Bone marrow-derived monocyte infusion improves hepatic fibrosis by decreasing osteopontin, TGF-β1, IL-13 and oxidative stress

Veruska Cintia Alexandrino de Souza, Thiago Almeida Pereira, Valéria Wanderley Teixeira, Helotonio Carvalho, Maria Carolina Accioly Brelaz de Castro, Caroline Guimarães D’assunção, Andréa Ferreira de Barros, Camila Lima Carvalho, Virgínia Maria Barros de Lorena, Vlúadia Maria Assis Costa, Álvaro Aguiar CoelhoTeixeira, Regina Celia Bressan Queiroz Figueiredo, Sheilla Andrade de Oliveira

Veruska Cintia Alexandrino de Souza performed the majority of experiments, analyzed the data and wrote the paper; Carvalho CL and de Barros AF performed the immunologic investigations; D’Assunção CG participated in the treatment of animals; Pereira TA and Figueiredo RCBQ performed the immunohistochemistry assays; Carvalho H performed the biochemical investigations; Costa VMA, de Lorena VMB and de Castro MCAB performed the cellular isolation and characterization; Teixeira VW and Teixeira ÅAC performed the morphologic and morphometric investigations; de Oliveira SA designed and coordinated the research.

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Correspondence to: Sheilla Andrade de Oliveira, PhD, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Professor Moraes Rego s/n, Cidade Universitária, Recife,
CD11b’CD14’ monocytes in a murine model of liver damage. The results show that mice with transplants had 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.

INTRODUCTION
Abuse of alcohol, infections caused by hepatitis viruses B and C, and nonalcoholic steatohepatitis (NASH) are the main causes of liver tissue damage[11]. These risk factors can lead to focal or diffuse hepatocellular degeneration and necrosis. Persistent inflammatory stimuli 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 (ECM) components[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 components comprise various types of proteins, including osteopontin (OPN)[3], a pro-inflammatory 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 and plays an important role in the repair of acute and chronic liver damage, both in humans and experimental models[5,6].
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-\(\beta\)\(^1\)\(^{20}\) and galectin-3 expression\(^{19}\).

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 exhibited similar behavior to those hepatic cells obtained from the liver culture\(^{25}\). 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.

MATERIALS AND METHODS

**Animals**

Male C57BL/6 mice (4-6 wk of age), weighing 20-23 g were obtained from the Animal Breeding Center Laboratory 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 ± 2 °C) 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 was induced in the mice by orogastric administration of 200 µL of 20% 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 6 mo. 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, United States) at 1000 × g for 15 min. 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; Miltenyi Biotec™, Bergisch Gladbach, Germany), washed and passed through a magnetic column (MACS; Miltenyi Biotec™), where CD11b\(^+\) monocytes were retained and recovered in a buffer [0.5% PBS/0.5% bovine serum albumin (BSA) + 2 mmol/L 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 immunomagnetic separation were first incubated with Anti-CD11b (PE Rat Anti-Mouse CD11b, M1/70 clone, BD Pharmingen™, San Jose, CA, United States), Anti-CD14 (FITC Rat Anti-Mouse CD14, rmCS-5 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, D8 clone; BD Pharmingen™). After 30 min of incubation, cells were washed with 2 mL of PBS wash solution (PBS with 0.5% BSA + 0.1% sodium azide), centrifuged at 400 × g for 5 min and then resuspended in 300 µL of the PBS wash solution.

The samples were then phenotypically characterized by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, United States). A minimum of 10000 events/sample were collected. The cell population
Bone marrow mononuclear cells (BMMCs) and analyzed using the Image Analysis Processing System Leica QWIN, version 2.6 MC (Leica, Cambridge, United Kingdom). Ten microscopic fields (100 × magnification) containing fibrous tissue areas were chosen for quantification. To detect and quantify Kupffer cells, the histological sections were stained with hematoxylin 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 × magnification).

**Hydroxyproline (Hyp) assay**
Liver samples (approximately 200 mg) were immersed in 6N HCl at approximately 120 °C for 18 h, followed by filtration. The hydroxyproline (Hyp) concentration was determined by a colorimetric assay at 558 nm as previously described 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 increasing concentrations of ethanol, incubated overnight with biotinylated antibody anti-α-SMA (Santa Cruz Biotechnology, Dallas, TX, United States), and then incubated with streptavidin-peroxidase for 10 min. For OPN staining, the samples were incubated overnight with primary anti-OPN antibodies (AF808; R&D Systems, Minneapolis, MN, United States), as previously described. Thereafter, a secondary
antibody bound to a synthetic polymer conjugate with peroxidase (horseradish peroxidase, HRP). 3,3' diaminobenzidine was used for staining. The sections were counterstained with Harris hematoxylin. The staining was measured in 10-fields/sections (200 × magnification) using the Image Analysis Processing System Leica QWIN, version 2.6 MC.

**Glutathione 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 min. GSH was detected using the Glutathione Assay Kit (Sigma Aldrich) and measured with a microplate reader at 415 nm (BioRad, Hercules, CA, United States).

**Enzyme-linked immunosorbent assay**

Frozen liver fragments (approximately 100 mg) were homogenized in a lysis buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L 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 min at 4°C 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 enzyme-linked immunosorbent assay assay following the manufacturers’ instructions (IL-13, IL-17, IL-23, MMP-9 and TIMP-1 by R&D Systems; TGF-β1: Human/Mouse TGF-beta1 by e-Bioscience, San Diego, CA, United States; TNF-α, IL-1β, IL-6 and IL-10 using the OptEIA Set for Mouse by BD Biosciences). Samples were read at a 450 nm wavelength using a microplate reader (Model 3550; Thermo Fisher Scientific, Waltham, MA, United States). 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’s). Differences were evaluated using the ANOVA test for parametric analysis, and the Kruskal-Wallis test with post-hoc Dunn’s test for non-parametric analysis. Statistical analyses were performed using Prism Software (version 5.0; GraphPad Software, San Diego, CA, United States) 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, 2 mo 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; Figure 3B, D and E). This decrease was also found in mice treated with BMMCs (P < 0.05; Figure 3B, C and E). A marked reduction in the amount of Hyp 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 were decreased in the mice that received monocytes (P < 0.01; Figure 4D and E) as well as in the BMMC-treated group (P < 0.05;
Figure 4C and E) compared with the group treated with saline (Figure 4B and 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 cytokines 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-C). IL-13 (Figure 6D) and TGF-β1 (Figure 7A), important fibrogenic mediators, were significantly lower compared to those 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 < 0.05; 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 animals treated with CD11b⁺CD14⁺ monocytes and BMMCs (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.

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. However, the results obtained in the present study demonstrated an improvement in these parameters on BMMC-derived CD11b⁺CD14⁺ monocyte infusion, with an almost
2-fold 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\(^\text{[27]}\). 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 pro-inflammatory cytokines (TNF-\(\alpha\), IL-1\(\beta\) and IL-6)\(^\text{[28]}\). Previous studies have reported the role of macrophages in mediating liver fibrogenesis, and have proposed using macrophage subpopulations during liver damage and repair\(^\text{[23,29]}\). Treatments carried out in experimental models have shown that the infusion of bone marrow-derived macrophages decreases fibrous tissue, and enhances hepatic regeneration\(^\text{[22,30,31]}\).

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\(^\text{[3,4]}\). In this regard, some studies have reported a decrease in the number of \(\alpha\text{-SMA}\) cells in murine models of liver damage treated with BMMCs. This decrease is probably due to an alteration in the modulation of HSCs by specific cytokines and growth factors, including TGF-\(\beta\)\(1\), TNF-\(\alpha\) and ROS, produced by hepatocytes in a damaged liver\(^\text{[32]}\). As activation of HSCs is mediated by autocrine and paracrine signaling and these cells not only secrete cytokines but also respond to them\(^\text{[32]}\), it was hypothesized that BMMC-derived CD11\(b\)CD14\(+\) monocytes modulate the activity of HSCs by regulating the secretion of cytokines and growth factors.

Production of the pro-inflammatory cytokine profiles of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 were inhibited in mice submitted to liver damage and treated with CD11\(b\)CD14\(+\) monocytes. Furthermore, there was an increase in the synthesis of IL-10 cytokine, which is known for its T helper (Th)2 profile and anti-
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. 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. 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, thereby down-regulating the inflammatory response and reducing activated HSCs, which ultimately leads to...
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 1 collagen[44]. 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[45]. 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 effect on pro-inflammatory cytokine, produced by CD4^+ T cells[37]. This mediator induces the recruitment of inflammatory factors into liver cells and also directly activates natural hepatic immunity systems, such as those mediated by neutrophils and dendritic cells, to release cytokines that perpetuate chronic inflammation[39]. Previous reports have reported that Th17 cells are able to participate in the pathogenesis of hepatic lesions associated with hepatitis B virus[39]. 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[39].

OPN is a glycoprotein expressed in a variety of tissues, mainly found in ECM and sites of healing wounds[40]. Studies have shown that this protein is highly expressed in fibrotic liver tissue and influences the function of hepatic progenitors[41]. Under this condition, increases in the level of TGF-β and activation of HSCs could be also observed[6,41]. 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[42,43]. Reduced levels of this molecule have been found in preclinical fibrosis models and in human fibrotic diseases[42]. 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[44] 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[19]. The presence of high quantities of these inhibitors in chronically damaged hepatic tissue may contribute to the establishment of liver fibrosis[49]. IL-13 is a cytokine associated with severe forms of schistosomal liver fibrosis as well as non-schistosomiasis liver diseases[46]. IL-13 is considered one of the central mediators in liver pathogenesis and is involved in TGF-β1 production by liver cells, besides its ability to induce progenitor cells to transdifferentiate into myofibroblasts, which produce collagen[47]. 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[29,30,48]. Ramachandran et al[29] found that Ly6G⁺Ly6C⁺ 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 down-regulation 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 for 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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