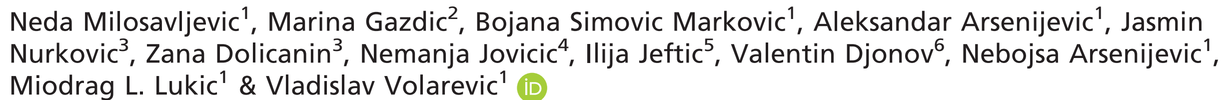
ORIGINAL ARTICLE

Mesenchymal stem cells attenuate liver fibrosis by suppressing Th17 cells – an experimental study



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SUMMARY

This study investigates molecular and cellular mechanisms involved in mesenchymal stem cell (MSC)-mediated modulation of IL-17 signaling during liver fibrosis. Mice received CCl_4 (1 μl/g intraperitoneally) twice/week for 1 month. MSCs (1×10^6), or MSC-conditioned medium (MSC-CM), were intravenously injected 24 h after CCl_4 and on every 7th day. Liver fibrosis was determined by macroscopic examination, histological analysis, Sirius red staining, and RT-PCR. Serum levels of cytokines, indoleamine 2,3-dioxygenase (IDO), and kynurenine were determined by ELISA. Flow cytometry was performed to identify liver-infiltrated cells. *In vitro*, $CD4^+$ T cells were stimulated and cultured with MSCs. 1-methyltryptophan was used for inhibition of IDO. MSCs significantly attenuated CCl_4-induced liver fibrosis by decreasing serum levels of inflammatory IL-17, increasing immunosuppressive IL-10, IDO, and kynurenine, reducing number of IL-17 producing Th17 cells, and increasing percentage of $CD4^+IL\text{-}10^+$ T cells. Injection of MSC-CM resulted with attenuated fibrosis accompanied with the reduced number of Th17 cells in the liver and decreased serum levels of IL-17. MSC-CM promoted expansion of $CD4^+FoxP3^+IL\text{-}10^+$ T regulatory cells and suppressed proliferation of Th17 cells. This phenomenon was completely abrogated in the presence of IDO inhibitor. MSCs, in IDO-dependent manner, suppress liver Th17 cells which lead to the attenuation of liver fibrosis.

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Key words
$CD4^+$ T cells, fibrosis, IL-17, liver, mesenchymal stem cells

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Introduction

Interleukin (IL)-17 plays an important pro-fibrogenic role in hepatic fibrosis [1,2]. During chronic liver inflammation, IL-17 is mainly produced by liver-infiltrated Th17 cells which are in increased number present in livers of patients suffering from hepatic fibrosis, developed after alcohol-induced liver injury, autoimmune, acute, and chronic hepatitis [3–6]. IL-17, produced by Th17 cells, stimulates hepatic stellate cells (HSCs) to increase synthesis of collagen-1, α-smooth muscle actin (α-SMA), and transforming growth factor (TGF)-β1 and promote liver fibrosis [1,7]. Mice deficient in IL-17 are resistant to liver fibrosis [1,2]

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suggesting that therapeutic approaches which attenuate IL-17 production in liver would have beneficent effects in the therapy of hepatic fibrosis.

Advanced IL-17-driven liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation. However, liver transplantation has several limitations, including lack of donors, complications of surgical interventions, side effects of immunosuppressive drugs, and high medical costs. Accordingly, the alternative approaches, such as stem cell transplantation, have been suggested as an effective alternate therapy to liver transplantation [8].

Among stem cells, mesenchymal stem cells (MSCs) are due to their immunomodulatory ability and capacity for differentiation into hepatocytes, tested in many preclinical and clinical studies as possible new therapeutic agents for the treatment of acute and chronic liver diseases including fibrosis [9,10]. MSCs can alter immune response and regulate the proliferation, activation, and effector function of all immune cells, including $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes [11]. MSC-induced suppression of T cells is mediated through cell-to-cell contact and through the production of soluble factors [11,12]. Interaction between inhibitory molecule programmed death 1 (PD-1), with its ligands (PD-L1 and PD-L2), is responsible for MSC-dependent inhibition of T cells mediated by cell-to-cell contact [13]. Among soluble factors, indoleamine 2,3-dioxygenase (IDO) is the most important for suppression of effector T cells by human MSCs [14]. IDO promotes the degradation of tryptophan into kynurenine and toxic metabolites (quinolinic acid and 3-hydroxy-anthranilic acid) which suppress proliferation or induce apoptosis of activated T cells.

Although it is known that MSCs may attenuate chronic liver inflammation and are able to suppress proliferation of Th1 cells, their capacity to modulate function of Th17 cells in hepatic fibrosis, is still unknown.

Using well-established murine model of liver fibrosis, induced by carbon tetrachloride (CCl_4), we investigated molecular and cellular mechanisms involved in MSC-mediated modulation of IL-17 signaling during the pathogenesis of liver fibrosis. Herewith, we provide the evidence that MSCs attenuate liver fibrosis by reducing liver infiltration of Th17 cells in IDO-dependent manner.

Materials and methods

Cells

Mouse MSCs isolated from bone marrow of C57Bl/6 mice were purchased from Gibco (Catalog No. S10502-01). Mouse fibroblasts (STO cells) were purchased from American Type Culture Collection (Catalog No. CRL-1503). MSCs and STO cells (passage 6) were cultured in complete *Dulbecco's Modified Eagle Medium* (DMEM) under standard conditions. MSC-conditioned medium (MSC-CM) was generated as previously described [15].

Animals

Eight- to ten-week-old male wild-type C57Bl/6 mice were used. All animals received humane care, and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Induction of liver fibrosis

Mice received CCl_4/corn oil [ratio 1:3; 1 μl/g intraperitoneally (i.p.); Sigma-Aldrich, St-Louis, MO, USA] twice per week for 1 month [16]. Mice were sacrificed 1 week after the last CCl4/corn oil challenge.

Administration of MSCs or STO cells

1×10^6 MSCs or STO cells were intravenously (i.v.) injected via the tail vein 24 h after the first administration of CCl_4/corn oil and on 7th, 14th, and 21st day of experiment [17].

Pharmacological inhibition of IDO

Mesenchymal stem cells were cultured for 48 h in culture medium containing 1 mM 1-methyltryptophan, (1-MT; Sigma-Aldrich), an inhibitor of IDO activity [18]. CCl_4/corn oil and CCl_4/corn oil + MSC-treated mice continuously received 1-MT (2 mg/ml in drinking water) for *in vivo* inhibition of IDO activity [19].

Transplantation of MSCs in established CCl_4-induced liver fibrosis

One week after the last injection of CCl_4/corn oil, mice i.v. received either 1×10^6 MSCs or saline once per week for next 3 weeks.

Histopathological analyses

Histological analysis of liver injury was performed as previously described [20]. Quantification of fibrosis in mouse liver sections stained with PicroSirius red (10×)

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was performed using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA), on 10 fields/section, as described [21].

Measurements of cytokines in serum

The commercial enzyme-linked immunosorbent (ELISA) sets (R&D Systems, Minneapolis, MN, USA) were used to measure concentration of IL-17 and IL-10 according to the manufacturer's instructions [22].

Measurement of IDO in serum

Indoleamine 2,3-dioxygenase content was determined in mice sera using mouse IDO specific ELISA kit (Catalog No. MI0064; NeoBioLab, Cambridge, MA, USA).

Detection of kynurenine

As IDO catalyzes the metabolism of tryptophan in the kynurenine pathway, IDO activity in serum of control, CCl_4 and CCl_4+MSC-treated mice was determined by spectrophotometric assay for kynurenine [18].

Isolation of liver-infiltrated immune cells and analysis with flow cytometry

Mononuclear cells (MNCs) were removed from liver of each mouse and analyzed by flow cytometry, as described previously [23].

Detection of MSCs in the livers of CCl_4-treated mice

Mesenchymal stem cells were fluorescence-labeled using pre-incubation with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Thermo Fisher Scientific, Stockholm, Sweden) according to the manufacturer's instructions, as described [24]. For homing assays, 1×10^6 CFSE-labeled MSCs were injected into the tail veins of mice 24 h after the first administration of CCl_4/corn oil and on 7th, 14th, and 21st day of experiment. Single cell suspensions were prepared from liver tissues on 2nd, 11th, 18th, and 27th day of the experiment and analyzed by flow cytometry. Absolute numbers of homed MSCs per organ were calculated according to the formula $n = m_g/m_a \times v_1/v_2 \times 100\,000/\underline{x}$, with m_g indicating total mass of the organ analyzed; m_a, mass of the analyzed proportion of the organ; v_1, total volume in which the entire organ-cell suspension was contained; v_2, volume of sample aspirated/analyzed; $\underline{x}$, number of positive events acquired per 100 000 events, as described [25].

Isolation of $CD4^+$ cells

$CD4^+$ cells were isolated from hepatic MNCs by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions, as described [26].

Co-culture of $CD4^+$ cells and MSCs *in vitro*

$CD4^+$ cells, *in vitro* stimulated with concanavalin A (2.5 μg/ml) [27] and IL-2 (50 ng/ml) [28], were cultured alone, in direct contact with MSCs (ratio 10:1), or physically separated from MSCs by 0.4 μm porous transwell system (Corning Incorporated; Life Sciences, Chorges, France). $CD4^+$ T lymphocytes were placed in the lower chamber, and MSCs were seeded in the transwell inserts, at ratio 10:1, as described previously [29]. After 48 h of culture, supernatants were collected and frozen at −20 °C until cytokine concentrations were measured by ELISA kits (R&D Systems).

Isolation of hepatic stellate cells

Hepatic stellate cells were isolated from the mouse liver as described previously [30,31]. Isolated HSCs were then used in the co-culture experiments.

Co-culture of MSC-primed $CD4^+$ cells and HSCs

Mesenchymal stem cells, cultured in the complete medium in the presence or in the absence of 1 mM 1-MT, were seeded in 0.4 μm porous transwell system with *in vitro* stimulated $CD4^+$ cells (ratio 10:1) [29]. After 48 h, $CD4^+$ cells were harvested and cultured with HSCs (ratio 20:1) for additional 48 h [32]. The expression of collagen-1 in HSCs was evaluated by real-time PCR analysis.

RNA isolation and real-time PCR analysis

Total RNA from livers and HSCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The fold change of mRNA gene expression for collagen-1, α-SMA, TGF-β, and β-actin as a housekeeping gene (Invitrogen) was calculated as described [33].

Biostatistics

Statistical analysis was performed using SPSS 20.0. Data are presented as means ± SEM (standard error of the mean). Statistical significance was determined by Student *t*-test and, where appropriate, using

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Mann–Whitney *U*-test. Statistical significance was assumed at $P < 0.05$.

Results

Intravenously injected MSCs migrate in the CCl_4-injured livers and ameliorate fibrosis

It is well known that intravenously injected MSCs migrate into livers [25] and that tissue damage and inflammation enhance the efficacy of their homing [34]. Accordingly, 24 h after intravenous injection, MSCs were detected in CCl_4-injured livers and their presence in damaged livers had been increasing after each application (Fig. 1a).

Mesenchymal stem cells managed to significantly attenuate CCl_4-induced liver fibrosis, as determined by macroscopic examination (Fig. 1b), histological analysis (Fig. 1c), Sirius red staining (Fig. 1d), and quantitative RT-PCR (Fig. 1g).

An irregular contour with one or several nodules was found macroscopically on the surface of the livers of CCl_4-treated mice (Fig. 1b). On contrary, livers obtained from CCl_4+MSC-treated mice had a smooth surface with uniform and soft textures, similar to the livers of control animals (Fig. 1b), indicating that CCl_4-induced macroscopic changes were significantly attenuated in MSC-treated animals.

H&E staining of liver tissues obtained from CCl_4-treated mice revealed damage of liver cells and centrilobular congestion with infiltration of inflammatory cells. Specifically, single cell necrosis and ballooning degeneration of hepatocytes were observed in central and midlobular regions (Fig. 1c, black arrows), often associated with fibrosis and inflammatory cell infiltration. Organized hepatic architecture was replaced by regenerative nodules connected with fibrous septa. On contrary, there were only several solitary areas of necrotic tissue in CCl_4-treated mice that received MSCs resulting with significantly reduced total size of liver fibrosis (Fig. 1c).

Sirius red staining of liver tissues obtained from CCl_4-treated mice revealed extensive collagen deposition and pseudolobular formation (Fig. 1d, black arrows), suggesting development of liver fibrosis. MSCs treatment resulted in a remarkable reduction in the size of the stained area of fibrous dense tissue (Fig. 1d).

In line with these findings, percentage of fibrotic liver tissue ($P = 0.007$, Fig. 1e) and increase of AST and ALT levels ($P = 0.038$ for AST and $P = 0.043$ for ALT, Fig. 1f) were significantly lower in CCl_4+MSC-treated mice compared to mice that received CCl_4 only.

In accordance with the histological and biochemical analysis, quantitative RT-PCR showed that MSC treatment significantly decreased expression of pro-fibrogenic markers: collagen-1 ($P = 0.043$) and α-SMA ($P = 0.034$) as well as TGF-β ($P = 0.036$) known as major pro-fibrogenic cytokine associated with organ fibrogenesis [1,7] (Fig. 1g).

Attenuation of liver fibrosis was not noticed in CCl_4-treated mice that received STO cells (Fig. 1b–g), confirming specificity of MSC-based beneficent effects.

Importantly, MSCs efficiently attenuated completed CCl_4-induced liver fibrosis, as well (Fig. S1), confirming their therapeutic potential.

MSCs treatment decreased serum levels of IL-17 and reduced infiltration of IL-17 producing $CD4^+$ T cells in the livers of CCl_4-treated mice

An attenuated liver fibrosis, noticed in CCl_4+MSC-treated mice, correlated with the decreased levels of inflammatory and pro-fibrogenic IL-17 ($P = 0.006$, Fig. 2a left panel) and increased levels of immunosuppressive and hepato-protective IL-10 in sera of CCl_4-treated mice which received MSCs when compared to animals that received CCl_4 only ($P = 0.042$, Fig. 2a right panel).

Interestingly, serum levels IDO ($P = 0.02$, Fig. 2b left panel) and kynurenine ($P = 0.048$, Fig. 2b right panel) were significantly increased in CCl_4+MSC-treated mice in comparison with CCl_4 only-treated animals, suggesting the importance of IDO for MSC-mediated attenuation of IL-17-driven liver fibrosis.

To dissect out cellular target of MSC-dependent modulation of liver fibrosis, flow cytometry analysis of liver-infiltrated MNCs was performed. Intracellular staining of liver infiltrating MNCs revealed significantly lower number of IL-17-producing $CD4^+$ T cells ($P = 0.009$, Fig. 2c) and significantly higher number of IL-10-producing $CD4^+$ T lymphocytes ($P = 0.024$, Fig. 2d) in CCl_4+MSC-treated mice compared to animals treated with CCl_4 only. On contrary, there was no significant difference in the total number of liver infiltrating IL-17-producing $CD8^+$ T lymphocytes between these groups (Fig. 2e) suggesting that $CD4^+$ T cells were major targets of MSC-mediated modulation of IL-17 production in liver-infiltrated T cells.

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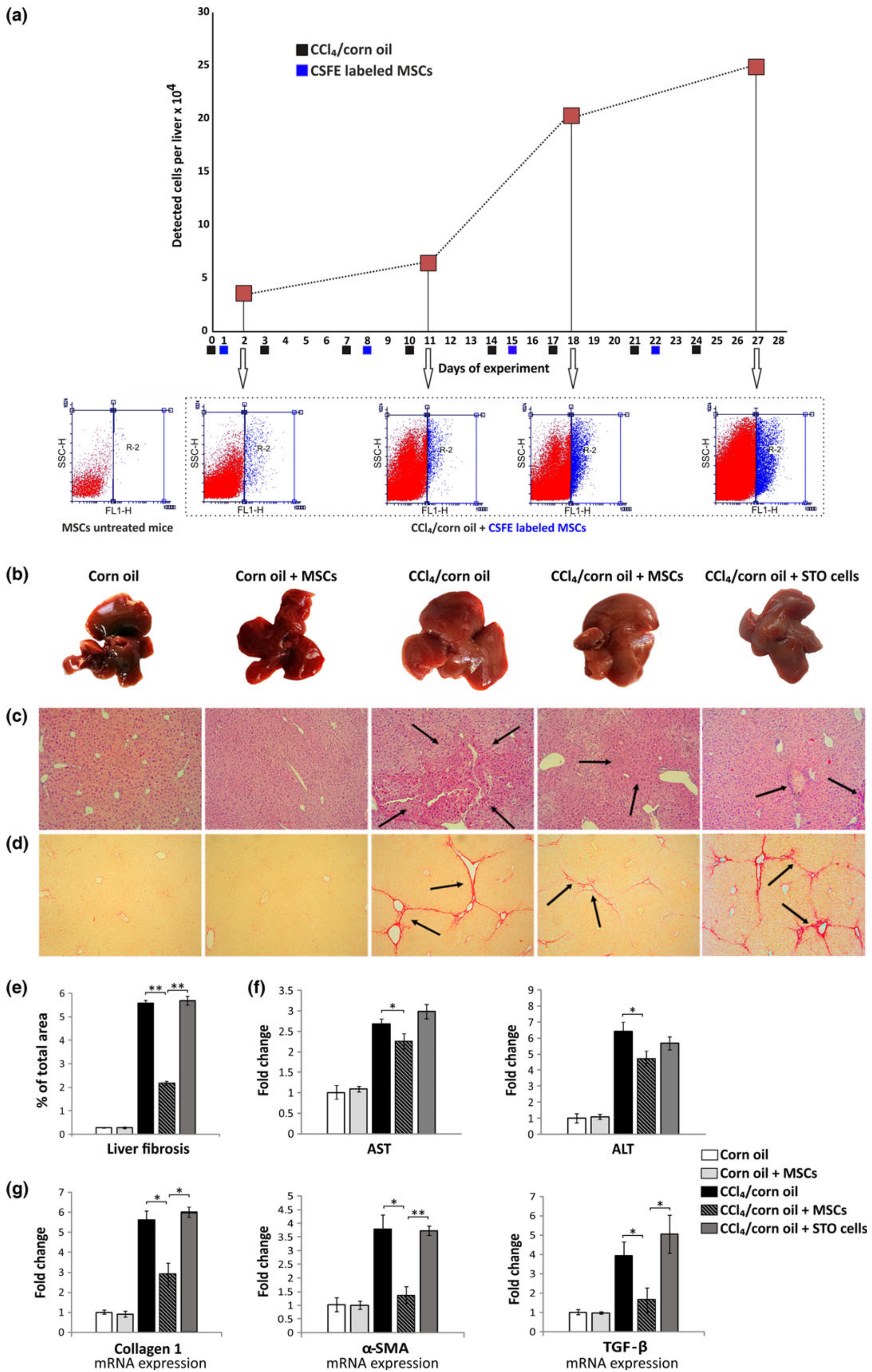

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MSCs suppress Th17 cells in paracrine manner

To investigate whether cell-to-cell contact was required for MSC-dependent suppression of liver-infiltrated Th17 lymphocytes, MSCs and liver $CD4^+$ T cells were cultured with or without transwell systems, which was used to separate cells by a membrane permeable to soluble molecules.

Intracellular staining revealed significantly lower number of IL-17 producing *in vitro* activated liver $CD4^+$ T cells, cultured with MSCs in transwell system, when compared to stimulated $CD4^+$ T lymphocytes that were culture without MSCs ($P = 0.003$, Fig. 3a left panel). On contrary, MSC-derived products induced significant increase in total number of IL-10 producing $Foxp3^+$ $CD4^+$ T cells ($P = 0.002$, Fig. 3a left panel). Similar results were observed when MSCs and *in vitro* activated liver $CD4^+$ T cells were cultured without transwell systems ($P = 0.001$ for IL-17-producing $CD4^+$ T cells and $P = 0.001$ for IL-10 producing $Foxp3^+$ $CD4^+$ T cells, Fig. 3a right panel) indicating that MSCs were able to induce proliferation of $CD4^+FoxP3^+$ regulatory T cells (Tregs) and suppression of Th17 cells in paracrine manner.

Interestingly, as it is shown in Fig. 3a, this phenomenon was completely abrogated in the presence of IDO inhibitor (1-methyl tryptophan, 1-MT). There was significant increase in Th17 cells ($P = 0.017$, Fig. 3a left panel and $P = 0.002$ right panel) and decrease in IL-10 producing Tregs ($P = 0.013$, Fig. 3a left panel and $P = 0.001$ right panel) when activated $CD4^+$ T cells were cultured in MSC-CM that contained 1-MT, suggesting that MSCs attenuate Th17/Tregs ratio in IDO-dependent manner.

In line with these findings, significantly lower expression of collagen-1 ($P = 0.001$, Fig. 3b) was noticed in HSCs that were cultured with MSC-primed liver $CD4^+$ T cells when compared with HSCs which were cultured with MSC+1-MT-primed liver $CD4^+$ T cells, confirming the importance of IDO for MSC-dependent modulation of $CD4^+$ T-cell capacity for HSC activation.

MSC-CM-mediated proliferation of IL-10 producing $CD4^+$ T cells and suppression of Th17 cells was abrogated by IDO inhibitor

In line with results obtained *in vitro*, injection of MSC-CM significantly down-regulated serum levels of liver enzymes (Fig. 3c, $P = 0.037$ for AST; $P = 0.042$ for ALT) and cytokine production in liver-infiltrated $CD4^+$ T cells (Fig. 3d), indicating that MSCs attenuate IL-17-driven liver fibrosis in paracrine manner.

Intracellular staining of liver immune cells revealed that MSC-CM treatment managed to significantly reduce infiltration of IL-17 producing $CD4^+$ T cells ($P = 0.004$, Fig. 3d upper panel) and to significantly increase percentage of IL-10 producing T cells ($P = 0.031$, Fig. 3d lower panel) in livers of CCl_4-treated mice. This was accompanied with decreased serum levels of pro-fibrogenic IL-17 ($P = 0.028$, Fig. 3e left panel) and elevated serum levels of hepatoprotective IL-10 ($P = 0.042$, Fig. 3e right panel).

Importantly, 1-MT completely diminished antifibrotic and immunomodulatory effects of MSC-CM. Injection of MSC-CM that contained 1-MT did not attenuate liver enzymes (Fig. 3c) and IL-17 in sera (Fig. 3e left panel) and did not increase serum levels of IL-10 (Fig. 3e right panel). In line with these findings, IDO inhibition reduced capacity of MSC-CM to increase percentage of IL-10 producing $CD4^+$ T cells in the

Figure 1 Intravenously injected mesenchymal stem cells (MSCs) migrate in the carbon tetrachloride (CCl_4)-injured livers and ameliorate fibrosis. (a) Total number of CSFE-labeled MSCs detected in the livers of CCl_4-treated mice. MSCs were detected in CCl_4-injured livers 24 h after intravenous injection, and their presence in damaged livers had been increasing after each application. (b) Macroscopic appearance of the livers showing an irregular contour with one or several nodules on the surface of the livers obtained from CCl_4-treated and CCl_4+STO cell-treated mice. Livers obtained from CCl_4+MSC-treated mice had a smooth surface with uniform and soft textures, similar to the livers of control animals (corn oil only-treated and MSCs only-treated mice). (c) H&E staining of liver tissues obtained from CCl_4-treated and CCl_4+STO cell-treated mice revealed single cell necrosis and ballooning degeneration of hepatocytes in central and midlobular regions (black arrows), associated with inflammatory cell infiltration. Organized hepatic architecture was replaced by regenerative nodules connected with fibrous septa. On contrary, there were only several solitary areas of necrotic tissue in CCl_4-treated mice that received MSCs. Liver architecture was unaltered in control animals (magnification ×10). (d) Sirius red staining of liver tissues obtained from CCl_4-treated and CCl_4+STO cell-treated mice revealed extensive collagen deposition and pseudolobular formation (black arrows). Stained area of fibrous dense tissue was reduced in CCl_4+MSC-treated mice and absent in control animals (magnification ×10). (e) Percentage of fibrotic liver tissue, estimated by ImageJ software, was significantly lower in CCl_4+MSC-treated mice compared to mice that received CCl4 ($P = 0.007$). (f) Fold change of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) levels to corn oil only-treated mice was significantly lower in CCl_4+MSC-treated mice compared to mice that received CCl_4 ($P = 0.038$ for AST and $P = 0.043$ for ALT). (g) Quantitative RT-PCR revealed significant decreased in expression of pro-fibrogenic markers: collagen-1 ($P = 0.043$), α-SMA ($P = 0.034$), and TGF-β ($P = 0.036$) in the livers of CCl_4+MSC-treated mice compared to mice that received CCl_4. Data presented as mean ± SEM; $n = 10$ mice per group. *p < 0.05; **p < 0.01

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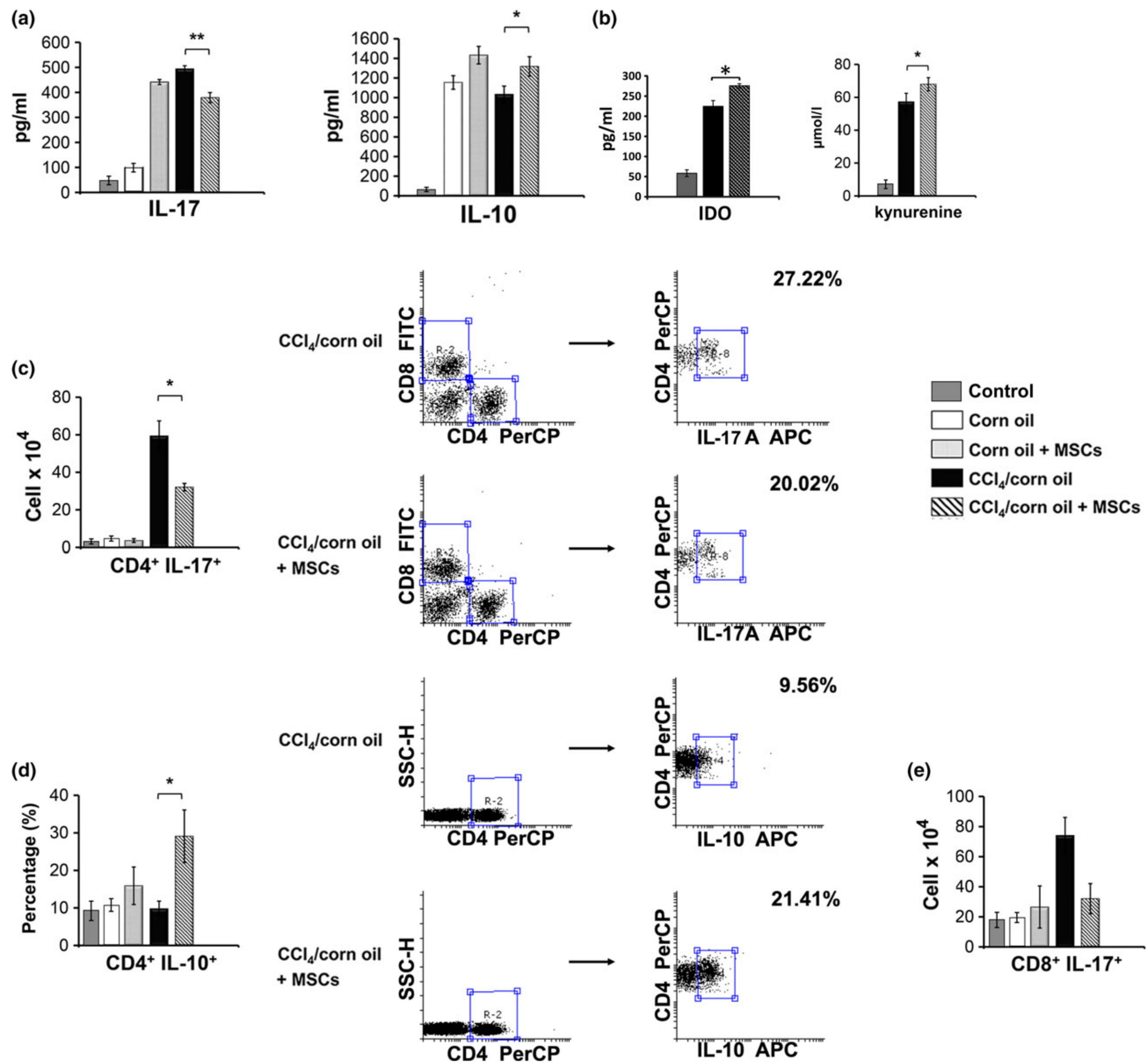

Figure 2 Mesenchymal stem cells (MSCs) attenuate IL-17-driven inflammation and significantly reduce total number of IL-17-producing $CD4^+$Th17 cells in the livers of carbon tetrachloride (CCl_4)-treated mice. (a) The level of IL-17 was significantly decreased, and levels of IL-10 and kynurenine were significantly increased in serum of CCl_4+MSC-treated mice compared to mice that received CCl_4 only ($P = 0.006$ for IL-17; $P = 0.042$ for IL-10). (b) Serum levels of indoleamine 2,3-dioxygenase (IDO) and kynurenine were significantly higher in of CCl_4+MSC-treated mice compared to mice that received CCl_4 only ($P = 0.02$ for IDO; $P = 0.048$ for kynurenine). (c and d) Intracellular staining and flow cytometry analysis with representative dotplots revealed significantly lower number of IL-17-producing $CD4^+$ T cells ($P = 0.009$, Fig. 2b), and significantly higher number of IL-10-producing $CD4^+$ T lymphocytes ($P = 0.024$, Fig. 2c) in the livers of CCl_4+MSC-treated mice compared to animals treated with CCl_4 only. (e) There was no significant difference in the total number of liver infiltrating IL-17-producing $CD8^+$ T lymphocytes between CCl_4+MSC-treated and CCl_4 only-treated mice. Data presented as mean ± SEM; $n = 10$ mice per group. *p < 0.05; **p < 0.01

fibrotic livers ($P = 0.048$, Fig. 3d lower panel). Moreover, total number of IL-17 producing $CD4^+$ T cells in CCl_4+MSC-CM+1-MT was significantly higher when compared to CCl_4+MSC-CM-treated mice ($P = 0.02$, Fig. 3d upper panel), indicating that MSC-mediated attenuated infiltration of Th17 cells in the fibrotic liver is IDO-dependent.

In line with these findings are data obtained from CCl_4+MSC-treated mice in which IDO activity was inhibited *in vivo* (Fig. 4). Macroscopic examination, histological analysis, and Sirius red staining revealed significant differences between CCl_4+MSC and $CCl_4^{1\text{-}MT}$+MSC-treated animals (Fig. 4a–c). Significantly higher percentage of fibrotic tissue ($P = 0.001$, Fig. 4d),

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elevated serum levels of AST and ALT (Fig. 4e, $P = 0.001$ for AST; $P = 0.006$ for ALT), increased concentration of IL-17 in sera ($P = 0.001$, Fig. 4g), and increased number of liver-infiltrated IL-17 producing $CD4^+$ T cells ($P = 0.003$, Fig. 4h), decreased serum concentration of IL-10 ($P = 0.033$, Fig. 4g), and significantly lower number of liver-infiltrated IL-10-producing $CD4^+$ T cells were observed in CCl_4+MSC-treated mice in which IDO activity was inhibited *in vivo* when compared with CCl_4+MSC-treated animals, confirming the importance of IDO for MSC-dependent attenuation of CCl_4-induced liver fibrosis.

Discussion

Th17 cells play an active and important role in liver fibrosis [1,2]. IL-17, produced by Th17 cells, directly stimulates HSCs to express collagen-1 and promotes their activation into fibrogenic myofibroblasts, directly contributing to the development and progression of liver fibrosis [1]. Increased expression of IL-17 was detected in livers of patients with hepatic fibrosis and correlated with increased numbers of circulating Th17 cells and with the severity of disease [1], suggesting IL-17 and Th17 cells as an attractive target for antifibrotic therapy.

Herewith, we demonstrated that transplantation of MSCs as well as injection of MSC-CM reduces liver infiltration of Th17 cells resulting with attenuation of liver fibrosis (Figs. 1 and 3). Decreased serum levels of IL-17 (Fig. 2a) and reduced number of Th17 in the livers (Fig. 2c), noticed in CCl_4+MSC-treated mice, correlated with decreased expression of HSC-derived fibrotic markers: collagen-1, α-SMA, and TGF-β (Fig. 1g). Additionally, MSCs managed to reduce established CCl_4-induced liver fibrosis (Fig. S1), indicating efficacy of MSC-based therapy for the treatment of liver fibrosis.

During the progression of liver fibrosis, IL-10 producing $CD4^+$ T cells have been recruited to the liver where they suppress hepatic inflammation [35]. Dynamic changes in the frequencies of IL-17 and IL-10 producing $CD4^+$ T cells significantly affect outcome of chronic liver injury [7], and their imbalance is usually linked to the progression of inflammation and fibrosis [7,36]. Herewith we showed that MSC-dependent suppression of Th17 cells in the liver was accompanied with elevated serum levels of immunosuppressive IL-10 (Fig. 2a) followed with an increased percentage of hepato-protective IL-10 producing $CD4^+$ T cells (Fig. 2d) indicating that beneficent effects of MSCs in CCl_4-induced fibrosis were closely related to the modulation of the balance between IL-17 and IL-10 producing $CD4^+$ T cells within liver.

Interestingly, serum concentration of IL-10 in control (corn oil-untreated mice) was lower than those measured in corn oil and corn oil + MSC-treated animals, while percentage of IL-10 producing $CD4^+$ T cells were similar between these groups. IL-10, one of the most important immunoregulatory and anti-inflammatory cytokines, is produced by $CD4^+$ T cells, alternatively activated macrophages and MSCs. MSCs may produce IL-10 even in the absence of fibrosis, after stimulation by IL-10-inducing factors, such as corn oil. As corn oil potentiates production of IL-10 in macrophages [37,38], we assumed that the higher serum concentration of IL-10, noticed in corn oil-treated mice compared to corn oil-untreated mice was a consequence of corn oil-induced increased production of IL-10 in macrophages and that this difference was not related to the production of IL-10 in $CD4^+$ T cells.

Similar as transplanted MSCs, injection of MSC-CM managed to efficiently attenuate liver fibrosis (Fig. 3c–e). The importance of MSC-CM-mediated beneficial effects in attenuation of CCl_4-induced fibrosis is an important finding having in mind that engrafted MSCs could be a major source of functional myofibroblasts which have detrimental effects in liver fibrosis [39]. After intrahepatic injection, MSCs permanently remain in the injured livers and can differentiate into myofibroblasts rather than in hepatocytes [40,41].

Mesenchymal stem cell-CM-mediated attenuation of CCl_4-induced fibrosis was accompanied with reduced number of liver-infiltrated Th17 cells and increased percentage of IL-10 producing $CD4^+$ T cells in the fibrotic livers (Fig. 3d), decreased serum level of IL-17 and increased serum concentration of IL-10 (Fig. 3e), indicating that MSC modulate cytokine production of $CD4^+$ T lymphocytes, through the release of soluble factors.

Among MSC-derived products, IL-6, insulin-like growth factor binding protein-2 (IGFBP-2), IL-1 receptor antagonist (IL-1Ra), hepatocyte growth factor (HGF), neural growth factor (NGF), IL-10, and TGF-β3 may contribute to the beneficent effects of MSCs in the attenuation of liver fibrosis [42–45]. MSC-derived IL-6 has anti-apoptotic effects, IGFBP-2 regulates insulin-like growth factor-I that has fibrinolytic activity while IL-1 Ra, as natural inhibitor of pro-inflammatory IL-1, is involved in MSC-dependent suppression of inflammation [42]. MSC-produced HGF and NGF promote apoptosis of HSCs, while IL-10 and TGF-β3 are responsible for MSC-dependent suppression of collagen synthesis in HSCs [43–45]. Additionally, MSCs can

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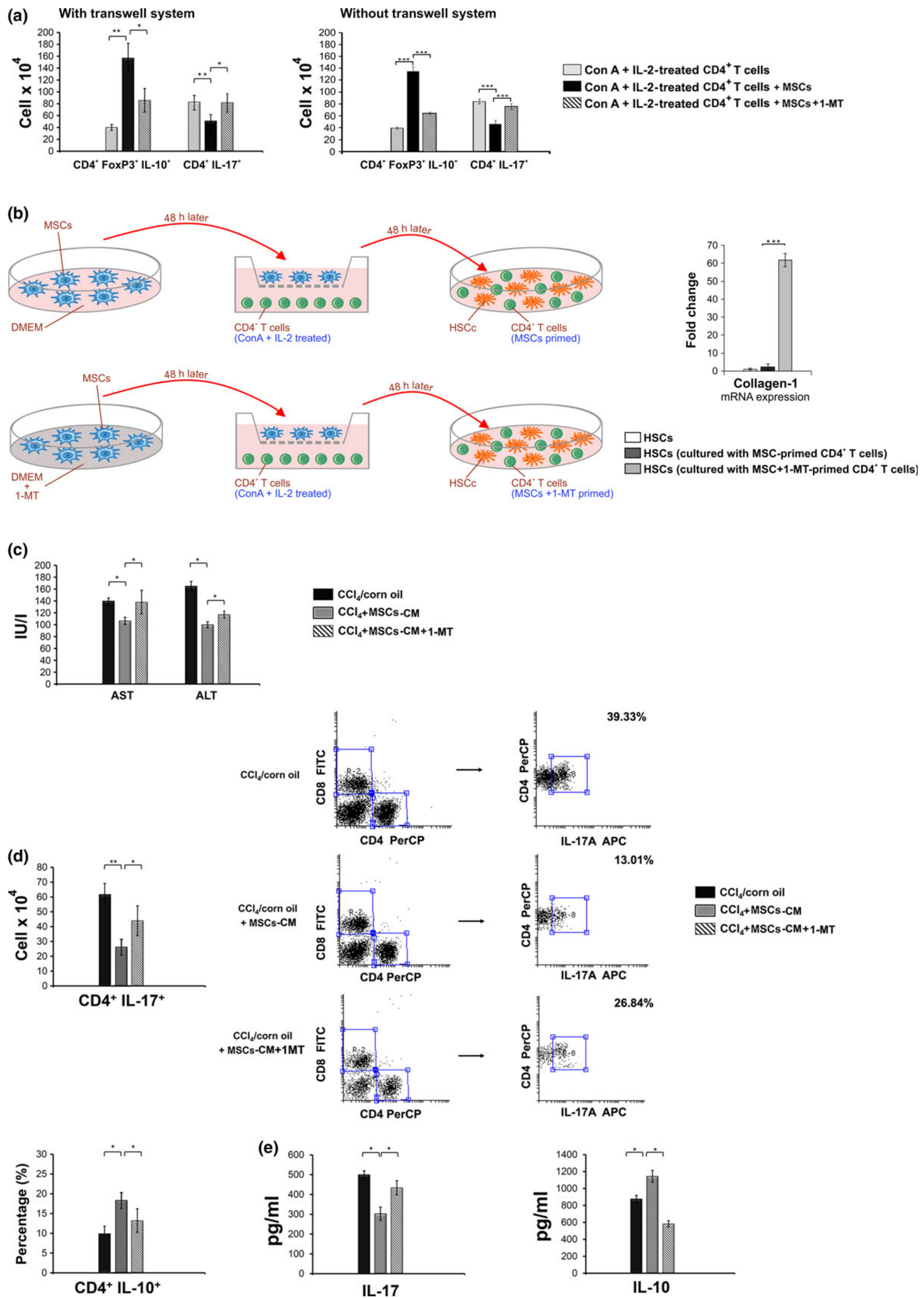

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Figure 3 Mesenchymal stem cell-conditioned medium (MSC-CM)-dependent proliferation of IL-10 producing $CD4^+$ T cells and suppression of Th17 cells were abrogated by 1-MT indoleamine 2,3-dioxygenase (IDO inhibitor). (a) Intracellular staining revealed significantly lower number of IL-17 producing *in vitro* (with concanavalin A and IL-2) activated liver $CD4^+$ T cells, cultured with MSCs in the presence (left panel) or in the absence (right panel) of transwell system, when compared to concanavalin A and IL-2 stimulated $CD4^+$ T lymphocytes that were culture without MSCs ($P = 0.003$ left panel and $P = 0.001$ right panel). MSCs induced significant increase in total number of IL-10 producing $Foxp3^+$ $CD4^+$ T cells ($P = 0.002$ left panel and $P = 0.001$ right panel). This phenomenon was completely abrogated in the presence of 1-MT. There was significant increase in Th17 cells ($P = 0.017$ left panel and $P = 0.002$ right panel) and decrease in IL-10 producing Tregs ($P = 0.013$ left panel and $P = 0.001$ right panel) when activated $CD4^+$ T cells were cultured in MSC-CM that contained 1-MT. (b) Real-time PCR analysis revealed significantly lower expression of collagen-1 ($P = 0.001$) in hepatic stellate cells (HSCs) that were cultured with MSC-primed liver $CD4^+$ T cells when compared with HSCs which were cultured with MSC+1-MT-primed liver $CD4^+$ T cells. (c) Comparison between liver enzymes obtained from CCl_4 only-treated and CCl_4+MSC-CM-treated mice showed that injection of MSC-CM significantly down-regulated serum levels of aspartate aminotransferase (AST) ($P = 0.037$) and alanine aminotransaminase (ALT) ($P = 0.042$). Injection of MSC-CM that contained 1-MT did not attenuate liver enzymes in CCl_4-treated animals. (d) Intracellular staining and flow cytometry analysis with representative dotplots showing significantly reduced infiltration of IL-17 producing $CD4^+$ T cells ($P = 0.004$, upper panel) and significantly increased percentage of IL-10 producing T cells ($P = 0.031$, lower panel) in livers of CCl_4+MSC-CM-treated mice when compared to CCl_4 only-treated animals. Total number of IL-17 producing $CD4^+$ T cells was significantly lower ($P = 0.02$, upper panel), and percentage of IL-10 producing T cells was significantly higher ($P = 0.048$, lower panel) in the livers of in CCl_4+MSC-CM-treated mice when compared to CCl_4+MSC-CM+1-MT-treated animals. (e) Serum levels of pro-fibrogenic IL-17 were significantly lower ($P = 0.028$, left panel), and serum levels of hepatoprotective IL-10 were significantly higher ($P = 0.042$ right panel) in CCl_4+MSC-CM-treated mice when compared to CCl_4 only-treated animals. Injection of MSC-CM that contained 1-MT did not significantly alter serum levels of IL-17 and IL-10 in CCl_4-treated animals. Data presented as mean ± SEM; $n = 10$ mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

regulate the expression of matrix metalloproteinase (MMP)-9 that has been associated with resolution of liver fibrosis [46].

Among all MSC-derived soluble molecules, IDO plays the most important role in MSC-mediated suppression of IL-17 signaling pathway [47]. Under Th1 and Th2 conditions, human MSCs most usually use IDO and its metabolites (kynurenine, quinolinic acid, and 3-hydroxy-anthranilic acid) to attenuate proliferation or to induce apoptosis of activated T cells [14], while murine MSCs have lower IDO activity and usually use inducible nitric oxide synthase (iNOS)-mediated immunomodulation [14,18,48]. However, under Th17 conditions, murine MSCs do not produce nitric oxide and their immunosuppressive effects are mediated through the production of other mediators, including IDO [35]. MSCs are able to suppress generation of effector Th17 cells in IDO-dependent manner, and IDO inhibitors could be used to restore MSC suppression of Th17 differentiation [49].

Protective and immunomodulatory role of IDO has been recently described in CCl_4-induced liver fibrosis [50]. Increased infiltration of immune cells and increased expression of pro-inflammatory cytokines was noticed in fibrotic livers of CCl_4-treated IDO-deficient mice. Aggravated liver fibrosis was also seen in mice that received IDO inhibitor [50].

In line with these findings, attenuated liver fibrosis noticed in CCl_4+MSC-treated mice (Fig. 1d and e) was accompanied with an increased serum levels of IDO and kynurenine (Fig. 2b) and reduced liver infiltration of Th17 cells (Fig. 2c). Inhibition of IDO completely diminished MSC-mediated suppression of Th17 proliferation (Fig. 3a) and enhanced infiltration of Th17 cells in the fibrotic livers (Fig. 3d). *In vitro*, capacity of MSCs to suppress Th17 cell-dependent induction of collagen-1 synthesis in HSCs was attenuated by IDO inhibitor (Fig. 3b). Additionally, *in vivo* inhibition of IDO activity completely abrogated MSC-based suppression of Th17 cells and delineate antifibrotic effects of MSCs (Fig. 4), suggesting important role of IDO for MSC-mediated suppression of Th17-driven liver fibrosis.

Indoleamine 2,3-dioxygenase has been identified as a critical molecular switch that stimulates immunosuppressive properties of IL-10 producing Tregs and simultaneously blocks re-programming of Tregs into IL-17 producing effector T cells [47]. IDO inhibition or genetic deletion led to the reduced generation of Tregs and an increase in Th17 differentiation both *in vitro* and *in vivo* [47]. Accordingly, we found that MSC-CM induced proliferation of IL-10 producing $CD4^+FoxP3^+$ Tregs (Fig. 3a), a phenomenon that was completely abrogated by IDO inhibitor (Fig. 3a).

We propose that MSCs produce IDO, which suppress proliferation of Th17 cells and their infiltration in fibrotic livers, reduce IL-17, and promote proliferation and liver infiltration of immunomodulatory IL-10 producing $CD4^+$ T cells creating an immunosuppressive microenvironment in which HSCs due to the lack of IL-17-dependent activation reduce production of collagen-1,

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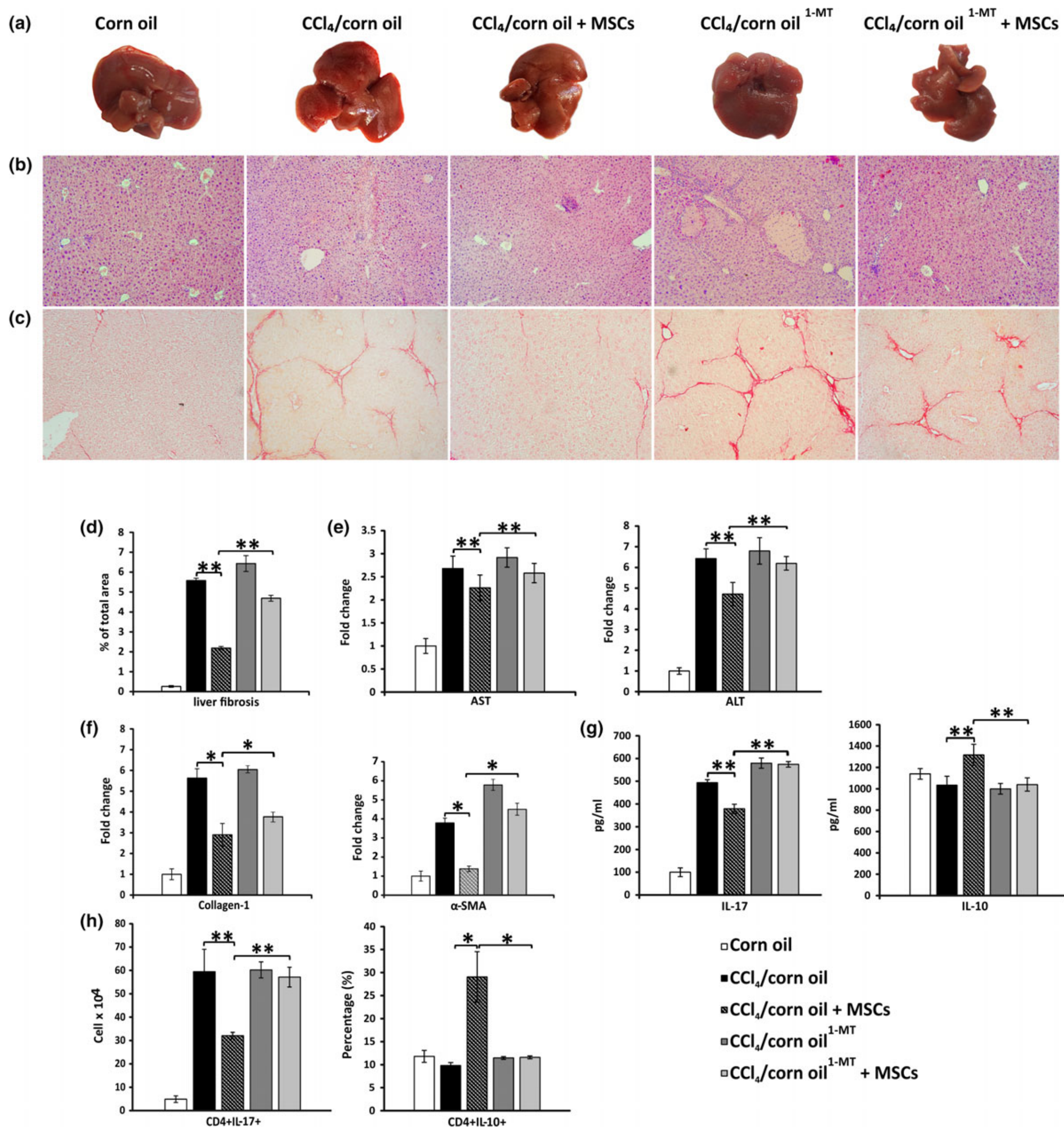

Figure 4 *In vivo* inhibition of indoleamine 2,3-dioxygenase (IDO) activity completely diminished mesenchymal stem cell (MSC)-based beneficent effects in carbon tetrachloride (CCl_4)-induced liver fibrosis. (a) One or several nodules were noticed on the surface of the livers obtained from CCl_4, $CCl_4^{1\text{-}MT}$-treated, and $CCl_4^{1\text{-}MT}$+MSC-treated mice, while livers obtained from CCl_4+MSC-treated mice had a smooth surface with uniform and soft textures. (b) H&E staining of liver tissues obtained from CCl_4, $CCl_4^{1\text{-}MT}$-treated, and $CCl_4^{1\text{-}MT}$+MSC-treated mice revealed regenerative nodules connected with fibrous septa, while there were only several solitary areas of necrotic tissue in CCl_4-treated mice that received MSCs. (magnification ×10). (c) Sirius red staining of liver tissues obtained from CCl_4+MSC-treated mice revealed reduced area of fibrous dense tissue when compared to CCl_4 and $CCl_4^{1\text{-}MT}$+MSC-treated animals (magnification ×10). (d) Percentage of fibrotic liver tissue was significantly lower in CCl_4+MSC-treated mice compared to $CCl_4^{1\text{-}MT}$+MSC-treated animals ($P = 0.001$). (e) Fold change of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) levels to corn oil only-treated mice was significantly lower in CCl_4+MSC-treated mice compared to mice that received $CCl_4^{1\text{-}MT}$+MSCs ($P = 0.001$ for AST and $P = 0.006$ for ALT). (f) Quantitative RT-PCR revealed significant decreased in expression of pro-fibrogenic markers: collagen-1 ($P = 0.022$), α-SMA ($P = 0.000$) in the livers of CCl_4+MSC-treated mice compared to mice that received $CCl_4^{1\text{-}MT}$+MSCs. (g) The level of IL-17 was significantly decreased, and levels of IL-10 were significantly increased in serum of CCl_4+MSC-treated mice compared to mice that received $CCl_4^{1\text{-}MT}$+MSCs ($P = 0.001$ for IL-17 and $P = 0.041$ for IL-10). (h) Intracellular staining and flow cytometry analysis revealed significantly lower number of IL-17-producing $CD4^+$ T cells and significantly higher number of IL-10-producing $CD4^+$ T lymphocytes in the livers of CCl_4+MSC-treated mice compared to $CCl_4^{1\text{-}MT}$+MSC-treated animals ($P = 0.003$ for IL-17-producing $CD4^+$ T cells and $P = 0.033$ for IL-10-producing $CD4^+$ T lymphocytes). Data presented as mean ± SEM; $n = 10$ mice per group for CCl_4 and CCl_4+MSC-treated mice and $n = 7$ per group for $CCl_4^{1\text{-}MT}$ and $CCl_4^{1\text{-}MT}$+MSC-treated animals. $^*p < 0.05$; $^{**}p < 0.01$

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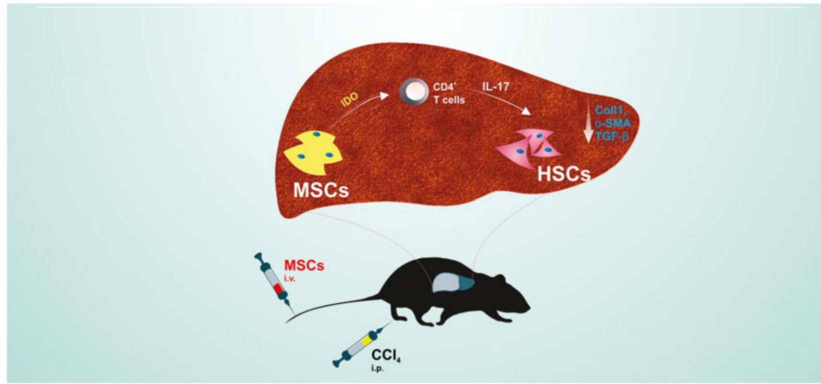

Figure 5 Schematic diagram describing mechanism responsible for mesenchymal stem cell (MSC)-mediated suppression of Th17 cells and attenuation of liver fibrosis. MSCs, in paracrine manner, through the release of indoleamine 2,3-dioxygenase (IDO), suppress proliferation of liver-infiltrated Th17 cells and attenuate IL-17-driven inflammation. Due to the lack of IL-17-dependent activation, hepatic stellate cells (HSCs) reduce production of collagen-1 (Coll1), α-smooth muscle actin (α-SMA) and TGF-β, resulting with the attenuation of liver fibrosis.

α-SMA and other pro-fibrotic molecules, resulting with the attenuation of liver fibrosis (Fig. 5).

The capacity of MSCs to suppress Th17 cells in IDO-dependent manner may be used as new therapeutic approach for the treatment of IL-17-driven liver fibrosis.

Authorship

NM, MG, BSM and AA: performed experiments and wrote manuscript. JN, ZD, NJ and IJ: performed experiments. VDj, NA and MLL: analyzed data and wrote manuscript. VV: designed and performed experiments, analyzed data and wrote manuscript. All authors: contributed to the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest related to this study.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Intravenously injected MSCs reduce already established CCl_4-induced liver fibrosis.

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