogenic cytokines (TGF-β) and chemokines (MCP-1, CCL5); and restorative macrophages involved in resolution of inflammation (anti-inflammatory cytokines) and liver fibrosis (MMP-9), with low expression of 6C lymphocyte antigen surface molecule (Ly6C) in mice (25).

It can be considered that, in relation to chronic liver diseases, the removal of aggressive stimulus associated with anti-fibrotic therapy can promote promising results.

Monocyte therapy isolated from bone marrow associated with Praziquantel chemotherapy was shown to be promising for liver fibrosis induced in mice by chronic *S. mansoni* infection. These findings corroborate results obtained previously (22) where it was verified indicative of improvement in several parameters observed in present study, after monocyte therapy, in liver fibrosis model CCl_4-induced. In addition, this study aimed to identify predominance of possible macrophages subtypes after bone marrow monocyte therapy, by evaluation of specific markers.

Morphometric study of fibrous tissue as well as volume of liver granulomas showed that cell therapy proposed here was capable of producing anti-fibrogenic effects in murine model of chronic schistosomiasis. Reduction of liver fibrosis levels corroborates the results obtained in liver mensurament of pro-fibrotic (TIMP-1, IL-13, TGF-beta) and anti-fibrotic factors (MMP-9). In our investigation, decrease in activated HSCs after monocyte transplantation was identified by reduction of α-SMA liver expression, evaluated by RT-qPCR, suggesting that macrophages may contribute to decrease fibrogenic activity attributed to HSCs. Previous work has associated BMCs transplantation with reduction of liver fibrosis and decrease in number of activated HSCs, positive for α-SMA (12).

Decrease in TNF-α, IL-1β and IL-6 cytokines was observed in liver protein extracts from monocyte transplant recipients. Several inflammatory cytokines collaborate to establish liver fibrosis pathogenesis by promoting myofibroblast differentiation (26). TNF-α and IL-1β are important mediators of fibrosis: TNF-α plays a critical role in liver fibrosis murine model induced by CCl4 (26); IL-1β is a potent inflammatory mediator that promotes the activation of myofibroblasts through TGF-β-dependent mechanisms (27). Both cytokines also induce the production of IL-6, which exhibits autocrine activity in fibroblasts, and is involved in the induction of hepatic fibrosis induced by CCl_4 (26).

Our study evidenced increase in IL-10 cytokine production after monocyte therapy. This anti-inflammatory cytokine plays a central role in regulating immune response, attenuating inflammation and thus, preventing host lesions, and has been strongly associated with M2reg profile (13,28). In liver, it has a protective function during establishment of chronic injury (29). In addition, it has been reported as a cytokine that supress the pro-fibrogenic activity of HSCs (30), and has been shown to inhibit collagen production and TGF-β secretion (29). These results corroborate the description by Ju & Tacke (16), which suggests the participation of restorative macrophages in modulating the inflammatory and fibrogenic response.

Immunological and molecular assays have shown that monocyte treatment promoted anti-fibrogenic effects by significantly reducing liver TGF-β1 in Schistosomiasis murine model. These data complement the data obtained by morphometric and biochemical analyzes of liver fibrosis, since TGF-β1 is characterized as one of the main pro-fibrogenic mediators in chronic liver injury, acting on phenotypic conversion of HSCs to an active and highly active state. (31), as well as contribute to downstream between synthesis and degradation of healing tissue (32). Previous studies in cell therapy have reported hepatic improvement associated with decreased TGF-β levels (11,33).

Our research also identified that monocyte therapy was also able to interfere in IL-13 production. Considered a potent pro-fibrotic Th2 cytokine, it has been extensively reported in schistosomiasis murine model, directly associated with granuloma formation and subsequent development of liver fibrosis (39). Anti-fibrosing therapies that act on your block may prevent liver fibrogenesis processes.

IL-17 and IL-23 had their levels reduced following monocyte therapy. Previous work has associated high IL-17 production to the immunomodulation processes of liver granuloma,

characteristic of pathogenesis of mansonic schistosomiasis (34), as well as HSCs activation (35). Our findings corroborate previous studies that identified IL-23 as an important proinflammatory cytokine inducing the Th17 fibrogenic response (26,36); in addition to its known Participation in the response produced by inflammatory macrophages M1 (18).

Interestingly, increase in fibrolytic enzyme MMP-9 levels were observed in this study. This result indicates, once again, anti-fibrogenic role that monocyte infusion can exert against liver fibrosis, as reported in previous studies (22; 37). A study conducted by Ramachandran et al. (25) identified that a population of $Ly6C^{low}$ macrophages have an anti-fibrotic role in liver, since they secrete MMPs. Research proposes increasing production and fibrolytic activity of MMPs should be considered for development of anti-fibrogenic therapeutic approaches (38).

The present investigation also showed that monocyte therapy promoted significant reduction in Gal-3 gene expression. Galectin-3 consists of pleiotropic protein, which plays an important role in cell proliferation, adhesion, differentiation, angiogenesis and apoptosis, and more recently has been associated with pathogenesis of hepatic fibrosis (39). During helminth infection by *S. mansoni*, Gal-3 is directly involved in modulation of inflammatory response (40). Although gal-3 is known to participate in HSC activation, it is still unclear whether observed effects are related to a direct action of Gal-3 on HSC, or whether there is a side effect responsible for change in liver cytokines that promote the liver fibrosis establishment (41). Pre-clinical studies have reported that the use of Gal-3 inhibitors promotes resolution of liver fibrosis in different experimental models (19,42). Oliveira et al. (11) demonstrated that BMCs therapy was able to promote reduction of liver fibrosis associated with decreased liver expression of Gal-3 in murine model of cirrhosis induced by CCl4. Based on these findings it can be said that monocyte therapy acted once again in an important fibrogenic pathway.

Several investigations have sought to identify and correlate distinct functional macrophages subgroups in tissue repair processes (21, 30,43). In the present study, molecular markers associated with M1 and M2 macrophages profiles were evaluated after monocyte therapy in parasite model of chronic liver injury. In our findings, a significant reduction in CCl5 and IL-12β expression, markers associated with the classic profile macrophages activation (M1), was observed. These results corroborate findings of immunological analyzes, where significant reductions in inflammatory mediators levels associated to M1 profile were observed after monocyte transplantation. Wynn and Ramalingam (44) describe that M1 macrophages are associated with proinflammatory functions, which promote initiation of pro-fibrotic processes through the activation of myofibroblasts.

The molecular study also indicated that cell transplantation promoted the reduction of Arg-1, YM-1 and CD206 markers, associated to M2 profile, more specifically to the M2a or M2 fibrogenic profile (37,45). Arginase-1 (arg-1) is considered a marker prototype of M2 macrophages, and it is an enzyme that uses the amino acid L-arginine as the substrate for the production of L-ornithine. This serves as a raw material for collagen biosynthesis, and therefore, several studies have suggested that arg-1 is involved in fibrogenesis processes (46), and that M2a arg-1 + macrophages are directly associated with Fibrosis (47). In schistosomiasis, a large number of arg-1+ macrophages are located around liver granulomas (48). Ym1, is a lectin widely considered a M2 macrophages marker in mice, and its expression is highly regulated by IL-4, IL-13 and TGF-β (49); CD206 is a well known marker for both mouse M2 macrophages and human M2 macrophages (37). Beljaars et al. (50) associated high CD206 and YM-1 expression to the areas of hepatic fibrosis induced by CCl4.

Interestingly, evaluation of expression levels in a murine model of chronic schistosomiasis showed a significant increase in Fizz1 gene expression after monocyte therapy. The role of Fizz1 in Th2 immune response as well as in fibrogenesis is not yet fully

elucidated. There is no consensus regarding the relationship of this marker to an M2 macrophage subtype. Some publications associate the high expression of Fizz1 to pro-fibrogenic M2 macrophages (51). Murthy et al. (52) reported that macrophages with pro-fibrotic phenotype, positive for arg-1, had reduced expression levels of Fizz1.

The present study also showed significant reduction in liver expression of CCR2 after monocyte transplantation. The CCR2 chemokine receptor is involved in recruitment of monocytes to lesion areas, and is highly expressed in $Ly6C^{hi}$ monocytes / macrophages, induced during fibrosis progression (53). A study conducted by Mitchell et al (53) demonstrated that mice CCR2 knockout had significantly lower levels of CCl4-induced liver fibrosis compared to wild-type animals. This decrease in liver fibrosis was accompanied by reduction in number of CD11b+ F4/80+ cell populations. Recently, a new subtype of macrophage has been identified, which has been considered a critical element for the resolution of liver fibrosis. Ramachandran and colleagues (25) found in their investigations that $Ly6C^{low}$ macrophages secrete large amounts of fibrolytic MMPs, such as MMP-9 and MMP-13, as well as IL-10. Increase in MMP-9 and IL-10 levels were also observed in our study, suggesting that monocyte therapy influenced the regulation of activation pathways of macrophages involved in chronic inflammatory response.

Based on results obtained in present study, we can infer that monocyte therapy in murine models of chronic liver injury seems to contribute to regression of liver fibrosis, by combination of mechanisms, which involves ECM remodeling constituent of fibrous tissue by MMPs expression, suppression of pro-fibrogenic liver microenvironment and induction of inhibition of activated HSCs.

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Author contributions

Souza VCA performed the majority of experiments, analyzed the data and wrote the paper; Moura DMN, Bozza PT and Costa LAP performed the molecular and immunologic investigations; Castro MCAB performed the cellular isolation and characterization and immunologic assays; Figueiredo RCBQ and Araujo RE performed the morphologic and morphometric investigations; Oliveira SA designed and coordinated the research.

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Figure legends

Figure 1. Photomicrographs of histological sections of liver stained with picro-Sirius, showing liver fibrous tissue in animals of (A) control (B) vehicle, (C) CMMO and (D) monocytes groups, (100X), two Months after cell transplantation. (E) Quantification of fibrous tissue by Morphometry in parasitic model of chronic liver

injury, seven days and two months after therapy. (F) Hydroxyproline Quantification levels in parasite model of chronic liver injury, seven days and two months post-therapy (a = P <0.05; b = P <0.01).

Figure 2. (A) Numerical density measurement of liver granulomas by Morphometry in chronic liver injury model, seven days and two months after cell therapy. (B) Measurement of hepatic granuloma volume in chronic liver injury model, seven days and two months post-cell therapy (a = P <0.05; b = P <0.01).

Figure 3. Effects of cellular therapy on liver profile of proinflammatory cytokines (A) TNF-α, (B) IL-6 and (C) IL-1β, in chronic model of schistosomiasis, measured by sandwich ELISA. (A = P <0.05, b = P <0.01, c = P <0.001).

Figure 4. Effects of cell therapy in mediators (A) TGF-β1, (B) IL-13, (C) IL-17 and (D) IL-23 in chronic model of schistosomiasis, measured by ELISA sandwich . (A = P <0.05, b = P0.01).

Figure 5. Effects of cellular therapy on liver profile of (A) MMP-9, (B) TIMP-1 and (C) IL-10, in chronic model of schistosomiasis, measured by sandwich ELISA. (A = P <0.05, b = P <0.01).

Figure 6. Effects of cell therapy on liver expression levels of (A) α-SMA, (B) TGF-β1 and (C) Galectin-3, in chronic model of schistosomiasis, seven days and two months after cell therapy. The targets were quantified by qPCR (a = P <0.05; b = P <0.01).

Figure 7. Effects of cell therapy on liver expression levels of macrophages markers M1 (A) CCL5, (B) IL-12β and (C) CCR2. (A = P <0.05; c = P <0.001).

Figure 8. Effects of cell therapy on liver expression levels of M2 macrophages markers in chronic schistosomiasis model: (A) Arginase-1, (B) YM-1, (C) CD206 and (D) Fizz1. The targets were quantified by qPCR (a = P <0.05; b = P <0.01; c = P <0.001).

APÊNDICE C - Low transformation growth factor-β1 production and collagen synthesis correlate with the lack of hepatic periportal fibrosis development in undernourished mice infected with *Schistosoma mansoni*

Low transformation growth factor-β1 production and collagen synthesis correlate with the lack of hepatic periportal fibrosis development in undernourished mice infected with *Schistosoma mansoni*

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Undernourished mice infected (UI) submitted to low and long-lasting infections by Schistosoma mansoni *are unable to develop the hepatic periportal fibrosis that is equivalent to Symmers' fibrosis in humans. In this report, the effects of the host's nutritional status on parasite (worm load, egg viability and maturation) and host (growth curves, biology, collagen synthesis and characteristics of the immunological response) were studied and these are considered as interdependent factors influencing the amount and distribution of fibrous tissue in hepatic periovular granulomas and portal spaces. The nutritional status of the host influenced the low body weight and low parasite burden detected in UI mice as well as the number, viability and maturation of released eggs. The reduced oviposition and increased number of degenerated or dead eggs were associated with low protein synthesis detected in deficient hosts, which likely induced the observed decrease in transformation growth factor (TGF)-β1 and liver collagen. Despite the reduced number of mature eggs in UI mice, the activation of TGF-β1 and hepatic stellate cells occurred regardless of the unviability of most miracidia, due to stimulation by fibrogenic proteins and eggshell glycoproteins. However, changes in the repair mechanisms influenced by the nutritional status in deficient animals may account for the decreased liver collagen detected in the present study.*

Key words: *Schistosomiasis mansoni* - undernutrition - liver fibrosis - cytokines

Mice that were experimentally infected by *Schistosoma mansoni* can develop two distinct and sometimes mixed histopathological patterns during the chronic stage of schistosomiasis: scattered small periovular granulomas with mild nonspecific reactive hepatitis and/or a concentration of circumoval granulomas causing fibrotic expansion and the development of thin fibrous tracts connecting portal spaces (murine periportal fibrosis). This fibrosis mimics the human disease known as Symmers' fibrosis or "clay pipestem" fibrosis (Andrade & Warren 1964, Warren 1966, Andrade & Cheever 1993).

Henderson (1993), working with inbred male CBA/J mice chronically infected with *S. mansoni*, reported the development of two syndromes that pathologically and immunologically imitate the intestinal and hepatosplenic clinical forms of the human disease. Later, Silva (2004) found that these two histological patterns (scattered granulomas and periportal fibrosis) had similar immunological profiles in well-fed BALB/c mice.

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The periportal fibrosis is due to massive egg laying by *S. mansoni* females and the development of periovular granulomas around intrahepatic portal veins, leading to the obstruction and destruction of the vessel walls (Andrade 1987). Due to fibrogenic stimuli from antigens derived from miracidia bodies (Hang et al. 1974) and, to a lesser extent, from the eggshells themselves (de Walick et al. 2012), inflammatory cells and macrophages are activated and secrete fibrogenic cytokines such as transformation growth factor (TGF)-β1, interleukin (IL)-13 and other mediators, which contribute to the regulation of the inflammatory response as well as stimulate the activation of hepatic stellate cells (HSCs) also known as pericytes, fat-storing cells or Ito's cells (Geerts 2001, Gressner et al. 2002).

The HSCs are located in the perisinusoidal space (Friedman 2008). They are actin-containing cells with high plasticity and the capacity to become transformed into myofibroblasts, an important matrix synthesising cell also involved in vascular remodelling of the fibrous tissue (Baptista & Andrade 2005). In response to chronic hepatic injury, HSCs differentiate into myofibroblasts, expressing intermediary filaments of alpha-smooth muscle actin (α-SMA) and collagen and secrete components of the extracellular matrix (Friedman 2008), whose stability is regulated by metalloproteinases and their tissue inhibitors, according to Hemmann et al. (2007).

Investigations of the interrelationships between schistosomiasis and host nutritional status have demonstrated that mice infected by *S. mansoni* and fed a low-protein diet develop, in the acute stage of the disease, small periovular granulomas with fewer inflammatory cells, reduced amounts of fibrous tissue in the liver and an inability to develop the murine "pipestem"-like fibrosis or periportal fibrosis (Coutinho et al. 1997, 2003, 2007) as was seen in 30-50% of well-fed animals submitted to low and long-lasting infections (Warren 1966, Andrade & Cheever 1993). Experiments on shifting from a deficient to a balanced diet and *vice-versa*, repeated infections or the use of different strains of inbred mice have been all unsuccessful (Coutinho 2004).

Previous studies on the development of the hepatic periportal fibrosis in well-nourished mice infected (WI) have demonstrated the importance of immunological mechanisms, emphasising the role of the T-helper (Th)2 response (Pearce & MacDonald 2002) and of IL-4, IL-10 and IL-13 as key cytokines for fibrogenesis (Hilkens et al. 1997). Undernourished-infected mice (UI) produce low levels of IL-4 (Oliveira et al. 2004) and IL-13 (Coutinho et al. 2010).

The host's nutritional status interferes with the course of *S. mansoni* infection in undernourished mice (UI), with negative effects on the growth and development of the parasites (Neves et al. 2001, 2002, Oliveira et al. 2004) and on egg release.

In this paper, the effects of the nutritional status on the biology of UI mice (growth curves) and parasites (worm load, egg viability and maturation), as well as on the collagen synthesis and the characteristics of the immunopathological response, were studied.

All these variables (nutritional status, egg unviability, low fibrogenic cytokine response, low collagen synthesis) are likely interdependent factors that influence the amount and distribution of fibrous (collagen) tissue in the liver periovular granulomas and portal spaces, explaining the absence of development of the murine type of periportal schistosomal fibrosis in UI mice.

MATERIALS AND METHODS

Animals and experimental infection - Sixty C57BL/6 mice and 100 Swiss Webster mice [for soluble egg antigen (SEA) preparation] at 21 days of age, weighing 11-15 g, were kept in individual wire-bottomed cages. They were raised and maintained at the animal facilities of Aggeu Magalhães Research Centre [Oswaldo Cruz Foundation (Fiocruz), Recife, state of Pernambuco, Brazil]. Water and food were provided *ad libitum*. Mice were maintained in a temperature (23ºC) and light-controlled environment.

Ethics statement - Animal experiments were performed in accordance with the Animal Care and Use Committee of Fiocruz (state of Rio de Janeiro), under license L 0028/07.

Experimental design and infection - Mice were infected percutaneously with 40 recently shed *S. mansoni* cercariae obtained from laboratory-raised *Biomphalaria glabrata* snails (Belo Horizonte strain). The animals were distributed into the following groups: group 1 [undernourished non-infected (UNI)], group 2 [undernourished-infected (UI)], group 3 [well-nourished non-infected (WNI)] and group 4 [well-nourished infected (WI)]. Infection was confirmed in each mouse by the detection of *S. mansoni* eggs in the faeces 50 days after cercarial exposure. Animals were fed their respective diet from 30 days before infection to the end of the experiment and were euthanised 60 and 150 days after infection.

Diets - Undernutrition was induced in mice by feeding them with a multi-deficient and essentially low-protein diet (7-8% protein), planned to simulate a diet usually ingested by low-income individuals living in endemic areas of Manson's schistosomiasis in Northeast Brazil (Coutinho et al. 1997) and is thus referred to as the regional basic diet. The control diet (NUVILAB) was a pelleted commercial balanced chow for mice produced by Nuvital Nutrientes Ltda (Colombo, PR, Brazil), with 22% protein content.

Evaluation of nutritional status - Body weight was weekly recorded and food consumption was measured every day. As this experimental model of mouse undernutrition has been extensively studied in previous investigations (Coutinho 1980, Teodósio et al. 1990, Coutinho et al. 1992, 1997, 2003), it was considered unnecessary to evaluate other parameters.

Parasitological studies - Parasites recovery and counting mice were killed by intraperitoneal injection of ketamine 115 mg/kg and xylazine 10 mg/kg. They were perfused after 60 (acute) and 150 (chronic) days of infection, according to the Duvall and DeWitt technique (1967). Worms recovered after perfusion of the portal system were counted and separated according to sex. Eggs in the liver and intestine were quantified after digestion with 4% potassium hydroxide (Cheever 1970).

Oogram procedures - Fragments of the small intestine (terminal ileum), of 1 cm length, were removed from all animals. They were later transferred to Petri dishes containing isotonic saline, opened lengthwise with scissors to remove excess mucus, partially dried on absorbent paper, weighed and placed between a glass slide and a plastic cover. The preparation was pressed on a rubber surface padded with absorbent paper (Pellegrino et al. 1963, Pellegrino & Faria 1965). The terminal ileum was used because this segment is the most frequent intestinal site for oviposition of several strains of *S. mansoni* in mice (Valadares et al. 1981). Using a light microscope at a magnification 100X or 400X in questionable cases, all eggs on each slide were counted and classified according to their developmental or maturation stage, following specific features of each stage. A qualitative and quantitative oogram evaluation was performed and in each fragment an average of 100 eggs were counted and classified as viable (mature, immature in 4 different development stages) or dead (calcified, with retracted miracidium, semi-transparent) according to the following features: mature eggs (containing an already developed miracidium); immature first stage (embryo at one-third of the diameter of the egg); immature second stage (embryo at one-half of the length of the egg); immature third

stage (embryo at two-thirds of the length of the egg); immature fourth stage (embryo almost entirely occupying the eggshell) (Pellegrino et al. 1963).

Morphological studies - The livers were removed, rinsed with phosphate buffered saline, weighed and divided into several portions. One section of the liver was fixed in 10% formaldehyde for histologic examination. Tissues were embedded in paraffin and the 5 µm-thick sections obtained were stained with haematoxylin-eosin and picrosirius-red method for collagen (Junqueira et al. 1979). Another portion of the liver was placed in 4% potassium hydroxide for egg counting (Cheever 1970). A liver portion weighing 100-200 mg was used for hydroxyproline determination, according to Bergman and Loxley (1963).

Morphometry - Randomly sampled 5 µm-thick liver histological sections, stained with picrosirius-red for collagen, were examined by semiautomatic morphometry using the LEICA Q500 MC Image Processing and Analysis System (Leica Cambridge, Cambridge, England). For morphometric measurements, a total sectional area of 6.6 mm^2 per animal was evaluated. All periovular granulomas were included. A spherical shape and normal size distribution were assumed. The following granuloma parameters were calculated: size, volume density and numerical density. The granuloma volume density was calculated as the quotient of the total granuloma profile area to the total sectional area studied per animal. The number of granulomas per unit volume of liver was assessed by applying Weibel's formula. The sectional area of red-stained fibrous tissue was directly measured and calculated as a percentage of the total area examined, as previously described (Coutinho et al. 1997, Barbosa-Júnior 2001).

Biochemical study - From each animal, a fresh liver sample was used for the determination of collagen, measured as hydroxyproline by the Bergman and Loxley (1963) spectrophotometric method B. Values for hepatic collagen were expressed in micromoles of hydroxyproline per gram of liver and corrected for intensity of infection by dividing the hydroxyproline content in the whole liver by the number of eggs per liver, results being expressed for 10,000 eggs. Calculations for this correction were made using a simplified electronic spreadsheet developed by Cheever (1987) and used in subsequent papers.

Immunofluorescence - Formalin-fixed paraffin-embedded 5 µm-thick sections were used for detection of α-SMA and type-I collagen by indirect immunofluorescence. Sections were deparaffinised and heat-induced antigen retrieval in citrate buffer (pH 6.0) was performed. α-SMA filaments were labelled using a biotinylated anti-α-SMA antibody, 1:100, overnight (Novotec, Lyon, France), followed by Streptavidin Alexa-Fluor 647, 1:200, for 1 h (Molecular Probes, Carlsbad, CA). For type-I collagen labelling, sections were treated with rabbit polyclonal anti-type-I collagen, 1:100, overnight (Santa Cruz, Biotechnology), followed by anti-rabbit IgG conjugated with Alexa-Fluor 488, 1:200, for 1 h (Molecular Probes). For the detection of α-SMA, sections were mounted in VECTASHIELD® HardSet™ Mounting Medium with DAPI H-1500 (4, 6-diamidino-2-phenylindole, Vector Laboratories): cell nuclei stained blue and cytoplasms, red. For type-I collagen, sections were stained with TO-PRO®-3 (carbocyanine monomer nucleic acid): nuclei stained red and type-I collagen, green. Ten random fields per mouse were studied with a Fluoview 1000 confocal microscope using a 40X objective (Olympus, Tokyo, Japan). Morphometric analysis was performed with Image ProPlus v.7.0 software (Media Cybernetics, Inc, Rockville, MD, USA).

Cell culture - After 60 and 150 days of infection, mice from all experimental groups were killed and their spleens removed under sterile conditions. For each group of mice, splenocytes were extracted from a pool of three spleens. The splenocytes were resuspended in RPMI-1640 medium (Cultilab, São Paulo, Brazil), to which 10 mL of 1% penicillin and streptomycin solution, 1% L-glutamine and 10% foetal bovine serum (Gilson) were added. Cell viability was observed after staining with 10% Trypan Blue. Splenocytes were cultured in 24-well plates at a final concentration of 5 x 10^6 cells/mL in a 5% CO_2 oven (Forma Scientific, Inc) and later stimulated with 20 µg/mL of SEA (Gazzinelli et al. 1983) or 5 µg/mL of concanavalin A. The levels of supernatant TGF-β1 were determined after cultures of 24 h, 48 h, 72 h and 120 h, in both acute and chronic phases of schistosomal infection.

TGF-β1 cytokine detection - The levels of TGF-β1 were determined as ng/mL, through ELISA, using the ELISA Ready-Set-Go Development System kit (e-Bioscience, San Diego, CA, USA) and according to the manufacturer's instructions. Each result is representative of three experiments in duplicate. The kinetics of this cytokine (24 h, 48 h, 72 h and 120 h) showed that the peak of secretion was detected in the supernatant of 24 h cultures, the peak of secretion observed in the WI group used as a reference. Readings were performed in the Bio-Rad Laboratories model 3550 microplate reader at a wavelength of 450 nm. The levels of TGF-β1 in the samples were calculated utilising the Microplate Manager software v.4.0, using standard curves of their respective recombinants. The results are shown as arithmetic mean and standard error of the mean.

Statistical analysis - The data are presented as the means ± standard error of the means. The statistical analyses performed using BioEstat software, v.5.0 (Belém, Pará, Brazil, 2007), included the Student´s *t* test or the non-parametric Mann-Whitney *U* test, when appropriate. For all experiments, p-values < 0.05 were considered statistically significant.

RESULTS

Host nutritional status is related to low body weight and low parasite burden - UI mice had lower body weight curves than WI animals, in both acute and chronic phases of schistosomal infection. However, schistosomiasis did not significantly affect the growth and development of the mice (data not shown).

These mice showed a low worm recovery during the course of the experiment (Fig. 1A, B) with significant differences than the well-fed group (acute phase: p = 0.0275; chronic phase: p = 0.0090).

Host undernutrition interferes on the amount of egg release, egg viability and evolution or maturation - Egg counts in the liver and intestine were also lower in UI animals, at both the acute and chronic phases (Fig. 1C-F). A greater number of dead eggs or remnants of eggshells were found in fragments of intestine from UI mice during both phases of the disease, with significant differences between the UI and the WI groups at the chronic phase (p = 0.0472). During the acute phase, the oogram of UI mice showed a significant reduction in the number of immature eggs in the first (p = 0.0018), third (p = 0.0009) and fourth (p = 0.0007) stages of maturation. During the chronic stage, the percentage of mature viable eggs of *S. mansoni* was significantly lower in UI animals (p = 0.009) when compared to WI controls and a marked increase in the number of dead eggs was detected in these deficient mice.

UI C57BL/6 mice develop an immunopathology characterised by small hepatic periovular granulomas, show the reduced ability to produce hepatic collagen and do not develop periportal fibrosis.

Histopathology - In the acute phase of infection, the livers of WI mice showed many exudative periovular granulomas within medium-sized and large portal spaces

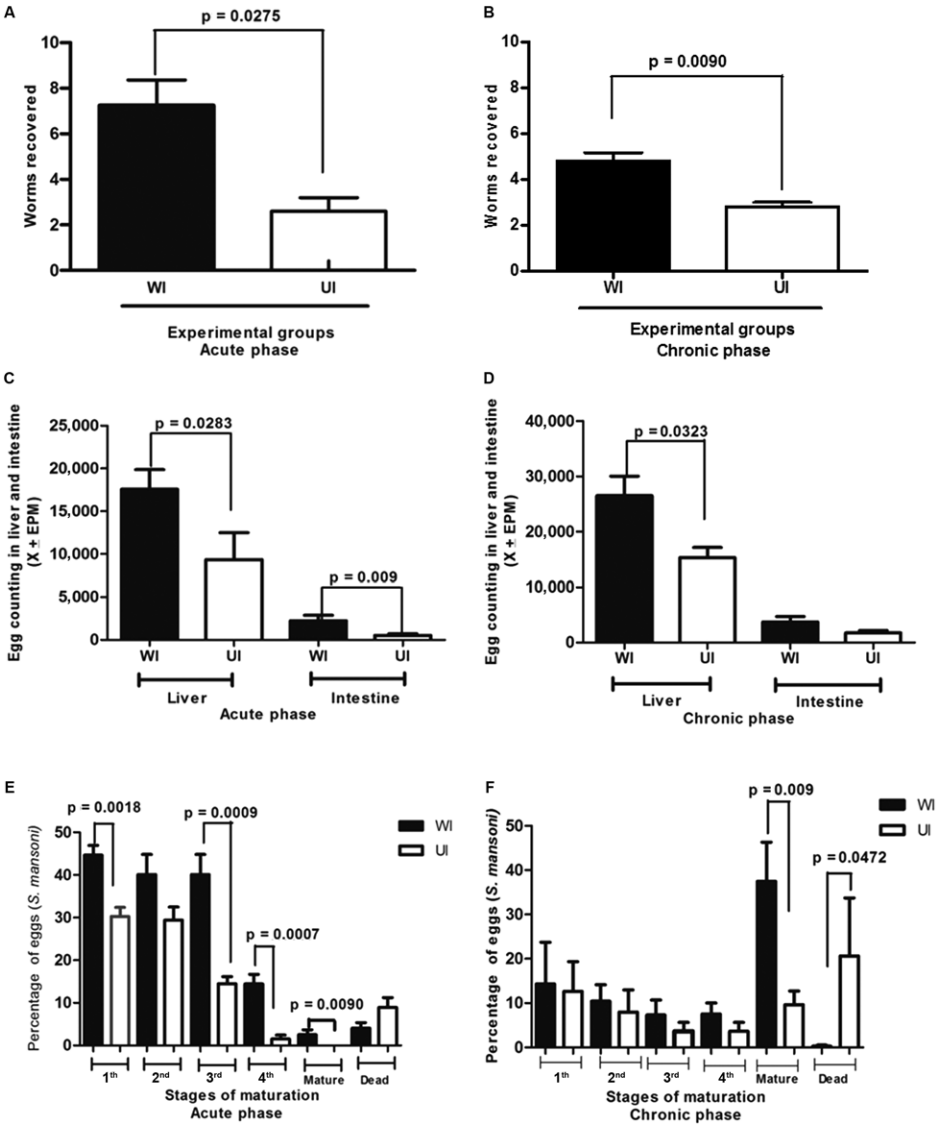

Fig. 1: worms recovered in well-nourished (WI) and undernourished-infected mice (UI) in both acute (A) and chronic (B) phases of *Schistosoma mansoni* infection. Egg counting in the liver and intestine of WI and UI with *S. mansoni* in the acute (C) and chronic (D) phases of the infection. Percentage of *S. mansoni* eggs in different stages of maturation in the intestinal wall of WI and UI in the acute (E) and chronic (F) phases of schistosomiasis. EPM: eggs per minute.

or scattered in the hepatic parenchyma. They appeared isolated or in aggregates (Fig. 2A) and predominantly composed of polymorphonuclear cells, sometimes intensely infiltrated by eosinophils. A loose connective neoformation could be observed around *S. mansoni* eggs, housing inflammatory cells of the acute phase in addition to low numbers of lymphocytes and eventually macrophages. A few foci of acute coagulative necrosis were detected. UI mice showed isolated small-sized granulomas in the proximity of dead eggs or the remains of eggshells throughout the liver parenchyma (Fig. 2B).The fibrous tissue was less conspicuous and almost always absent and low numbers of scattered acute inflammatory cells were observed around some immature or dead eggs.

During the chronic phase, the livers of WI mice displayed isolated granulomas or aggregates of these structures around mature eggs and an annular or lamellar fibrous tissue with low numbers of cells (lymphocytes, plasmocytes and epithelioid cells). Only two animals out of a group of five showed a portal concentration of circumoval granulomas causing fibrotic expansion and neovascularisation of medium-sized to large portal spaces (Fig. 2C), in addition to thin fibrous tracts connecting portal spaces (murine "pipestem"-like fibrosis).

UI mice, however, showed only very small granulomas with low numbers of inflammatory cells and reduced collagen synthesis (Fig. 2D). None were able to develop the "pipestem"-like fibrosis as described in the group of WI mice.

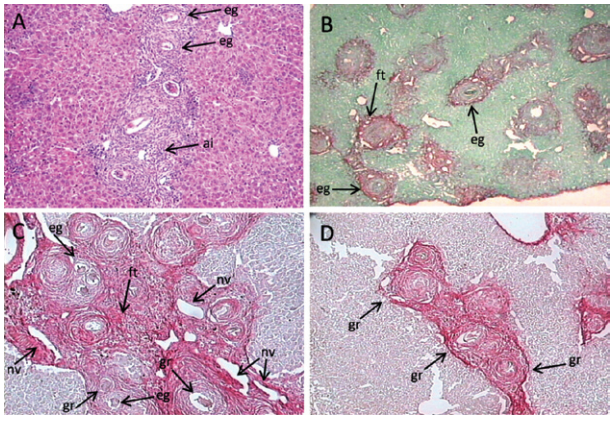

Fig. 2: liver histopathology. A: well-nourished infected mouse with *Schistosoma mansoni*. Conglomerates of well-developed periovular granulomas around mature eggs, acute inflammatory cellular infiltration and scarce collagen deposition (60 days of infection) (haematoxylin-eosin 50X); B: undernourished-infected mouse with *S. mansoni*. Small scattered periovular granulomas with a poor infiltrate of polymorphonuclear cells around remnants of eggs (60 days of infection x 100, Picrosirius-red); C: well-nourished mouse. Periportal liver fibrosis ("pipestem"-like fibrosis) showing concentration of periovular granulomas, fibrous enlargement, increased vascularisation and chronic inflammatory infiltration of the portal space (150 days of infection, x 100, Picrosirius-red); D: undernourished mice. Groups of small periovular granulomas around empty eggshells, but absence of "pipestem"-like fibrosis (150 days of infection, x 100, Picrosirius-red); ai: acute inflammatory infiltrate; eg: eggshells (remnants); ft: fibrous tissue; gr: circumoval granulomas; nv: neovascularisation.

Morphometric analysis (picrosirius-red staining) revealed that during the acute phase, the percentage of fibrous tissue in the liver of UI mice was similar to that observed in WI animals (Fig. 3A). At the chronic phase, however, the synthesis of collagen in the UI mice was less than in the WI group ($p = 0.0204$) (Fig. 3B).

The liver collagen measured biochemically as hydroxyproline also showed significant differences between UI and WI mice at both the acute ($p = 0.0163$) and chronic ($p = 0.009$) phases of the infection (Fig. 3C, D). Infected mice had higher levels of hydroxyproline when compared with control non-infected groups, regardless of their nutritional status.

Morphometric analysis also revealed that the mean volume of the periovular granulomas was significantly lower in the UI group, in both acute ($p = 0.009$) and chronic ($p = 0.0283$) phases of the schistosomal infection (Fig. 4A).

Differences were not detected between UI and WI mice with regard to periovular granuloma volume density or numerical density at either phase of schistosome infection (Fig. 4B, C).

Host undernutrition had no influence on the activation of HSC, but interfered with the production of TGFβ-1 and hepatic type-I collagen - During the chronic phase, HSCs were activated to similar levels, in both UI and WI mice, as demonstrated by the presence of Alexa-Fluor 647-labelled cells (Fig. 5A-E). α-SMA-positive cells (red) were detected in periovular granulomas and in blood vessel walls. Although significant differences were found between the WI and WNI groups ($p = 0.0001$) and between the UI and UNI groups ($p = 0.0001$), no differences were detected between the WI and UI groups, at either phase of infection ($p = 0.286$ and $p = 0.1029$, respectively).

Striking differences in type-I collagen were detected between UI and WI mice ($p = 0.0001$) at the chronic phase (Fig. 6A-E) with the increased deposition of collagen in periovular granulomas, vessel walls and portal spaces observed in the infected WI animals. In UI mice, the green fluorescence of collagen fibres was less evident and was restricted to a few granulomas, with portal spaces showing no evidence of increased fibrous deposition.

The mean levels of TGF-β1 were significantly lower in the UI mice at the acute phase of infection ($p = 0.0039$), which decreased drastically as the disease progressed to the chronic phase, when titres were detected at approximately five times lower ($p = 0.0039$) than those of the WI mice (Fig. 7A, B).

DISCUSSION

Previous investigations on the interactions between the host nutritional status and infection by *S. mansoni* have called attention to the role of undernutrition on the biology and differentiation of the parasites, inducing phenotypic changes in adult worms of both genders (Ferreira & Coutinho 1999, Neves et al. 2002, Oliveira et al. 2003), most likely owing to the lack of essential nutrients for their adequate growth and development. Nutritional deficiency also influences the immune response

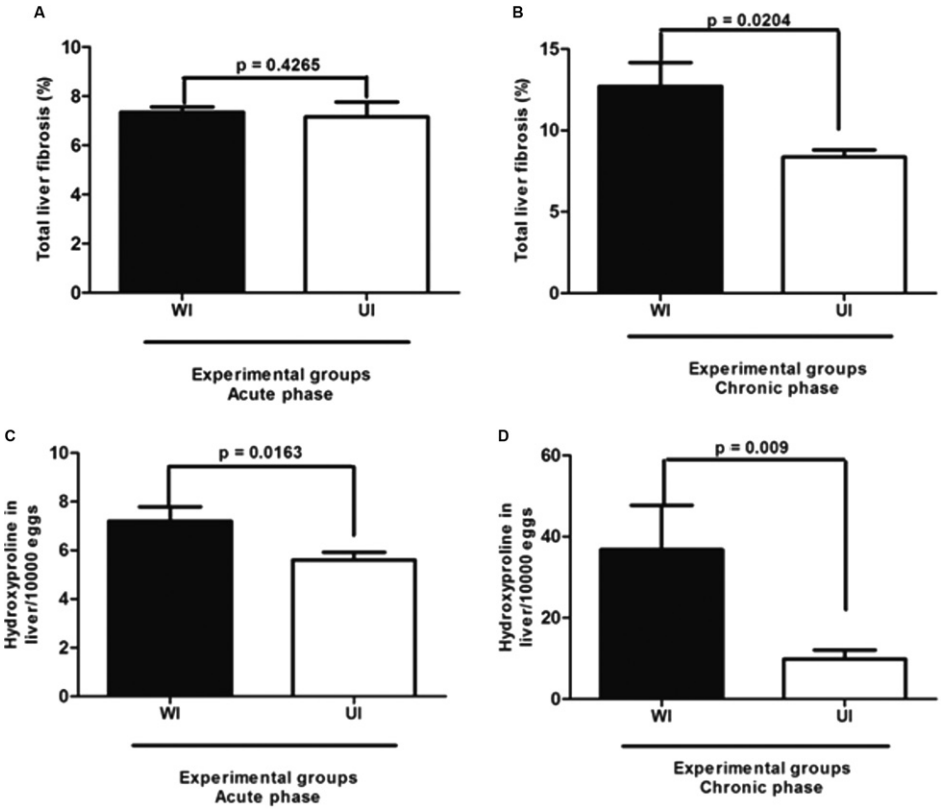

Fig. 3: morphmetric (A, B) and biochemical (C, D) measurements of liver fibrous tissue in the acute and chronic phases of *Schistosoma mansoni* infection in undernourished-infected (UI) and well-nourished (WI) C57BL/6 mice.

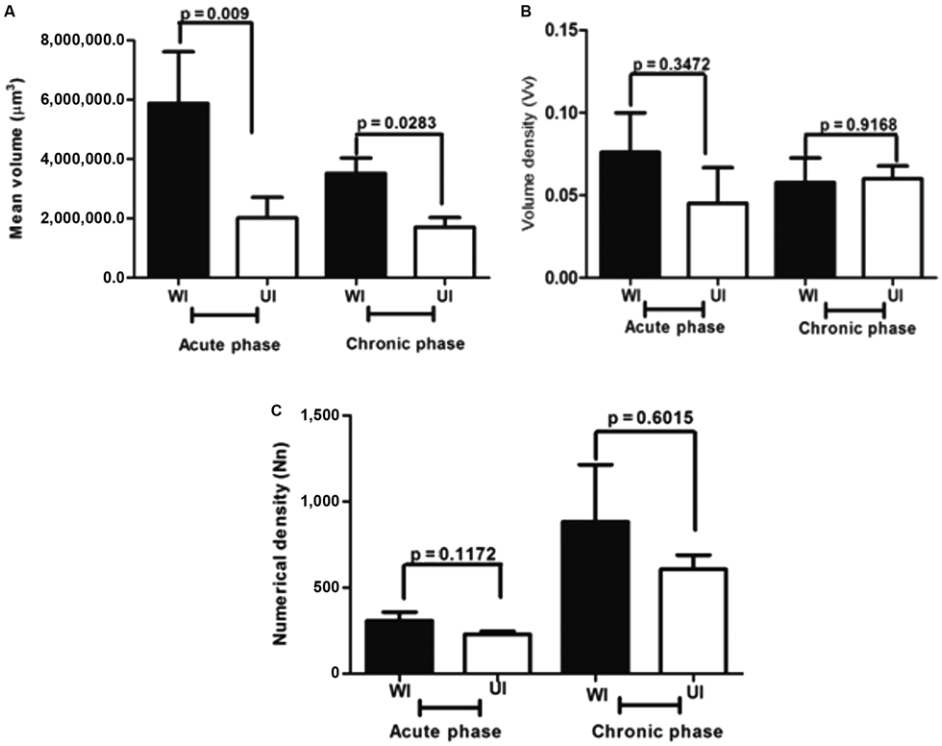

Fig. 4: morphometric measurements of the mean volume (A), volume density (B) and numerical density (C) of egg granulomas in undernourished-infected (UI) and well-nourished (WI) mice in the acute and chronic phases of *Schistosoma mansoni* infection.

and immunopathology of schistosomiasis in the murine experimental host (Coutinho 2004, Oliveira et al. 2004, Coutinho et al. 2007, 2010).

Schistosomal hepatic fibrosis (Symmers' fibrosis) is a chronic lesion involving essentially portal spaces, whose etiopathogenesis is still incompletely understood. In humans, both periovular granulomas and a diffuse progressive periportal fibrosis contributing to the increased amount of fibrous tissue develop within the liver.

The experimental "pipestem"-like liver fibrosis (Symmers' fibrosis of humans) that develops in 30-50% of mice infected by *S. mansoni* (Andrade 1987, Andrade & Cheever 1993) cannot be reproduced in UI outbred and/or inbred mouse models (Coutinho 2008). Different experimental approaches that have tried to reproduce the lesion in UI mice have been unsuccessful thus far (Coutinho 2004). However, further studies on the immunological profile of these animals provided promising results regarding the role of some cytokines secreted at both the acute (60 days) and chronic stages (150 days) of murine schistosomiasis (Coutinho et al. 2010).

It has been previously reported that schistosome-undernourished infected mice are able to develop a humoral immune response, but antibody titres are much lower than in control well-fed animals (Oliveira et al. 2004, Coutinho et al. 2010). The production of the cytokines interferon (IFN)-γ, IL-4 and IL-10 is lower in the UI mice, but in INOS-knockout mice, as infection progresses to the chronic phase, the kinetics of IFN-γ runs an antagonistic course when compared to that of WI animals (Ramos et al. 2006). On the other hand, the secretion of IL-13, a pro-fibrogenic cytokine, at the chronic phase, has been found to be at alternatively low and high levels of concentrations in splenocyte culture supernatants (Coutinho et al. 2010). The Th2 cytokines and, in particular, IL-13 and the IL-13 receptor (IL-13Rα2) appear to be crucial for the hepatic fibrogenesis associated with schistosomal infection (Wynn et al. 2004).

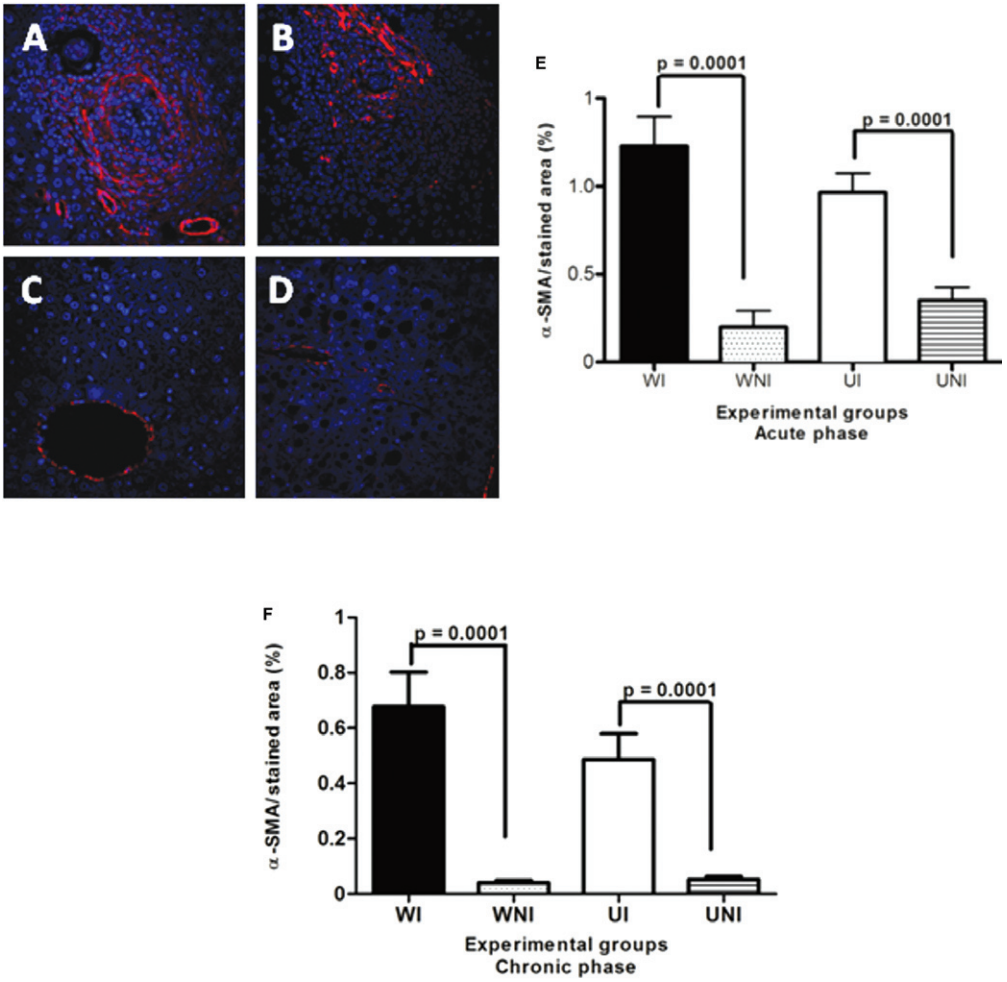

Fig. 5: immunolabelling by Alexa-Fluor 647 for alpha-smooth muscle actin (α-SMA) on myofibroblasts (red) and vascular walls in the liver of mice chronically infected by *Schistosoma mansoni* using the indirect fluorescence technique. Hepatic cells nuclei were stained with DAPI (blue). Data represent the mean and standard error of 10 sections per mouse in groups of five animals. Differences regarding the amount of hepatic stellate cells between UI and WI mice were not statistically significant in both phases. A: well-nourished infected (WI); B: undernourished-infected (UI); C: well-nourished non-infected (WNI); D: undernourished non-infected (UNI) (400X).

In this study, the host nutritional status was related to low body weight curves, low parasite burdens and low egg loads detected in UI mice. It was found that host protein undernutrition negatively interfered with tissue egg release, as well as on egg viability and the degree of maturation (oogram), as a high metabolism appears to be necessary for a perfect oogenesis by *S. mansoni* adult females (Tempone et al. 2002).

UI C57BL/6 mice infected with *S. mansoni* developed a $CD4^+$ T-cell mediated immunopathology characterised by small hepatic granulomas surrounding parasite eggs. This pathological finding is in line with previous reports (Cheever 1987, Rutitzky et al. 2009). The high expression of type-I collagen was detected in the periovular granulomas of WI mice at the early and/or final collagenous stages (productive granulomas), as well as on blood vessel walls. In UI animals, however, type-I collagen deposition was less evident and restricted to a few granulomas with statistically significant differences between the UI and WI groups. In the chronic stage of schistosomiasis, the amount of collagen (hydroxyproline) was approximately four times less in the UI group when compared to well-fed animals.

The data gathered thus far suggest that nutritional deficiency interferes directly with connective tissue changes occurring in murine hepatic schistosomiasis (Coutinho et al. 2003).

Recently, HSCs have gained increasing attention, not only as contractile cells, but also as obligatory regulators of vascular development (Lee et al. 2007), stabilisation, maturation and remodelling of the connective matrix. This is strongly indicative of the role played by these capillary-associated actin-containing cells in the remodelling of the extracellular matrix and associated vascular lesions (Andrade 2009). The consistent morphological association between HSCs and extracellular matrix reinforces their important role in hepatic fibrogenesis. α-SMA, a cytoskeletal protein, is one of the six actin isoforms expressed in mammalian tissue and is the single most reliable marker of stellate cells activation and differentiation into myofibroblasts (Friedman 2008). UI and WI mice were able to activate HSCs to similar levels, as shown by Alexa-Fluor 647 positively labelled cells expressing α-SMA in their cytoplasms. These positive cells (red) could be observed in periovular granulomas and blood vessel walls at both the acute and chronic phases of schistosomal infection. Despite this, the production

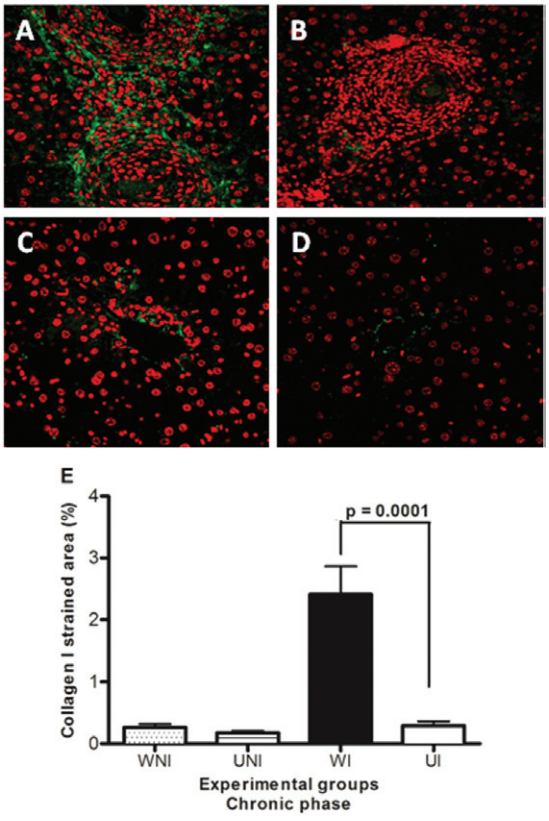

Fig. 6: immunolabelling by Alexa-Fluor 488 for collagen I (green) in the liver of mice chronically infected by *Schistosoma mansoni* using the indirect fluorescence technique. Hepatic cells nuclei were stained with TO-PRO 3 (red). Data represent the mean and standard error of 10 sections per mouse in groups of five animals. Significant results were detected between UI and WI mice (p = 0.0001). A: well-nourished infected (WI); B: undernourished-infected (UI); C: well-nourished non-infected (WNI); D: undernourished non-infected (UNI) (400X).

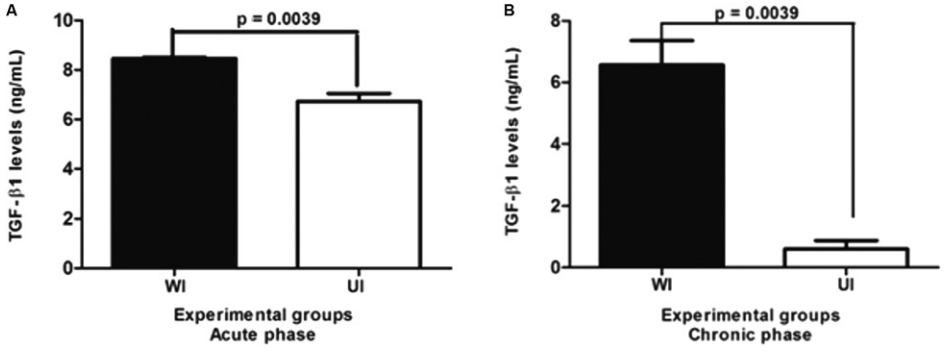

Fig. 7: levels of transformation growth factor (TGF)-β1 in the supernatant of splenic cells (24 h culture) in undernourished-infected (UI) and well-nourished infected (WI) mice with *Schistosoma mansoni* in the acute (A) and chronic (B) phases of the infection.

of type-I collagen in the chronic phase was significantly lower in UI mice, but this difference appeared to be related to the interference by dietary protein deficiency on the synthesis of collagen and likely other elements of the extracellular matrix (Coutinho et al. 2003).

Studies on the cellular kinetics of hepatic fibrosis have demonstrated the involvement of different cell types, in addition to HSCs, including eosinophils and macrophages and their relationships to the development of the granulomatous response and the production of profibrotic cytokines. The deposition of eggs by *S. mansoni* followed by the release of SEA incites a granulomatous response in which $CD4^+$ T cells (Mathew & Boros 1986) and eosinophils (Rumbley et al. 1999) play an important role. Additionally, $CD8^+$ T cells, B cells and M2 macrophages (alternatively activated macrophages) play a role in the formation and regulation of hepatic granulomas (Herbert et al. 2004, Hams et al. 2013). It has been found that M2 macrophages induced by Th2 responses promote collagen synthesis and fibrogenesis via the metabolism of L-arginine to proline and polyamine by arginase-1 (Song et al. 2000).

In this study, the levels of TGF-β1 in UI were significantly lower at the beginning of the infection (acute phase), falling drastically to levels approximately five-times lower than those detected from WI animals at the chronic phase.

The reduced egg laying in tissues and the increase in the amount of degenerated and dead eggs detected in UI mice did not entirely impair the activation of HSCs because fibrogenic stimuli from the remaining miracidia bodies and even from immunogenic cellular proteins and glycoprotein molecules derived from the eggshells (de Walick et al. 2012) were sufficient to induce the differentiation of HSCs (Ito's cells, perisinusoidal cells, pericytes) into myofibroblasts. Thus, TGF-β1 was produced, although at lower concentrations.

The results obtained in this experiment suggest that host malnutrition is directly responsible for the reduced egg laying and low maturation of *S. mansoni* eggs, leading to a decreased release of immunogens and thus affecting the expression of fibrogenic cytokines such as TGF-β1, in addition to IL-4, IL-10 and IL-13, as previously reported (Coutinho et al. 2010). The low expression of fibrogenic cytokines, however, is sufficient to activate HSCs, but these cells in UI mice are unable to adequately synthesise type-I collagen. Dietetic protein deficiency, together with the low immunogenic stimulus generated by the reduced oviposition and egg unviability, may account for the low liver collagen production and the inability of UI mice to develop the murine type of liver periportal fibrosis.

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APÊNDICE D - In vivo study of schistosomicidal action of 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one

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In vivo study of schistosomicidal action of 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one

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ABSTRACT

Praziquantel has been the drug most widely used therapy against different forms of schistosomiasis around the world. However, this treatment has shown ineffective in humans and in experimental models of *Schistosoma mansoni*. New therapeutic alternatives have been tested, including the imidazolidine derivative LPSF/PT-09, which has shown high therapeutic potential *in vitro*. In this work, we tested the schistosomal activity of this derivative in doses of 250 mg/kg and 200 mg/kg in mice experimentally infected with a high parasite load of *S. mansoni*. Parasitological evaluations related to the number of *S. mansoni* worms and their oviposition were performed during the acute phase of the disease and have demonstrated moderate effectiveness of 30–54,4%. However, LPSF/PT-09 did not influence oviposition of the parasites or the embryonic development of the eggs. The results obtained in this model showed that the imidazolidine derivative LPSF/PT-09 presented significant antischistosomal activity *in vivo*, posing as a potential candidate for this class of drugs. However, a better understanding of the pharmacokinetics and pharmacodynamics of the imidazolidine derivative LPSF/PT-09 is needed.

1. Introduction

Schistosomiasis is the second most prevalent infectious, parasitic disease in tropical and subtropical areas in terms of socio-economic importance and public health. It is endemic in some 78 countries with an estimated infection rate of more than 200 million people worldwide [1,2]. Schistosomiasis mansoni is caused by a trematode, *Schistosoma mansoni* and clinical course of the disease can vary from asymptomatic forms up to the extremely severe [3].

The pathology of schistosomiasis is initially due to granulomatous inflammation around the eggs deposited by *S. mansoni* with subsequent formation of fibrosis. Although variation of severity occurs in the development of pathological forms in schistosomiasis [4,5], all individuals who come into contact with the infective, larval form of the parasite (cercaria) have great susceptibility to worm infection regardless of gender, age or ethnic group [3].

In Brazil the treatment of schistosomiasis is based mainly on chemotherapy, using oxamniquine or praziquantel [6]. Oxamniquine is a drug that belongs to the family of tetrahydroquinoline, which is active only against *S. mansoni* and, after his administration side effects have been observed in the central nervous system, as well as carcinogenic and mutagenic effects [3,7]. In addition, there is a limitation in the production of this medicine by the pharmaceutical firm which owns the registration of products containing oxamniquine [8].

Praziquantel, in turn, is derived from pyrazinoisoquinoline, which came to be used as the main drug in the treatment of schistosomiasis due to the following factors: its effectiveness against all species of *Schistosoma* which infect humans; minor side

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effects presented by individuals after treatment; its low cost [7,9–12].

The action mechanisms responsible for the therapeutic efficacy of praziquantel have not yet been fully clarified [13]. However, it is already known that praziquantel acts on the homeostasis of calcium ion flow causing spasms, damage to the integument and muscular paralysis in adult worms [12,14–17].

In recent years resistance of *Schistosoma* to praziquantel has been described in some endemic areas, where a decrease in the effectiveness of the treatment has been observed in humans [16,18–20] and also in experimental studies with mice [21] and with cercariae [22].

As the action of praziquantel in schistosomiasis is not completely known, it becomes more difficult to understand the mechanisms responsible for the resistance of strains of *S. mansoni*. However, the unexpected resistance of the parasite to this drug justifies further studies that can generate knowledge about the mechanisms of action of compounds against the parasite, as well as the need to search for new drugs for the treatment of *S. mansoni* [23].

Imidazolidines are bioactive compounds which have stood out for their broad biological action in terms of antimicrobial, anticonvulsant [24], antihypertensive [25], antineoplastic [26] and schistosomicidal properties [27].

Based on the promising results of the imidazolidine-derived 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one (LPSF/PT-09) *in vitro* (Fig. 1) [28], this study sought to assess the schistosomicidal potential of LPSF/PT-09 in an experimental model of mansonic schistosomiasis. Our results showed a moderate schistosomicidal effect of imididazolidine tested in acute infections of *S. mansoni*.

2. Materials and methods

2.1. Chemistry study

2.1.1. Compounds

The compound 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one (LPSF/PT-09) was obtained from Laboratório de Planejamento de Síntese de Fármacos (LPSF) at Universidade Federal de Pernambuco (Brazil) and was duly identified by nuclear magnetic resonance of hydrogen as well as infrared (IR) and mass spectroscopy (MS). Fig. 1 displays the synthetic route of LPSF/PT-09. The starting reagent was imidazolidine-2,4-dione which was reacted with benzyl chloride under basic conditions to obtain the intermediate 3-(4-bromo-benzyl)-imidazolidine-2,4-dione [29]. After that, the reaction of 3-benzyl-imidazolidine-2,4-dione with Lawesson's reagent in anhydrous dioxane gave rise to 3-benzyl-4-thioxo-imidazolidin-2-one. The reaction mixture was heated under reflux for 24 h [30]. Then, the compound 1-Benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one (LPSF/PT-09) was synthesized by a reaction with a diazonium salt. Reactions were monitored with analytical thin-layer chromatography in silica gel 60 F254 plates and visualized under UV light (254 nm). Melting points were determined on a Quimis 340 capillary melting point apparatus and were not corrected. Infrared spectra were recorded as KBr discs using a BRUKER (IFS66) infrared spectrophotometer. Nuclear magnetic resonance ^{1}H NMR were recorded in a VMMRS 300 MHz VARIAN spectrometer using tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as the solvent. Chemical shifts (δ, ppm) were assigned according to the internal standard signal of TMS in DMSO-d_6 (δ, ppm). Coupling constants (J) are reported in Hz. ^{1}H NMR spectra are reported in the following order: chemical shift, multiplicity, number and type of proton and coupling constant(s). Mass spectra with MALDI-TOF Autoflex III (Bruker Daltonics, Billerica, MA, USA). Laser Nd:YAG, 355 nm. Freq. laser: 100 Hz. The presence of the characteristic proton peak in ^{1}H NMR confirmed the presence of the final compound 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one (LPSF/PT-09). The IR spectrum of the compound showed characteristic peaks of the carbonyl and thiocarbonyl groups. For the preparation of 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one (LPSF/PT-09), equimolar amounts of 3-benzyl-4-thioxo-imidazolidin-2-one (200 mg) and 4-fluoro-phenylamine (107.75 mg) with sodium nitrite (0.15 mg) in the presence of sulfuric acid (0.3 mL) was reacted with absolute ethanol (8 mL) as solvent and morpholine (1 mL) as catalyst. The reaction mixture to 0 °C for 8 h and after left at room temperature. The solid that precipitated out was filtered under vacuum and washed with water and absolute ethanol. MF: $C_{16}H_{13}FN_4OS$; MW: 328.3640 g/mol; m.p.: 202–3 °C; yield: 44.73%; R_f: 0.56 *n*-hexane/ethylacetate 8:2. IR (KBr): ν max (cm^{-1}) 3420 (N—H), 1720 (C=O), 1500 (C = S). ^{1}H NMR (300 MHz, DMSO-*d*6): δ 11.66 (s, 1H, NH), 7.70 (s, 1H, NH), 7.48-7.26 (m, 4H, Ar), 7.20-6.97 (m, 5H, Ar), 5.09 (s, 2H, CH_2). Anal. Calcd for $C_{16}H_{13}FN_4OS$: C, 58.52; H, 3.99; N, 17.06; S, 9.77. Found: C, 59.54; H, 3.87; N, 16.87; S, 9.50. HRMS: 328.00.

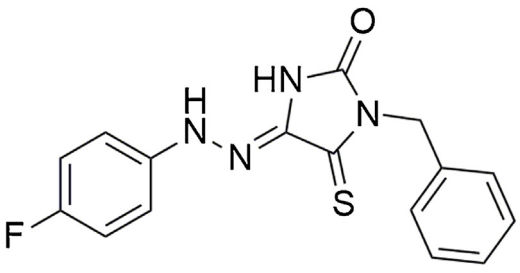

Fig. 1. Structure of derivative 1-benzyl-4-[(4-fluoro-phenyl)-hydrazono]-5-thioxo-imidazolidin-2-one (LPSF/PT-09).

2.2. Biological study

2.2.1. Parasites and hosts

Infection was performed percutaneously, using for each mouse 80 *S. mansoni* cercariae. LE (Belo Horizonte) strains of *S. mansoni* adult worms collected from *Biomphalaria glabrata* were obtained by the Departamento de Malacologia do Centro de Pesquisa Aggeu Magalhães (CPqAM). Fifty male albino *Swiss* mice (*Mus musculus*) were used, aged 25 days. Sixty days after infection, a parasitological examination was done from feces of mice to evaluate the positivity of the infection. This project was approved by the Ethics Committee on the Use of Animals of the Centro de Pesquisa Aggeu Magalhães/Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) authorized by license No. 21/2011.

2.2.2. *Assessment of* in vivo *susceptibility of adult worms of* Schistosoma mansoni *to the imidazolidine derivative*

For the assessment of schistosomicidal activity of the imidazolidine derivative, LPSF/PT-09, experimental and control groups consisting of eight mice were used. The allocation of mice to experimental groups was performed randomly. The mice were treated orally 60 days after infection. The recommended doses of the imidazolidine compound and PZQ were 250 mg/kg or 200 mg/kg being administered for five consecutive days. The groups were allocated as follows: Group I (LPSF/PT-09/250 mg/kg); Group II (LPSF/PT-09/200 mg/kg); Group III (praziquantel/250 mg/kg); Group IV (praziquantel/200 mg/kg); and the Group V (vehicle and PEG (polyethylene glycol).

Fifteen days after treatment with the compounds, the animals were anesthetized with an intraperitoneal injection of ketamine

hydrochloride (115 mg/kg) associated with xylazine hydrochloride (10 mg/kg). After anesthesia, the animals underwent perfusion of hepatic portal system for removal of the worms, which were separated in Petri dishes containing 0.85% saline and then the parasites were counted and classified according to sex and vitality [31].

2.2.3. Determination of the effectiveness of treatment

The evaluation of the effectiveness of the imidazolidine derivative LPSF/PT-09 and of PZQ was determined by reducing the percentage of parasitic load in each group treated using the following equation: reduction of worms (%) = $\frac{\#\text{ of worms in the control group} - \#\text{ of worms in the treatment group}}{\#\text{ of worms in the control group}} \times 100$ [18].

2.2.4. Percentage egg developmental stages

Three fragments of the distal portion of the intestine were washed in saline solution and slightly dried on absorbent paper. Subsequently, each intestinal fragment was squeezed between the slide and the cover slip and analyzed in a microscope to classify the eggs. For each fragment 100 eggs were counted and classified according to their developmental stage. The eggs were classified as immature, viable eggs (from the 1st to the 4th stage); mature, viable eggs; and non-viable eggs (calcified, with retracted miracidium, semitransparent) [32].

2.2.5. Counting the eggs in the liver and intestine

Fragments of the liver and the intestine of each mouse submitted to euthanasia were taken after perfusion and digested in 4% potassium hydroxide (KOH) [33]. The eggs found were quantified with the aid of a cell-counting "Sedgewick Rafter" camera (Graticules Limited: model S50, Tonbridge-England).

2.2.6. Morphometric evaluation

Samples of liver from each mouse was fixed in 10% formalin solution, included in paraffin blocks, sectioned and stained by Picro-sirius red. Ten microscopical fields from histological liver sections (5 μm), that presented large production of conective tissue, were selected and the collagen tissue was labeled and acquired as percentage using Image Processing and Analysis System LEICA Qwin 2.6 (Leica Cambridge, England). The measurement result from each animal was the mean percentual from these ten microscopical fields, and the ultimate result was the mean percentual per group. The system was calibrated for 10 x lens magnification using a Leica DM LB2 microscope.

2.2.7. Statistical analyses

A descriptive analysis was performed to display the results obtained. The presentation of the measured variables was done using tables, including also the use of some descriptive measures such as minimum, maximum, average and standard deviation. For comparative analysis of quantitative variables with more than two groups, the following tests were used: Bartlett to test the assumption of homogeneity of variance; Kruskal-Wallis was applied when an assumption of homogeneity was seen. All findings were taken at a significance level of 5%. The software used was GraphPad Prism 5.

3. Results

3.1. Effectiveness of treatment

The derivative LPSF/PT-09, at a dose of 250 mg/kg, presented significant efficacy in the treatment of mansonic schistosomiasis in mice, reducing the number of adult worms after treatment by 54.4%, with 60% being represented by female worms when used at a dose of 250 mg/kg. The use of the lower dose, 200 mg/kg, also had good efficacy, being able to cut down on the number of worms by 30.4%, with 59% being represented by female worms. PZQ, in turn, had efficacy of 100% in both the doses evaluated (Table 1).

3.2. Oogram

In the assessment of stages of eggs found in the intestine, the imidazolidine-derived LPSF/PT-09 proved to be active only in relation to mature eggs, not causing significant changes in the number of immatures eggs or of inviable eggs. Conversely, PZQ proved to be significantly effective in reducing the immature stages (primary, secondary, tertiary and quaternary) (Table 2).

3.3. Counting the eggs in the liver and intestine

In the evaluation of liver tissue, no significant differences were observed between the number of eggs found in animals treated with LPSF/PT 09, with PZQ or not treated. While in the evaluation of the intestine, PZQ was effective in reducing the number of eggs at both doses, with the 200 mg/kg dose, interestingly, more effective in this reduction (Table 3).

3.4. Morphometric analysis of collagen percentage of mice infected with S. mansoni

In assessing the percentage of fibrous tissue in samples of livers of infected mice with *S. mansoni*, there was a decrease in the percentage of collagen in the treated groups with imidazolidine derivative LPSF/PT-09 at both doses. However, there was no significant difference between the groups treated with PZQ and untreated control group (Table 3).

4. Discussion

The study present showed that the imidazolidine derivative LPSF/PT-09 showed significant antischistosomal activity *in vivo*. It is known that the imidazolidines, considered a class of bioactive compounds represent a class of molecules of great importance in medicinal chemistry. Besides the already known antischistosomal activity [34], the imidazolidines have pharmacological activities such as anticonvulsant [35], antiarrhythmic [36], in the treatment of chronic diabetic complications [37], antitumor properties [38], anti-inflammatory [39], antihypertensive [34] and antimicrobial [40,41].

Once proven the therapeutic efficacy high of imidazolidine derivative LPSF/PT-09 against mature worms of *S. mansoni* [42], was evaluated in this work schistosomicidal activity *in vivo*. As demonstrated *in vitro* study [28,42], where the LPSF/PT-09 showed mortality rates of up to 100%, *in vivo* assessment he also proved effective, with an overall reduction in the number of worms adults was 54.4%, with 60% represented by female worms when used at a

Table 1
Effectiveness of treatment of the animals infected with *S. mansoni* and the treated with imidazolide derivative PT-09 and PZQ.

Animal groups[#]	Number of worms	Efficacy of treatment (%)
I	36	54.4
II	55	30.4
III	0	100
IV	0	100
V	79	–

#Group I: LPSF/PT-09/250 mg/Kg; group II: LPSF/PT-09/200 mg/Kg; group III: PZQ/250 mg/Kg; group IV: PZQ/200 mg/Kg; group V: untreated.

Table 2
Percentage of egg developmental stages in mice infected with *S. mansoni* untreated and treated with imidazolidine derivative LPSF/PT-09.

Animal groups#	% egg developmental stages		
	Immature eggs	Mature eggs	inviable eggs
I	77.38 ± 15.23	10.08 ± 8.14	12.52 ± 15.69
II	71.98 ± 10.35	15.18 ± 6.31	12.97 ± 6.41
III	11.38 ±17.30	63.98 ± 16.95**	24.62 ± 20.35**
IV	0.61 ± 1.22*	61.67 ± 13.70*	37.71 ± 13.07***
V	48.22 ± 8.37	51.36 ± 7.56	0.41 ± 1.16

*Significant difference from infected untreated control at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$.
#Group I: LPSF/PT-09/250 mg/Kg; group II: LPSF/PT-09/200 mg/Kg; group III: PZQ/250 mg/Kg; group IV: PZQ/200 mg/Kg; group V: untreated.

dose of 250 mg/kg. This result indicates a moderate schistosomicidal activity. The effectiveness of the schistosomicidal activity is considered high if the compound is capable of generating a mortality rate of over 80% [43]. In the group treated with LPSF/PT-09 at a dose of 200 mg/kg, the total reduction in the number of worms was 30.4%, and 59% represented by female worms. The effectiveness of total load of the worms is considered a modest result, since according to literature data, a compound has a low efficiency when a reduction in the number of worms is less than 30% [44].

Treatment of mice infected with *S. mansoni* 50, 100 or 200 mg/kg mefloquine resulted in a total load reduction of *S. mansoni* adult worms 44.1%, 64% and 93.4%, respectively. The dihydroartemisinin also exhibited *in vivo* with a single oral dose of 600 mg/kg or continuous administration at a dose of 400 mg/kg, an activity against of *S. mansoni* adult worms infection after 49 days of presenting more sensitive against the female adult worms with a reduction of 94%, while males were significantly less likely to dihydroartemisinin and survival of worms was higher in the groups administered [45].

The decrease of the load of the female adult worms in the two doses tested was of great importance, indicating that the female may be more susceptible to pharmacological target the imidazolidine derivative LPSF/PT-09. As PZQ, the imidazolidines not have a mechanism of action elucidated [28]. This route was identified in *S. mansoni*, and proved to be the justification responsible for causing the death of these parasites [34,40]. There is evidence that imidazolidines have influence on the levels of cholinergic receptors. And since the pharmacological and physiological system *S. mansoni* is different from its host, it is likely that these receptors are good biological targets [46].

Furthermore, chemical groups can be essential for expression and intensity of many biological reactions, are considered functional group [47]. The imidazolidines have the same pharmacophore group that niridazol 1-(5-nitro-thiophene-3-yl)-imidazolidin-2-one. They can act as chelating agents due to imidazolidines and arylazo group nitrogen, leaving the most reactive molecule [47]. The biofunctional groups of LPSF/RZS-2 (5-(4-chloro-benzylidene-3-(4-nitrobenzyl)-4-thioxo-imidazolidin-2-one) and LPSF/RZS-5 (5-(4-fluoride-benzylidene-3-(4-nitrebenzyl)-4-thioxo-imidazolidin-2-one) imidazolidines have alkyl radicals, chlorine and fluorine atoms, respectively. These radicals can change the alkalinity or lipophilicity of the compounds inducing changes in interactions with its receptor and the phenomenon of absorption by different cell behaviors. Thus, it is believed that these features can be responsible for the biological responses of the imidazolidines derivatives *in vitro* studies [48].

As noted in this study, the difference in efficacy of treatment *in vitro* and *in vivo* can be supported by the possibility of different forms of migration of *S. mansoni* in the body, so that it can escape the action of the drug [49]. Discrepancies were also observed in studies using mefloquine against schistosomula and adult worms of *S. mansoni*. Pharmacokinetic studies more specific, responsible for measuring the actual concentration of the drug in the body or directly on the target in question, may help to elucidate the differences observed *in vitro* and *in vivo* studies [45].

Differences between *in vitro* and *in vivo* activity may also occur between jobs without using the imidazolidine derivative LPSF/PT-05 (1-benzyl-4-[(4-chlorophenyl)-hydrazono]-5-thioxo-imidazolidin-2-one) which showed 100% mortality of adult worms of *S. mansoni* in the first 24 h at a concentration of 174 μM. However, *in vivo* evaluation in mice infected with *S. mansoni*, the imidazolidine derivative LPSF/PT-05 showed an efficacy of 70.5% in the treatment of parasitic load [50].

It is known that mature eggs remain viable in the intestine for about twelve days. Thus, if oviposition decrease with administration of the drug, oogram should provide a high amount of mature eggs due to the occurrence of oviposition previous treatment at the same time a high decrease or absence of the immature stages [51]. Contrary to the one observed *in vitro*, in which the derivative LPSF/PT-09 proved to be a potent inhibitor of oviposition [28], *in vivo* work he seems to have an effect. Although data in literature show a high variation in the number of eggs obtained by pairs of worms *in vitro* studies [52] and *in vivo* [33], the results shown in our study indicate that LPSF/PT −09 did not exercise any activity in the immature stages or oviposition. Although the activity *in vivo* have demonstrated a significant change in decreasing the number of worms in the treated groups, an absence of changes in oogram related to other stages of the eggs of *S. mansoni* was observed.

On the number of eggs found in liver tissue, contrary to what one might imagine, no significant differences were observed between treated and untreated groups, even those treated with PZQ, variation also supported by the literature. In the intestine, however, the results corroborate the well demonstrated in the literature, where PZQ is shown to reduce the number of tissue eggs [33].

Table 3
Morphometric measured on collagen content in hepatic tissue and egg count in hepatic and intestinal tissue in mice untreated and treated with imidazolidine derivative LPSF/PT-09.

Animal groups#	Morphometry of tissue collagen (%) (image analysis system)	Tissue egg loads × 10^3	
		Hepatic	Intestinal
I	14.89 ± 3.87	5.02 ± 3.69	1.63 ± 1.42
II	12.27 ± 4.14	3.65 ± 2.31	1.78 ± 1.06
III	20.90 ± 6.68	3.58 ± 3.57	0.34 ± 0.11***
IV	13.89 ± 3.07	2.29 ± 1.16	0.12 ± 0.08
V	16.63 ± 4.18	6.58 ± 4.59	2.27 ± 2.12

*Significant difference from infected untreated control at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$.
#Group I: LPSF/PT-09/250 mg/Kg; group II: LPSF/PT-09/200 mg/Kg; group III: PZQ/250 mg/Kg; group IV: PZQ/200 mg/Kg; group V: untreated.

5. Conclusions

Based on these results, we conclude that the imidazolidine derivative LPSF/PT-09 showed significant antischistosomal activity *in vivo*, posing as a potential candidate for this class of drugs. However, the investigation of the mechanism of action at effects the imidazolidine derivative LPSF/PT-09 against worms of *S. mansoni* as well as further studies on the increased solubility and pharmacodynamics are still needed.

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ANEXO A – parecer da Comissão de Ética

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o Projeto intitulado: **"INVESTIGAÇÃO DO POTENCIAL TERAPÊUTICO DE CÉLULAS-TRONCO NO TRATAMENTO DE LESÕES HEPÁTICAS CRÔNICAS."** protocolado sob o Nº 15/2011, coordenado pelo (a) pesquisador(a) ***SHEILLA ANDRADE DE OLIVEIRA***, está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/ Fundação Oswaldo Cruz (CEUA-CPqAM) em reunião de 26/05/2011. Na presente versão, este projeto está licenciado e tem validade até o mês de maio de 2015.

Quantitativo de Animais Aprovados	
Espécie - linhagem	**Nº de Animais**
CAMUNDONGO MUS MUSCULUS C57BL/6	**320**
CAMUNDONGO MUS MUSCULUS C57BL/6 E GFP	**480**
TOTAL	**800**

We certify that the project entitled "**INVESTIGAÇÃO DO POTENCIAL TERAPÊUTICO DE CÉLULAS-TRONCO NO TRATAMENTO DE LESÕES HEPÁTICAS CRÔNICAS**." *(CEUA* Protocol Nº 15/2011), coordinated by **SHEILLA ANDRADE DE OLIVEIRA** according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/ Fundação Oswaldo Cruz on may 26, 2011. In the present version this project is licensed and valid until may 2015

Recife (PE, Brazil) 30 may 2011.

Drª Eridan Medeiros Coutinho
Coordenadora da Comissão de Ética no Uso de Animais
Centro de Pesquisas Aggeu Magalhães – FIOCRUZ

Eridan M. Coutinho M. D. Ph.L
Pesquisadora Estatutária da FIOCRUZ
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ANEXO B: Termo aditivo

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisas Aggeu Magalhães

Comissão de Ética no Uso de Animais

Recife, 03 de julho de 2015

Termo Aditivo ao Certificado de Licença Nº 15/2011

A Comissão de Ética no Uso de Animais do Centro de Pesquisas Aggeu Magalhaes – CEUA/CPqAM, em atenção a solicitação do(a) pesquisador (a): **Sheilla Andrade de Oliveira**, autoriza o presente Aditivo que altera a vigência desta licença, referente ao Projeto intitulado: **Investigação do potencial terapêutico de células tronco no tratamento de lesões hepáticas crônicas.**

Ressaltamos que a nova data de validade desta licença é 30 de maio de 2016.

Drª Gerlane Tavares de Souza Chioratto
Vice-coordenadora CEUA/CPqAM

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