Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice

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Abstract

Mesenchymal stem cells (MSCs) are adult progenitor cells that contribute to stromal tissue renewal [1]. Originally found in the bone marrow [2], MSCs are present in all types of tissues [3] and were recently recognized as closely related to blood vessel pericytes [4]. MSC-based cell therapy is currently investigated with the aim to treat acute and chronic liver injury [5]. Indeed, it was suggested that MSCs might be able to transdifferentiate into hepatocytes [6]. Several other studies demonstrated that MSCs have immunosuppressive and anti-inflammatory properties [7–9], which could represent another mechanism by which MSCs improve chronic liver injury.

In experimental models, MSCs reduce liver fibrosis in rodents [10–14]. The mechanisms of action remain largely unknown but may involve the secretion of anti-inflammatory cytokines, such as IL-10 [15] or IL-1 receptor antagonist (IL-1Ra) [16], or the secretion of growth factors such as hepatocyte growth factor [7,17], vascular endothelial growth factor [18] or insulin-like growth factor-binding proteins [17]. Further, MSCs secrete matrix metalloproteinases that could mediate a fibrolytic effect [12].

Introduction

Mesenchymal stem cells (MSCs) are adult progenitor cells that contribute to stromal tissue renewal [1]. Originally found in the bone marrow [2], MSCs are present in all types of tissues [3] and were recently recognized as closely related to blood vessel pericytes [4]. MSC-based cell therapy is currently investigated with the aim to treat acute and chronic liver injury [5]. Indeed, it was suggested that MSCs might be able to transdifferentiate into hepatocytes [6]. Several other studies demonstrated that MSCs have immunosuppressive and anti-inflammatory properties [7–9], which could represent another mechanism by which MSCs improve chronic liver injury.

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Other studies showed that in vitro hepatocyte-like pre-differentiation of MSCs has a therapeutic effect in experimental liver fibrosis [19,20]. Consequently, it is currently not clear whether molecules secreted by MSCs are sufficient to mediate the anti-fibrotic effect or whether cell-cell interactions and/or the presence of hepatocyte-differentiated MSCs are necessary.

A further issue is phenotype stability: even if MSCs engraft in the injured liver and differentiate into hepatocyte-like cells, it is likely that induction of chronic injury (e.g., high levels of transforming growth factor-beta 1 (TGF-β)) precludes those cells from maintaining epithelial-like characteristics. It was shown that cells recruited from the bone marrow to an experimentally-induced fibrotic liver finally became collagen-producing fibrocytes [21].

In the present study, we investigated whether immunoprotection by microencapsulation prevents MSCs from participating to scar formation and allows MSCs to mediate an anti-fibrotic effect by releasing soluble molecules in vitro and in vivo. We found that MSC-conditioned medium (MSC-CM) reduced alpha smooth muscle actin (α-SMA) expression, a marker of hepatic stellate cell (HSC) activation (the key event in liver fibrosis).

We used recently developed alginate-polycrylamide glycol (alg-PEG) hybrid hydrogel to encapsulate MSCs. This hydrogel is permissive to soluble factors (e.g., O2, glucose, cytokines) but not to immune cells or antibodies, thus protecting MSCs from activation (the key event in liver fibrosis).

Formation of microspheres Microspheres were prepared under sterile conditions. We used a co-axial air-flow droplet generator as previously described [26]. Briefly, MSCs or EDX cells were detached using 0.25% trypsin-EDTA (Sigma) for about 30 sec and washed twice. The cell suspension was centrifuged (1200 rpm, 5 min, RT) and the supernatant discarded. The pellet was resuspended in Na-alg/PEG-8-20 solution (1.5% (w/v) Na-alg + 10% (w/v) PEG-8-20 in DMEM (special formulation without NaCl and KCl, Culture Technologies, Gravessano, Switzerland)) to a final concentration of 500,000 cells/ml. The mixture was extruded through a 400 μm needle into the sterile gelation bath prepared by dissolving CaCl2 and DTT, in DMEM (special formulation as indicated above) with osmolality adjusted to 300 mOsm/kg (30 ± 5 mM CaCl2). The receiving bath was incubated in a shaker (80 rpm) at 37 °C for 3 h to achieve optimal cross-coupling [27]. Microspheres were collected and cultured in IMDM 10% FCS. Microspheres without cells were prepared using the same protocol.

Fibrosis induction in mice All animal studies were approved by the animal ethics committee of the Geneva Veterinary Office and the University of Geneva, Geneva, Switzerland (protocol number 1043/1003/2). Eight to 10 week-old male DBA-1 mice were purchased from Janvier (Le Genest-St-Isles, France). All mice were maintained under standard conditions at the animal facility of the Geneva University. Water and food were provided ad libitum. Liver fibrosis was induced by BDL as previously described [28]. Briefly, mice were anesthetized with isoflurane and a midline laparotomy was performed in order to expose the hepatic hilum and to identify the common bile duct. We used a dissecting microscope to cut the common bile duct in between three ligatures. To obtain CCl4-induced liver fibrosis, 2 ml/kg of CCl4 was administered by intraperitoneal injection weekly for 4 weeks (to avoid intra-peritoneal damage of the encapsulated cells). Animals received intraperitoneally 1.5 million encapsulated MSCs in 1 ml alg-PEG microspheres, or 1.5 million encapsulated EDX cells in 1 ml alg-PEG, or 1 ml alg-PEG microspheres without cells. Sham operated mice were used as controls. The animals were sacrificed 15 days after BDL and blood (transaminases, IL-10, and IL-11Ra measurements) and liver samples (histology and RT-PCR) were collected.

Statistical analysis Results were expressed as mean values ± SEM. Differences between groups were analyzed using the Student t-test or Mann-Whitney U test (2 groups) and one-way analysis of variance with Bonferroni multiple testing correction (>2 groups). p <0.05 was considered statistically significant.

Results Isolation and characterization of mesenchymal stem cells MSCs were isolated from 11 adult donors. MSCs showed typical spindle-shape morphology and were expanded during 3–6 passages to reach about 15 population doublings (Supplementary Fig. 1A). Analysis of the surface antigens on MSCs by flow cytometry showed patterns that are typical of MSCs. Cells were negative for HLA class1, CD34, CD36, and CD45, and positive for CD44, CD54, CD90, CD105, and CD106 (Supplementary Fig. 1B).
Mesenchymal stem cell conditioned medium decreased HSC activation in vitro

The effect of MSC-CM on α-SMA expression, in immortalized (LX-2) and primary human HSCs, was studied. LX-2 cells were treated with control medium, MSC-CM, TGF-β (used as positive control), and MSC-CM together with TGF-β for 5 days, and analyzed by Western blotting (Fig. 1A). LX-2 cells cultured with MSC-CM showed decreased levels of α-SMA compared to untreated cells (Fig. 1A). The treatment of LX-2 cells with MSC-CM together with TGF-β also resulted in decreased expression of α-SMA compared to cells treated with TGF-β alone (Fig. 1A), demonstrating neutralization of the activation induced by TGF-β. Quantification of α-SMA levels is shown in Fig. 1B. In primary HSCs, levels of collagen type I and MMP-2 mRNA were increased upon TGF-β treatment, while the increase was statistically significantly lower in the presence of MSC-CM (Fig. 1C and D). These results indicate that MSC-CM was able to reduce LX-2 and primary HSC activation when cells were cultured in profibrogenic conditions (i.e., upon treatment with TGF-β).

Survival, proliferation, and differentiation of encapsulated mesenchymal stem cells in vitro

First, the quality of MSCs after microencapsulation was analyzed in vitro assays. MSCs were microencapsulated in recently developed alg-PEG hybrid microspheres [26, 27, 29] (Supplementary Fig. 2A). Cell viability was assessed at day 0 and at 5, 15, 45, and 218 days after microencapsulation (Supplementary Fig. 3A). Immediately after trypsinization and encapsulation, MSCs were round-shaped (Supplementary Fig. 2B) and viability was 66.8 ± 3.8%, compared to 74.4 ± 2.9% among free MSCs (after trypsinization) (mean ± SEM, n = 4 independent experiments, difference not statistically significant (p = 0.418)) (Supplementary Fig. 3B). Five days after microencapsulation, MSCs recovered their typical spindle-shaped morphology (Supplementary Fig. 3C), almost without any cell death (viability close to 100%). Viability of MSCs and integrity of alg-PEG microspheres were maintained up to 6 months of culture (Supplementary Fig. 3A). Similar levels of proliferation were observed in encapsulated MSCs (2.7 ± 0.6%) and free MSCs (2.4 ± 0.2%) (mean ± SEM, n = 4 independent experiments, difference not statistically significant (p = 0.673)), as shown by analysis of 24 h-EdU incorporation (Supplementary Fig. 3C and D). When cultured in the respective differentiation media, free MSCs and microencapsulated MSCs were able to differentiate into adipocytes storing large lipid droplets (oil-red-O staining), osteoblasts producing calcific depositions (Alizarin red staining), and chondrocytes producing cartilage matrices (Supplementary Fig. 3C and D).
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collagen type I to IV (Goldner's trichrome), (Supplementary Fig. 3E). These results indicate that microencapsulated MSCs remain fully functional in terms of viability, proliferation, and differentiation potential.

Cytokine profile of free and microencapsulated mesenchymal stem cells

The cytokine profile of MSCs was analyzed using a human cytokine antibody array, with the aim of identifying molecules that might implicate an anti-fibrotic or anti-inflammatory effect (Fig. 2C). Medium conditioned by free EDX cells was used as control for media from free MSCs and microencapsulated MSCs (Fig. 2A). MSCs secreted several cytokines (Fig. 2A, Supplementary Fig. 4A), with the most elevated ones being angiogenin, IGFBP-2, IL-6, and MCP-1 (Fig. 2B). With the exception of angiogenin, EDX cells secreted modest levels of cytokines when compared to MSCs (Fig. 2B, Supplementary Fig. 4B). Conditioned medium from microencapsulated MSCs and medium from free MSCs showed a similar cytokine profile in vitro (i.e., angiogenin, IL-6, MCP-1) (Fig. 2A and B).

Since it has been suggested that MSCs secrete interleukin 1 receptor antagonist (IL-1Ra) [16], this was investigated for 22 MSC-CM preparations using a human-specific IL-1Ra ELISA (Fig. 2C). IL-1Ra was detected at levels above background (i.e., 100 pg/ml) in 5 out of 22 (23%) MSC-CM preparations and was not detectable in control media (n = 4) [16]. These results revealed that both free and microencapsulated MSCs secreted cytokines such as IGFBP-2, IL-6, MCP-1, and IL-1Ra.

Survival of microencapsulated mesenchymal stem cells after transplantation into mice

At one and six months after transplantation in mice, viable MSCs were found, as shown by the presence of nucleated, spindle-shaped, and human vimentin-positive cells in microspheres (Supplementary Fig. 5A and B, Supplementary Fig. 6A and B). The fibrotic reaction around the microspheres visualized by Masson's trichrome staining was moderate, did not increase from one to six months, and was not different between empty microspheres and microspheres containing MSCs (Supplementary Fig. 6A). A cellular infiltration was observed around the microspheres with MSCs at one month (Supplementary Fig. 6A). The infiltration included IBA-1-positive macrophages and CD4 and CD8-positive T cells. The relative presence of CD4 and CD8-positive T cells (normalized to the total number of cells) was significantly higher around microspheres with MSCs than around empty microspheres (n = 3 mice per group, \( p = 0.042 \) and \( p = 0.011 \), respectively). Similar levels of IBA-1-positive macrophages, expressed with respect to the total number of nuclei, were present around microspheres, in animals receiving empty microspheres or microspheres with MSCs (n = 3 mice per group, difference not statistically significant (\( p = 0.176 \)). Quantification of F4/80-positive cells on cryosections gave similar results (data not shown). Together, these results indicated that encapsulated MSCs are durably protected from rejection in mice and cause a modest fibrotic reaction, with emergence of an immune infiltrate around microspheres.

Reduced liver fibrosis and increased MMP-9 expression in the liver of mice after bile duct ligation and transplantation with microencapsulated mesenchymal stem cells

DBA-1 mice were treated with BDL or CCl4 and underwent intra-peritoneal transplantation with empty microspheres, encapsulated EDX cells or microencapsulated MSCs. The extent of liver fibrosis was analyzed by Masson's trichrome staining and by measuring the expression of fibrosis-related genes. Quantification of collagen revealed significantly less liver fibrosis in mice transplanted with microencapsulated MSCs than in mice transplanted with microencapsulated EDX cells or empty microspheres (8.2% vs. 12.0% and 11.0%, BDL model; 1.0% vs. 1.4% and 4.4%, CCl4 model) (Fig. 3A–D). Histology revealed microencapsulated MSCs around the liver (Supplementary Fig. 5C). Liver collagen type I mRNA levels were increased compared to mice without BDL (>23-fold) or without CCl4 (10-fold). Collagen type I expression was reduced in the liver of mice treated with microencapsulated MSCs compared to mice treated with microspheres containing EDX cells or empty microspheres: the respective differences did not reach statistical significance (Fig. 3E and F). Compared with control mice that were not treated with BDL or CCl4, all three treatment groups showed a substantial increase in liver mRNA levels of \( \alpha \)-SMA (Fig. 3G and H), MMP-9 (Fig. 3I and J), and MMP-13 (Fig. 3K and L). In the BDL model, \( \alpha \)-SMA mRNA levels were significantly increased in the liver of mice treated with empty microspheres (8.1-fold) or microencapsulated EDX cells (9.1-fold), but not in mice treated with microencapsulated MSCs (1.3-fold): the difference reached statistical significance (Fig. 3G). No significant difference was observed in the CCl4 model (Fig. 3H). The number of liver \( \alpha \)-SMA and IBA-1 positive cells was reduced in mice treated with encapsulated MSCs compared to control (data not shown). Microencapsulated MSCs induced a substantial increase in MMP-9 expression in BDL and CCl4 models (7.6-fold and 8.3-fold, respectively) whereas microencapsulated EDX cells or empty microspheres gave a lower increase (1.8 and 2.0-fold, respectively (BDL) and 2.9 and 5.7-fold, respectively (CCl4)) (Fig. 3I and J). MMP-13 mRNA levels increased compared to mice without BDL or CCl4 and without significant differences between the three groups (Fig. 3K and L). We concluded that microencapsulated MSCs transplanted into bile duct-ligated mice have a protective effect during the development of liver fibrosis since collagen accumulation was reduced. The results further suggest that regulation of MMP-9 contributes to this effect.

Decreased ALT and AST levels and increased levels of IL-10 and IL-1Ra in mice transplanted with microencapsulated mesenchymal stem cells

Mice with bile duct ligation- or CCl4-induced liver fibrosis showed a substantial increase in ALT and AST after bile duct ligation, reflecting liver parenchymal damage (Fig. 4A–D). Liver enzymes were lower in mice receiving microencapsulated MSCs in both models, and the difference was statistically significant in the bile duct ligation model (\( p < 0.05 \)). Mice treated with microencapsulated EDX cells showed a lower reduction, which did not reach statistical significance in either model. Levels of IL-10 were significantly higher in mice after transplantation with microencapsulated MSCs than in control mice in which no bile duct ligation was performed (\( p < 0.05 \)); an increase in IL-10 was also seen in mice after bile duct ligation and transplantation with
microencapsulated EDX cells, but this difference did not reach statistical significance (Fig. 4E). In the bile duct ligation model, human IL-1Ra was detected in the serum of 4 out of 11 mice (36%) transplanted with microencapsulated MSCs, and in none of the mice transplanted with empty microspheres (n = 7) (Fig. 4F). These results showed that encapsulated MSCs reduced liver damage induced by bile duct ligation or CCl₄, promoted endogenous anti-inflammatory IL-10 secretion, and secreted anti-inflammatory cytokine IL-1Ra in vivo.

Discussion

MSC transplantation is currently investigated in 22 clinical trials as potential treatment for chronic liver diseases (www.clinicaltrial.gov) and several phase 1-2 studies are published[5,30,31]. These trials are based on pre-clinical studies where MSCs were shown to reduce liver fibrosis upon systemic injection[10,11,14]. The mechanisms underlying this beneficial effect are not well understood and may include MSC ability to differentiate into hepatocyte-like cells [32], to reduce inflammation [33] and to enhance tissue repair at the site of injury [34]. The aim of the present study was to analyze the potential anti-fibrotic effect of molecules secreted by MSCs. We used cell microencapsulation to avoid cellular interactions between recipient’s cells and transplanted MSCs, and to analyze solely the effect of released cytokines on liver fibrosis.

We first showed that conditioned medium from human bone marrow-derived MSCs impeded the activation of HSC in vitro. To analyze whether this effect persisted in vivo, MSCs were microencapsulated to avoid intercellular contact after transplantation into mice. We used newly developed alg-PEG microspheres [27] allowing reduced inflammation and better mechanical resistance, when compared to conventional calcium-alginate microspheres [29]. In vitro, microencapsulated MSCs continued to proliferate and kept their capacity to differentiate into adipocytes, osteocytes and chondrocytes, demonstrating that microencapsulated MSCs were fully functional after encapsulation.

To identify molecules implicated in the anti-fibrotic effects of MSCs, we performed a cytokine antibody array using MSC-CM. We compared medium from free and microencapsulated MSCs to verify that cytokine release from MSC was not inhibited by the microencapsulation procedure. The results confirmed that both free and microencapsulated MSCs secrete cytokines at similar levels. IL-6, IGFBP-2, and MCP-1 were among the cytokines showing the highest level of secretion. Of note, IL-6 has known anti-apoptotic effects and may contribute to the protective effect of MSCs [35,36]. IGFBP-2 regulates insulin-like growth factor-I, which is a potent cytoprotective and anabolic hormone produced by the liver; upregulated insulin-like growth factor-I was shown

Fig. 3. Effect of microencapsulated mesenchymal stem cells on liver fibrosis in mice and correlation with MMP-9 expression in the liver. (A–D) Livers from mice were collected 15 days after BDL or 4 weeks of CCl₄ treatment and transplantation with either empty microspheres (n = 8 (BDL), n = 8 (CCl₄)), microencapsulated EDX cells (n = 5 (BDL), n = 8 (CCl₄)), or microencapsulated MSCs (n = 4 (BDL), n = 8 (CCl₄)). Mice without BDL (n = 6) or mice treated with corn oil (n = 3) were used as controls. Samples were fixed in formalin and embedded in paraffin. Liver sections were stained with Masson’s trichrome. Liver parenchyma appears in red and fibrotic areas in blue. Scale bars 400 μm. (B and D) Morphometric quantification of fibrosis was performed on multiple liver sections and expressed as percentage fibrotic surface area. Data are presented as mean value ± SEM. p <0.05, **p <0.01, ***p <0.001.

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Fig. 4. Liver enzymes and IL-10 and IL-1Ra in serum of mice treated with microencapsulated mesenchymal stem cells or EDX cells, or with empty microspheres. (A–D) Serum levels of AST and ALT (expressed in international unit (IU)) in mice without BDL (n = 8) or treated with corn oil (n = 3), and in mice 15 days after BDL or 4 weeks after CCl4 treatment and transplantation of empty microspheres (n = 4 (BDL), n = 8 (CCl4)), microencapsulated EDX cells (n = 7 (BDL), n = 8 (CCl4)), and microencapsulated MSCs (n = 8 (BDL), n = 8 (CCl4)). (E) Serum levels of IL-10 in mice with BDL and after transplantation of empty microspheres (n = 6), microencapsulated EDX cells (n = 4), and microencapsulated MSCs (n = 5), expressed in fold change with respect to control mice without BDL (n = 5). (F) Human IL-1Ra in the serum of mice transplanted with empty microspheres (n = 7) and microencapsulated MSCs (n = 11). (A–C) Data are presented as mean value ± SEM, *p < 0.05.

that was similar for empty microspheres and microspheres with MSCs.

Second, we showed the effectiveness of microencapsulated MSCs in reducing BDL- and CCl4-induced liver fibrosis in mice. Fifteen days after BDL and transplantation of microencapsulated MSCs, a significant reduction of α-SMA expression was observed, compared to mice treated with encapsulated EDX cells or with empty microspheres. This confirmed the in vitro results where MSC-CM reduced α-SMA expression in HSCs. After 4 weeks of CCl4 treatment, this effect was not observed, possibly because the peak of α-SMA expression had passed, making potential differences difficult to detect.

We found that microencapsulated MSCs increased endogenous IL-10 secretion, confirming that MSCs promoted anti-inflammatory/anti-fibrotic signals in the mouse liver fibrosis model. Our results are in line with another study on liver fibrosis in mice, where mouse bone marrow MSCs reduced α-SMA expression in the fibrotic liver [10]. A direct cellular contact of MSCs with immune or inflammatory cells or liver cells was prevented by microencapsulation procedure in our experimental design, indicating that factors secreted by MSCs are responsible for the inhibitory effect on HSC activation.

We observed a reduced collagen deposition in the liver of mice treated with microencapsulated MSCs compared to mice treated with microencapsulated EDX cells or empty microspheres; this is in line with previous studies [10,11,42]. Further, reduced fibrosis could implicate matrix metalloproteinases, known to contribute to the matrix degradation during liver fibrosis and resolution of fibrosis [43]. The substantial increase of MMP-9 expression in the liver, induced by microencapsulated MSC, might represent a possible explanation for the reduced fibrosis in the mouse liver fibrosis model. Indeed, MMP-9 is overexpressed by lymphocytes, neutrophils, and Kupffer cells during chronic liver injury, to counteract massive extracellular matrix accumulation [43,44]; also, increased levels of MMP-9 have been associated with diminished liver fibrosis in rodents [12,45] or cardiac ventricular fibrosis [46].

The microencapsulation of human MSCs allows the study of the effect of human MSCs instead of murine MSCs, because the micropore provides protection against the xeno-immune reaction. We sought to specifically analyze human MSC effects with the aim of applying our findings to clinical research. This is relevant because murine MSC isolation is challenging and homogeneous populations showing similar properties compared to human MSCs are difficult to obtain, rendering the comparison between these two cell types difficult [47–50]. An alternative approach is to use immunodeficient mice unable to mount a xeno-immune reaction, but liver fibrosis may develop inadequately in such animals [51].

A clear advantageous observation in the present study was that microencapsulated human MSCs in immunocompetent mice induced minor inflammation, indicating that the observed effects on the liver are not likely to be due to a bystander effect of an activated immune system or inflammatory reaction. In order to exclude such an effect, we transplanted microencapsulated EDX cells as a control group. EDX cells had a marginal effect on fibrosis development in the BDL model; an intermediate effect was observed in the CCl4 model. Albeit to a lesser extent compared to MSCs, fibrosis and transaminases were reduced, following encapsulated EDX cell transplantation. Of note, EDX had no effect on MMP-9 in either model. The effect of EDX cells might be
explained by their basal secretion of soluble molecules (Supplementary Fig. 4) that could be responsible for an anti-inflammatory effect [52]. Moreover, the differences between the two models may be explained by the fact that liver fibrosis, induced by the subcutaneous injection of CCL4, is less severe and of different nature (mainly centrilobular vs. perportal) compared to BLD-induced fibrosis. This could explain why EDX cells have an intermediate effect in the CCL4 model and a marginal effect in the BDL model. Nevertheless, a bystander effect caused by xenogeneic MSCs or EDX cannot be totally ruled out. Overall, the fact that MSCs have a stronger effect compared to EDX suggests that the anti-fibrotic effect is likely to be due to the secreted factors and not to a “deviation” of inflammation to the peritoneal cavity.

As stated above, MSCs are investigated in several clinical trials to treat end-stage liver diseases caused by hepatitis B, C or alcoholic hepatitis [5,30,31]. Overall, evidence for efficacy in most of these clinical studies is quite poor, and there were few indications of a safety concern. Factors contributing to a low efficacy might be that the cells are often poorly characterized; and improvements are claimed where there are insufficiently powered experimental/control groups or lack of randomization to make this claim. Based on the present experimental animal study, we suggest to test clinically microencapsulated MSCs for liver diseases, such as the group of patients with alcoholic steatohepatitis and underlying cirrhosis that do not respond to a short-term course of steroids. For these patients, no therapy is currently available and the mortality is over 50% at 6 months; essentially, there is an unmet medical need for alternative therapeutic options [53]. Microencapsulated MSCs might provide anti-inflammatory effects and prevent progression to liver failure.

In conclusion, we here show that secreted factors from human bone marrow-derived MSCs have a direct effect on HSC activation in vitro. We further demonstrate that microencapsulated MSCs show anti-fibrotic and anti-inflammatory effects in BDL-induced liver fibrosis in mice and that soluble factors secreted by the microencapsulated cells have a key role in this effect. Potential mechanisms may include increased secretion and activity of MMP-9, an anti-apoptotic effect mediated by IL-6 or IGFBP-2, and inhibition of inflammation by IL-1Ra.

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Conflict of interest
The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors’ contributions
RPHM, RM, CW, CG, and LHB conceived and designed the experiments. RPHM, RM, JM, EM, and CG performed the experiments. PM, PC, CW, CG, and LHB contributed reagents/materials/analysis tools. RPHM, RM, PM, JM, EM, YDM, PC, CW, CG, and LHB contributed to the writing of the manuscript.

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Supplementary data
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Supplementary data to:

**Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice**

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Supplementary Methods

Flow cytometry
MSCs were detached using 0.25% trypsin-EDTA (Sigma, Switzerland) for about 30 s and washed twice. Cells were stained for CD11b (Hycult biotechnology, Uden, the Netherlands), CD31 (Dako, Baar, Switzerland), phycoerythrin (PE)-conjugated mouse anti-CD34, fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD36, PE-mouse anti-CD44, PE-mouse anti-CD54, mouse anti-CD90, mouse anti-CD106 (all from Becton Dickinson, San Jose, CA, USA), PE-mouse anti-CD45 (R&D Systems, Abingdon, UK), FITC-mouse anti-CD105 (Serotec, Oxford, UK), mouse anti-HLA-ABC (Chemicon Australia, Victoria, Australia), and mouse isotype control (Becton Dickinson), at saturating concentrations. Secondary antibodies were FITC-rat anti-mouse IgG1, PE-rat anti-mouse IgG1 (both from Becton Dickinson). Cells were washed with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and those labeled with unconjugated antibody were secondary labeled with FITC or PE secondary rat anti-mouse antibody. Negative controls were mouse isotype standard immunoglobulin and secondary antibody alone. Data were collected on a FACScanto and analyzed using Kaluza software (Becton Dickinson).

Viability assay
Fluorescein diacetate (FDA) living cell staining and propidium iodide (PI) dead cell staining (both from Sigma, Switzerland) were used to assess MSC cell viability. A mixture with FDA or PI was incubated for 2 min prior to evaluation. Cell viability was assessed immediately after encapsulation (day 0) and at days 5, 15, 45, and 218. 3,000 cells per time point were manually counted (4 independent experiments): the
number of viable cells was subsequently expressed as percentage of the total number of cells.

**Proliferation assay**
To analyze proliferation, 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analog of thymidine that is incorporated into DNA during active DNA synthesis, was added to the culture medium. After 24 h of culture with EdU, free or encapsulated MSCs were fixed with 4% paraformaldehyde for 15 min and permeabilized using 0.5% Triton X-100 for 5 min. Proliferating cells were detected using the histochemical assay kit as described by the manufacturer (Click-iT EdU Cell Proliferation Assays, Invitrogen). Hoechst 33342 (Sigma) was used to stain cell nuclei. A total of 1200 cells (out of three independent experiments) were manually counted and proliferation was expressed as percentage of EdU-positive cells with respect to the total number of cells (Hoechst-positive).

**Cytokine array of cell supernatants**
Supernatants of MSCs, encapsulated MSCs and EDX cells were prepared by collecting low serum medium (IMDM with 5% FCS) after 48 h culture of 5×10^6 cells. IMDM with 5% FCS incubated for 48 h without cells was used as control. The analysis was done for a panel of specified proteins using an antibody array (RayBio Human Cytokine Antibody Array 6 (60), RayBiotech Inc., Norcross, GA) according to the manufacturer protocol. Spots were quantified using Quantity-one software (Bio-Rad, Hercules, CA). Values obtained with control medium were subtracted from values obtained with conditioned medium.

**MSC differentiation into adipocytes, osteocytes and chondrocytes**
Adipogenic differentiation: Free MSCs cultured on coverslips and microencapsulated MSCs were differentiated into adipocytes as previously described [1]. Briefly, free and microencapsulated MSCs were cultured for 3 weeks in adipogenic differentiation media which consisted of IMDM supplemented with 10% rabbit serum, 0.5 mM 3-isobutyl-1-methylxanthin (IBMX), 1 mM hydrocortisone, 0.1 mM indomethacin (all from Sigma), and P–S. Media were changed every 3 days. Free and microencapsulated MSCs were fixed with cold 10% formalin for 1 h, washed twice with tap water, and cytoplasmic triglyceride droplets were stained with Oil-red-O solution (Sigma) for 2 h at room temperature (RT). Cells were washed and observed under an optical microscope (Zeiss Axiophot1, Carl Zeiss AG, Feldbach, Switzerland).

Osteogenic differentiation: free and microencapsulated MSCs were differentiated in osteocytes as previously described [1]. Briefly, free and microencapsulated MSCs were cultured for 3 weeks in osteogenic differentiation media consisted of IMDM supplemented with dexamethasone 0.1 mM, β-glycerolphosphate 10 mM, ascorbic acid 200 mM (all from Sigma), and P–S. Media were changed every 3 days. Free and microencapsulated MSCs were fixed with cold 10% formalin for 1 h, washed twice with tap water, and stained with 2% Alizarin Red S (Sigma) for 20 min at RT to reveal calcific depositions. Cells were washed, destained in 10% cetylpyridinium chloride monohydrate (Sigma) for 30 minutes and observed under an optical microscope (Zeiss Axiophot1).

Chondrogenic differentiation: Free and microencapsulated MSCs were differentiated into chondrocytes as previously described [1]. Briefly, free and encapsulated MSCs were cultured for 3 weeks in DMEM - high glucose (25 mmol/l of glucose),
supplemented with 0.1 mM dexamethasone, 50 mg/ml ascorbic acid, 10 mg/l insulin, 5.5 mg/l transferrin, 5 mg/l selenium (ITS)-premix, 40 mg/ml L-proline (all from Sigma), 10 ng/ml TGF-β 3 (PeproTech EC Ltd, London, UK), P–S. Media were changed every 3 days. Free and microencapsulated MSCs were fixed with cold 10% formalin for 1 h and washed twice with tap water. Microencapsulated MSCs were dehydrated and embedded in paraffin, and 5 µm-thick histological sections performed. Collagen deposition was detected using Goldner’s trichrome staining. Briefly, microsphere sections and free cells monolayers were washed with 2% acetic acid, stained successively with hematoxylin, phosphomolybdic acid, and light green (all from Sigma) with 2% acetic acid wash in between. Free and encapsulated MSCs were observed under an optical microscope (Zeiss Axiophot1).

**Transplantation of microencapsulated MSCs under the kidney capsule**

DBA-1 wild-type mice were anesthetized with isoflurane and side incision was performed to reach the kidney. 150,000 encapsulated MSCs in 100 µl alg-PEG microspheres or empty microspheres were transplanted under the kidney capsule via an Abbocath-T 18G catheter (Hospira, IL, USA). Mouse kidneys were collected at 1 month and 6 months after transplantation.

**Assessment of hepatic and kidney fibrosis**

Liver and kidney collagen content was determined using Masson’s trichrome histochemistry on samples fixed in formalin and embedded in paraffin. Liver and kidney sections of 5 μm thickness were incubated for 1 h at 60°C in Bouin’s solution. Slides were then washed in tap water for 5 min. Nuclei were stained with hematoxylin. Cytoplasm was stained with Biebrich scarlet for 5 min followed by rinsing in distilled water, then incubated in phosphomolybdic acid for 10 min and
thereafter in aniline blue for 5 min, rinsed in distilled water and then one minute in 2% acetic acid, rapidly dehydrated in ethanol/xylol and coverslipped. Images from tissue sections were acquired with an Axiocam color camera (Zeiss). The extent of hepatic fibrosis was determined using morphometric quantification (MetaMorph Software, Universal Imaging, West Chester, PA) of the blue area (collagen I to VI) normalized to the red area (hepatocytes) on Masson’s trichrome stained liver and kidney sections.

**Immunohistology**

Formalin-fixed tissue was embedded in paraffin, and sections of 5 μm thickness were prepared. The detection of human MSCs was performed using a human-specific mouse anti-vimentin antibody (Dako) at 1:1000 dilution. The detection of macrophages was performed using an anti IBA-1 antibody (Wako Chemicals, VA, USA) at 1:500 dilution. Kidney sections were incubated overnight at 4°C with primary antibody diluted in PBS containing 0.1% BSA, washed in PBS, and incubated for 1 h with a secondary antibody (Alexa Fluor 488 goat-anti mouse) diluted 1:1000 in PBS containing 0.1% BSA. Sections were stained with 0.09% Evans blue solution. Images were acquired using the Axiocam color camera. The percentage of positive cells for IBA-1 was determined using MetaMorph Software (Universal Imaging). Cells positive for IBA-1 staining were counted and normalized to the total number of nuclei in each section.

Cryostat sections were prepared from kidney tissue harvested one month after transplantation with empty microsphere or microencapsulated MSCs and stored frozen at -80°C. Sections were stained for CD4 (dilution 1:50, Becton Dickinson), CD8 (dilution 1:100, Serotec) and F4/80 (dilution 1:50, Serotec). Secondary
antibodies were anti-rat Alexa 555-conjugated (1:1000, Invitrogen). The slides were stained with Hoechst 33342 (Sigma), examined using a fluorescence microscope (Zeiss), and quantification were performed using MetaMorph Software (Universal Imaging).

**Real-time polymerase chain reaction (RT-PCR)**

RT-PCR was used to assess expression levels of fibrosis-related genes. Total RNA was extracted from liver samples or HSCs using Qiagen RNeasy Mini kit (Qiagen, San Diego, CA, USA) according to the manufacturer’s instructions. cDNA was synthesized from 1 mg of total RNA using SuperScript III reverse transcriptase (Invitrogen). RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems Inc, CA, USA), with 2ng cDNA and 300 nM of each primer according to the following protocol: 2 min at 50°C, 10 min at 95°C, and for 45 cycles of 15 sec at 95°C and 60 sec at 60°C, using a SDS 7900 HT machine (Applied Biosystems). The reactions were performed in triplicates on 384-well plates. Raw Ct values obtained with SDS 2.2 (Applied Biosystems) were imported in Microsoft Excel software, and normalization factor and fold changes were calculated using the GeNorm method [2].

The following primers were designed using Primer3 online software (http://frodo.wi.mit.edu/): mice matrix metalloproteinase (MMP) -9 forward: AGT TGC CCC TAC TGG AAG GT, reverse: GTG GAT AGC TCG GTG TT, mice MMP-13 forward: AGT TGA CAG GCT CCG AGA AA, reverse: AGT TCG TTT GGG ACC ATT TG, mice collagen type I alpha 1 forward: GCA TGG CCA AGA AGA CAT CC, reverse: CCT CGG GTT TCC ACG TCT C, mice α-SMA forward: CAG GAA TAC GAC GAA GCT GG, reverse: GCT GAT CCACAA AAC GTT CA. Human MMP-2 and collagen type I alpha 1 were purchased from Qiagen (San Diego, USA). mRNA
levels were normalized using the geometric mean of Cyclin G-associated kinase (GAK) and signal recognition particle 72kDa (SRP72) expression in mice liver samples, and eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in human cell extracts (housekeeping genes). All primers were tested with Amplifix Software (http://ifrjr.nord.univ-mrs.fr/AmplifX-Home-page) and blasted on http://www.ensembl.org. The efficiency of each primer was tested using positive control cDNA serial dilutions and a negative control.

Electrophoretic and immunoblot analysis
Proteins from lysed LX-2 cells were separated on polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Milipore, Billerica, MA). Membranes were blocked with 5% skim milk in wash buffer (20 mM Tris–HCl, pH 7.4, 140 mM NaCl, 0.1% Tween 20) and incubated with anti-α-SMA (1:500) (obtained from Dr. Christine Chaponnier, Geneva University [3]) or anti-vimentin (1:1000) (Dako) antibodies diluted in blocking solution. Following three washes, membranes were incubated with peroxidase-conjugated goat anti-mouse antibodies (Molecular Probes Inc., Eugene, OR) diluted 1:6000 in wash buffer. Proteins were revealed by chemiluminescence (ECL, Interchim Inc., Montluçon, France). Chemiluminescence was detected on photographic films (GE healthcare, Geneva, Switzerland) and signals were quantified using the Quantity One software (PDI, Inc., Huntington Station, NY) and normalized with respect to the expression of human vimentin that was set at 100%.

Measurement of aspartate aminotransaminase (AST) and alanine aminotransferase (ALT)
Serum AST and ALT were measured using UniCel DxC 800 Synchron Clinical Systems (Beckman Coulter, CA, USA), following the manufacturer’s instructions.

**Measurement of cytokines**

Serum IL-10 was measured using a cytometric bead array mouse inflammation kit (Becton Dickinson) performed on a FACSCanto II flow cytometer (Becton Dickinson) following manufacturer’s instructions. Raw data were analyzed with cytometric bead array Software (Becton Dickinson). Serum IL-1Ra contents was measured using mouse IL-1Ra ELISA kit (Ray Biotech) following the manufacturer’s instructions.
Supplementary figures and legends

Supplementary figure S1

Characterization of human mesenchymal stem cells. (A) Representative image of fibroblast-like plastic-adherent human MSCs showing typical spindle-shape morphology (Passage 3). Scale bar 100 µm. (B) Flow cytometry analysis of surface antigen expression on expanded human MSC (Passage 3).
Supplementary figure S2.

(A) Empty alg-PEG microspheres. (B, C) alg-PEG microspheres with MSCs, (B) immediately after encapsulation and (C) 5 days after encapsulation. Scale bars 400 µm.
Supplementary figure S3

A

MGs before encapsulation

Day 0  |  Day 5  |  Day 15  |  Day 45  |  Day 218

Encapsulated MGs

B

% of viable cells

Free MGs (day 0)
Encapsulated MGs (day 0)
Supplementary figure S3 (continued)

Supplementary figure S3.
Viability of microencapsulated mesenchymal stem cells and their capacity to differentiate and proliferate after microencapsulation in alg-PEG microspheres. (A) Representative bright field (BF) images showing free MSCs before and encapsulated MSCs, from 0 to 218 days. Viable cells are stained by fluorescein diacetate (FDA, green) and dying cells by propidium iodide (PI, red). Scale bars 100 µm. (B) Directly after microencapsulation, the percentage of viable MSCs was obtained by counting viable cells which were normalized to total number of data are mean values ± SEM obtained from 6 independent experiments. (C) Representative images showing...
proliferation of free and encapsulated MSCs assessed by 24h-EdU incorporation; bright field (BF), proliferating EdU positive MSCs (green), Hoechst stained nuclei (blue). Scale bars 100 µm. (D) Percentage of proliferating MSCs (EdU positive/all nuclei) upon culture as free or microencapsulated cells. Data are mean values ± SEM obtained from 3 independent experiments. (E) Representative images showing differentiation of MSCs cultured as free or microencapsulated cells into adipocytes (lipid droplets were stained by Oil-red-O staining (RO)), into osteoblasts (mineral matrix deposition was colored by Alizarin Red (Alizarin)), and into chondrocytes (collagen deposition was colored by Goldner’s trichrome). Scale bars 100 µm (top, encapsulated MSCs) and 20 µm (bottom, free MSCs).
Supplementary figure S4.
Relative quantification of the molecules secreted by (A) MSC and (B) control EDX cells. Quantification was done by Human Cytokine Antibody Array performed on 48h-conditioned medium from either free EDX cells or free MSCs. A representative experiment performed in duplicate is presented and data are mean values ± SEM.
Supplementary figure S5.

Histological sections of encapsulated MSCs under the kidney capsule of DBA-1 wild-type mice showing viable microencapsulated MSCs (A) one and (B) six months after transplantation. (C) Microencapsulated MSCs around the liver of DBA-1 mice 15 days after BDL and transplantation with microencapsulated MSCs by intraperitoneal injection. Hematoxylin and eosin staining.
Supplementary figure S6.
Survival of microencapsulated mesenchymal stem cells upon transplantation into mice. Microencapsulated MSCs were transplanted under the kidney capsule of mice and harvested one and six months later; empty microspheres were used as a control.
(A) Representative illustration of histology of the kidney capsule stained with hematoxylin and eosin (HE) or Masson’s trichrome. HE staining shows viable cells in the microspheres. Masson staining shows a moderate fibrotic reaction (stained in blue) around the microspheres, with a similar distribution between empty microspheres and microspheres with MSCs. Note that there was a mononuclear cell infiltrate around the microspheres, in particular for microspheres with MSC at one
month after transplantation. Scale bars 200 µm. (B) Frozen tissue section stained for human-specific vimentin, revealing a spindle shaped MSC one month after transplantation under the kidney capsule. Scale bars 100 µm (left panel) and 50 µm (right panel). (C) Frozen tissue section stained for IBA-1 (macrophages) and CD4 and CD8 positive T cells around empty microspheres or microspheres with MSCs one month after transplantation. Scale bars 100 µm. Also, mean percentages ± SEM are shown, n=3 mice per group, * P < 0.05.
References:

