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Hematopoietic stem cells and liver regeneration: Differentially acting hematopoietic stem cell mobilization agents reverse induced chronic liver injury

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ABSTRACT

Bone marrow (BM) could serve as a source of cells facilitating liver repopulation in case of hepatic damage. Currently available hematopoietic stem cell (HSC) mobilizing agents, were comparatively tested for healing potential in liver fibrosis. Carbon tetrachloride (CCl_4)-injured mice previously reconstituted with Green Fluorescent Protein BM were mobilized with Granulocyte-Colony Stimulating Factor (G-CSF), Plerixafor or G-CSF + Plerixafor. Hepatic fibrosis, stellate cell activation and oval stem cell frequency were measured by Gomori and by immunohistochemistry for a-Smooth Muscle Actin and Cytokeratin-19, respectively. Angiogenesis was evaluated by ELISA and immunohistochemistry. Quantitative real-time PCR was used to determine the mRNA levels of liver Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ), Interleukin-6 (IL-6) and Tumor Necrosis-alpha (TNFα). BM-derived cells were tracked by double immunofluorescence. The spontaneous migration of mobilized HSCs towards injured liver and its cytokine secretion profile was determined in transwell culture systems. Either single-agent mobilization or the combination of agents significantly ameliorated hepatic damage by decreasing fibrosis and restoring the abnormal vascular network in the liver of mobilized mice compared to CCl_4-only mice. The degree of fibrosis reduction was similar among all mobilized mice despite that G-CSF + Plerixafor yielded significantly higher numbers of circulating HSCs over other agents. The liver homing potential of variously mobilized HSCs differed among the agents. An extended G-CSF treatment provided the highest anti-fibrotic effect over all tested modalities, induced by the proliferation of hepatic stem cells and decreased hepatic inflammation. Plerixafor-mobilized HSCs, despite their reduced liver homing potential, reversed fibrosis mainly by increasing hepatic PPAR-γ and VEGF expression. In all groups, BM-derived mature hepatocytes as well as liver-committed BM stem cells were detected only at low frequencies, further supporting the concept that alternative mechanisms rather than direct HSC effects regulate liver recovery. Overall, our data suggest that G-CSF, Plerixafor and G-CSF + Plerixafor act differentially during the wound healing process, ultimately providing a potent anti-fibrotic effect.

Introduction

Liver transplantation is the only effective treatment for end-stage liver cirrhosis, but it is limited by donor shortage, postoperative morbidity and mortality, immune rejection, high costs and long-term side effects. In order to fulfill the unmet medical needs in the field, alternative, cell-based therapies for the treatment of end-stage hepatic diseases are under investigation.

Mature hepatocytes have been traditionally recognized as the major contributors to liver repair [1,2]. However, recent evidence suggests that intrahepatic stem cell populations, the so-called "*hepatic progenitor/stem cells*" or *oval cells* (OCs) become activated, expand and actively contribute to the regenerative responses by giving rise to hepatocytes and biliary epithelial cells when hepatocyte proliferation is overwhelmed

Abbreviations: a-SMA, a-Smooth Muscle Actin; BM, bone marrow; CCl_4, carbon tetrachloride; CK19, Cytokeratin-19; FCM, flow cytometry; FVIII, Factor VIII; G-CSF, Granulocyte-Colony Stimulating Factor; GFP, Green Fluorescent Protein; HSCs, hematopoietic stem cells; IL-6, Interleukin-6; LK, $Lin^-/c\text{-}Kit^+$; OCs, oval cells; panCK, pan Cytokeratin; PPAR-γ, Peroxisome Proliferator-Activated Receptor gamma; SDF-1, stromal cell-derived factor-1; SGPT, serum glutamic–pyruvic transaminase; TNFα, Tumor Necrosis Factor alpha; VEGF, Vascular Endothelial Growth Factor.

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by severe liver injury [3,4]. A third source of liver-repopulating cells, bone marrow (BM), has been shown to contribute to the liver healing process after tissue injury [5,6] and efforts were made to exploit this novel source for therapeutic purposes. The BM is the largest reservoir of pluripotent stem cells in adults, traditionally considered to give rise to only hematopoietic cell lineages. This concept was challenged by reports demonstrating that BM-derived stem cells can generate a variety of adult cell types that express non-hematopoietic cell markers [7–11]. Although it had been proposed earlier that BM cells could generate hepatocytes under tissue stress [12,13], the contribution of BM to liver regeneration, under either physiological or pathological conditions, was extremely low to replace even the physiological hepatocyte turnover. Thus, the concept of bone marrow-derived liver regeneration has been strongly questioned [14–16] and the current belief is that the clinical benefit observed in injured tissues after hematopoietic stem cell (HSC) therapies is produced by the activation of endogenous progenitor cells through paracrine signaling between donor and host cells providing cytokines and growth factors [16–18].

BM-resident HSCs can be mobilized into the peripheral blood at a low magnitude under specific stimuli such as tissue injury [19,20] or at high amounts after pharmacological priming with cytostatic drugs, chemokines or hematopoietic cytokines [21,22]. G-CSF is a hematopoietic growth factor that mediates HSC mobilization to peripheral blood and represents the most widely used mobilizing agent [23]. Several reports have suggested that G-CSF-mobilized HSCs contribute to liver repair in acute and chronic liver injury models [24,25]. However, it remains arguable whether liver repopulation is mediated through a paracrine signaling process from the recruited to the liver, HSCs, stimulating tissue progenitor cells or is a direct hepatotrophic G-CSF-effect.

A novel agent, Plerixafor (AMD3100, Mozobil), is a bicyclam molecule that reversibly antagonizes the binding of stromal cell-derived factor-1 (SDF-1) to its receptor CXCR4. Plerixafor results in the rapid mobilization of HSCs into the circulation and acts synergistically when combined with G-CSF, yielding large numbers of hematopoietic progenitor cells [29,30]. Plerixafor-alone or its combination with G-CSF have only been limitedly studied as liver regenerating factors in injury models [31].

In the present study, we comparatively investigated all currently available HSC mobilizing agents (G-CSF, Plerixafor or G-CSF + Plerixafor) as liver repopulating factors. In addition, the contributing cell subpopulations and the functional mechanisms involved in the regeneration process of the chronic liver damage were also evaluated.

Materials and methods

Animals and chronic liver injury model

All procedures were approved by and performed in accordance with the Animal Care and Use Committee of the Regional Veterinary Health Authority. Seven week old C57Bl6 mice were lethally irradiated (900 cGy) and injected *via* tail vein injection with 1×10^7 BM cells isolated from age-matched Green Fluorescent Protein (GFP) donors. After an 8-week period, chronic liver injury was induced to the recipients, by injecting 1.5 ml/kg carbon tetrachloride (CCl_4: Fluka, 1:1 dilution in corn oil: Sigma) intraperitoneally (i.p.), twice a week, up to a total of 23 doses.

Mobilization

Mice were mobilized with 250 μg/kg/day recombinant human G-CSF (Tevagrastim, TevaGenerics GmbH), administered i.p. for either 6 or 12 days or with 5 mg/kg Plerixafor (Mozobil, Sanofi-Genzyme) i.p. for three days. When the combination of G-CSF plus Plerixafor was used, Plerixafor was injected on the last 3 days of G-CSF administration. Control mice received saline injections. Animals were sacrificed one day after the last mobilization dose and 5 days after the last CCl_4 dose.

Flow cytometry

Donor GFP chimerism in the blood of transplanted mice was determined by flow cytometry (FCM), before the initiation of CCl_4.

At sacrifice, whole blood obtained by cardiac puncture was stained with APC-Mouse Lineage Cocktail (anti-CD3, anti-CD11b, anti-B220, anti-GR-1, anti-Ter-119) and PE-anti-c-Kit mAb (BD Pharmingen). The absolute numbers of Lin−/c-Kit$^+$ (LK) cells per ml of blood, were calculated based on the LK cell frequency by FCM and the absolute cell counts. Results were obtained on a FACSCalibur device (Becton Dickinson, BD) and analyzed with the CellQuest Pro6 software.

Assessment of liver injury

Serum glutamic–pyruvic transaminase (SGPT; Reflotron Roche) was measured using the Reflotron PLUS autoanalyzer.

Liver tissue samples were fixed in 10% formalin and embedded in paraffin (Paraplast Plus, Tissue Embedding Medium, Leica). Liver sections (2.5 μm) were stained with hematoxylin and eosin for morphological evaluation and connective tissue staining (Gomori and Masson, Bio-Optica). All samples were evaluated blindly by a pathologist and the total fibrosis score was based on the severity of parenchymal extinction, fibrosis and "arterialization" and "capillarization" of portal veins and sinusoides.

The extent of liver fibrosis was determined using a 4-scale injury grading score, similar to the one used in non-alcoholic steatohepatitis/cirrhosis [32], as follows: Grade I: periportal and mild perisinusoidal fibrosis, Grade II: periportal fibrosis with bridging to neighboring portal tracks, Grade III: collagen fibers connecting portal tracks and moderate fibrosis around the central vein, and Grade IV: collagen fibers connecting portal tracks and portal tracts with central veins.

Immunohistochemistry and double immunofluorescence

Immunohistochemistry of paraffin-embedded liver sections (3 μm) was performed with anti-a-Smooth Muscle Actin antibody (a-SMA, 1:800; Sigma), Cytokeratin-19 (CK19, 1:100; Abcam), ki-67 (MIB1, Clone TEC-3, 1:20; DAKO) and Factor VIII (FVIII, 1:800; Abcam) antibodies. Bound antibodies were visualized by Dako Real Envision Detection System Peroxidase/DAB+, and slides were then counterstained with hematoxylin, mounted and studied by light microscopy. For quantitation of positive cells, a minimum of 20 high power fields of view (×400) were evaluated. Double immunofluorescence was performed with FITC-labeled anti-GFP antibody (1:200; Abcam) and pan Cytokeratin (panCK: CK 4, 5, 6, 8, 10, 13, 18, 1:200; Sigma) or Cytokeratin-19 (CK19, 1:100; Abcam), followed by incubation with TRITC-labeled IgG antibody (1:40; DAKO). The slides were mounted in fluorescence media with 4′,6-diamidino-2-phenylindole (DAPI/Antifade, Q-BIOgene) and 10 randomly selected fields were screened by fluorescence microscopy.

Real-time quantitative reverse transcription PCR

Total RNA from liver tissues was extracted using the QIAmp RNA kit (Qiagen) and reverse transcribed with RT^2 First Strand kit (Superarray Biosciences) to make complementary DNA.

Hepatic gene expression of Tumor Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6) and Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ) were assessed by real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using predesigned gene expression assays (Applied Biosystems) in accordance with the manufacturer's protocol. GAPDH was used as endogenous control for relative quantification. All reactions were performed in duplicate.

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ELISA

ELISA was employed in liver homogenates to measure Vascular Endothelial Growth Factor (VEGF) expression by a quantitative sandwich enzyme immunoassay technique (R&D Systems). The amount of VEGF (pg/ml) was proportional to the optical absorption of the sample.

Transwell system culture assays

To evaluate whether mobilized HSCs alter the secretion profile of the pro-inflammatory cytokines TNFα and IL-6 in CCl_4 injured livers, 3×10^4 HSCs, mobilized with G-CSF, Plerixafor or G-CSF plus Plerixafor, were placed in the upper chamber of a transwell system. The cells were separated by a permeable membrane (pore size 0.4 μm; Corning Costar) from 50 mg liver tissue resected from C57Bl6 mice with CCl_4-induced chronic liver injury. The system was incubated for 48 h (37 °C, 5% CO_2) with complete alpha-MEM (Gibco) medium supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco). Normal and injured livers cultured only with medium in the upper chamber, were used as controls. Cytokine secretion in liver supernatants was quantitated by FlowCytomix Multiple Analyte Detection Bead Assay (eBioscience).

To investigate the spontaneous chemotactic migration of mobilized HSCs towards the CCl_4-injured liver, 2×10^5 GFP total blood cells from CCl_4-injured mice mobilized with G-CSF, Plerixafor or G-CSF plus Plerixafor, were placed in the input control wells and upper chambers of a transwell culture system separated by a permeable membrane (pore size 5 μm; Corning Costar) from liver homogenates (20 mg) derived from corresponding mobilized C57Bl6 animals. After 4-hour incubation (37 °C, 5% CO_2), the input and the migrated cells were collected, stained with APC-Mouse Lineage Cocktail (containing anti-CD3, anti-CD11b, anti-B220, anti-GR-1, and anti-Ter-119) and counted with BD Trucount (BD Biosciences). The percentage of Lin^-/GFP^+ cells that spontaneously migrated towards the injured liver was calculated as: number of cells migrated / number of input control cells × 100. Results were obtained on a FACSCalibur device (BD) and analyzed with the CellQuest Pro6 software.

Statistical analysis

Data are expressed as the means ± SEM. Analysis of variance general linear model (ANOVA) with a Tukey's post hoc test for multiple comparisons was performed. Values of $p < 0.05$ were considered statistically significant. Probability of survival was determined by Kaplan–Meier curve and statistical significance was estimated by log-rank test.

Results

Donor chimerism

Predominant donor chimerism was demonstrated by FCM after infusion of GFP^+ BM donor cells to C57Bl recipients; more than 90% of peripheral blood of the recipients expressed GFP, indicating that donor GFP^+ cells successfully reconstituted the depleted BM. Fully chimeric mice were then subjected to CCl_4-induced liver injury (Fig. 1A).

The liver homing potential of G-CSF- or plerixafor-mobilized HSCs is different

A chemotaxis assay was performed to elucidate the role of SDF-1/CXCR4 axis in the homing of mobilized HSCs to the injured and subjected to mobilization liver. A robust, spontaneous chemotactic migration of G-CSF-mobilized Lin^- cells towards CCl_4-injured liver derived from G-CSF-mobilized mice was observed, in contrast to Plerixafor or G-CSF + Plerixafor-mobilized HSCs (Fig. 1B). This suggests that the SDF-1 produced by the CCl_4-liver which had been subjected to G-CSF-mobilization, chemoattracts G-CSF-mobilized HSCs whereas Plerixafor, by interrupting the SDF-1/CXCR4 axis in the CCl_4-liver, significantly inhibits the HSC motility towards the injured liver.

Hematopoietic stem cell mobilization agents reduce mortality and ameliorate CCl_4-induced chronic liver injury

Successful mobilization of HSCs in injured mice was confirmed by the numbers of circulating LK cells. The G-CSF + Plerixafor combination yielded the highest numbers of LK cells over single-agent mobilization ($p < 0.001$, Table 1).

Survival was improved in all mobilized groups. In particular, 5/35 (14%) and 2/87 (2.3%) of CCl_4-treated and mobilized mice respectively, died from the initiation of treatment until sacrifice. Consequently, the probability of survival increased from 88% in the CCl_4-only group to 98% in mobilized animals with a trend to significance ($p = 0.06$, Fig. 1C).

Liver sections of the CCl_4-treated group demonstrated established cirrhosis. Masson and Gomori staining confirmed the presence of fibrous septa dividing the hepatic parenchyma into numerous, pleomorphic nodules. In contrast, mice mobilized by all modes, appeared with almost normal liver architecture and with only a slight increase of reticular fibers. The overall grading of fibrosis was significantly lower in mobilized mice over control animals ($p < 0.001$). The 12-day G-CSF treatment reduced by more than 50% the score of fibrosis over all other groups, however, this difference did not reach statistical significance (Figs. 2A–B and Table 1).

Hepatic fibrosis was also evaluated by immunohistochemistry for $a\text{-}SMA^+$ cells, which represent the activated hepatic stellate cells. The number of $a\text{-}SMA^+$ cells was significantly decreased in treated mice as compared to CCl_4-only mice, in good correlation with the total fibrotic index ($p < 0.001$, Figs. 2C–D and Table 1).

Fibrosis was also characterized by "arterialization" of small portal veins, in portal tracts and fibrous septa connecting portal tracts, and by the obliteration of adjacent portal veins, in sharp contrast to normal liver vascular network (Fig. 2E I, II). The sinusoidal endothelium of fibrotic liver expressed FVIII ("capillarization") (Fig. 2E III) accompanied by a-SMA staining of the sinusoidal wall, due to the transformation of perisinusoidal stellate cells into myofibroblasts (Fig. 2E IV). These features, namely "arterialization" of small portal veins and "capillarization" of sinusoidal epithelium were limited or absent in mobilized mice and in particular, in Plerixafor-treated mice (Fig. 2E V, VI).

CCl_4-treated mice developed transaminasemia and significantly increased SGPT values over mobilized animals which restored SGPT at almost normal levels ($p < 0.001$, Table 1).

Mobilization agents contribute to liver reconstitution through different mechanisms

qRT-PCR was used to evaluate whether the pattern of liver cytokine secretion is altered in mobilized CCl_4-treated mice. Compared to the CCl_4-group, G-CSF treatment for 6 or 12 days decreased liver TNFα and IL-6 expression at levels similar to normal liver ($p = 0.004$ and $p = 0.009$, respectively) whereas Plerixafor-involving treatments had no significant impact on the hepatic expression of these proinflammatory cytokines (Figs. 3A–B). Similarly, in a transwell culture system, G-CSF-mobilized but not Plerixafor or G-CSF + Plerixafor-mobilized HSCs, decreased proinflammatory cytokine secretion in culture supernatants of CCl_4-injured livers ($p < 0.001$ and $p = 0.003$, respectively: Figs. 3C–D).

On the other hand, the beneficial effect of Plerixafor or G-CSF + Plerixafor in ameliorating hepatic fibrosis was mostly mediated by the upregulation of liver PPAR-γ and VEGF expression ($p < 0.001$, Figs. 3E–F). In addition, higher mitotic activity, as assessed by the presence of $Ki67^+$ cells, was observed in liver sections of Plerixafor- and mainly G-CSF + Plerixafor-mobilized mice ($p = 0.008$, Figs. 4A–B).

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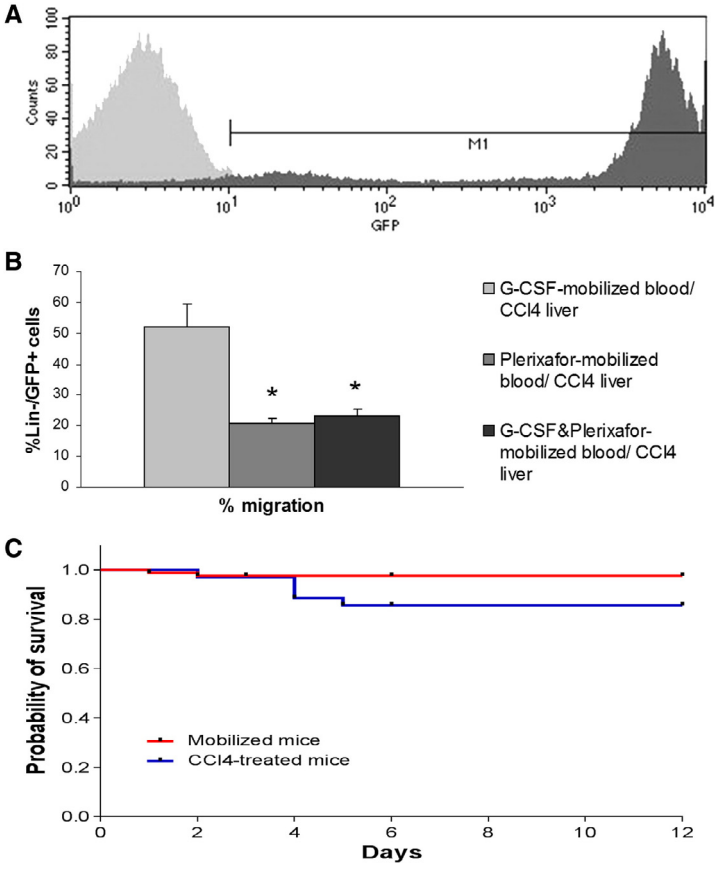

Fig. 1. Predominant donor chimerism, chemotaxis of mobilized HSCs and probability of survival. (A) GFP donor chimerism in C57Bl6 recipients. (B) Transwell culture system: % migration of Lin^-/GFP^+ mobilized HSCs towards CCl_4-injured livers from C57Bl6 mice subjected to mobilization ($^*p = 0.03$, by Tukey's test). (C) Probability of survival of mobilized (red line) vs CCl4-injured (blue line) animals ($p = 0.06$).

Cell populations participating in liver regeneration

In order to identify cell populations that participate in the regeneration process, we performed immunohistochemistry for CK19 and double immunofluorescence for GFP/CK19 and GFP/panCK. GFP positivity allowed us to trace the BM origin of cells expressing liver markers. The $GFP^+/panCK^+$ cells, $GFP^-/CK19^+$ cells and $GFP^+/CK19^+$ cells were characterized as BM-derived mature hepatocytes, endogenous OCs and BM-derived liver-committed stem cells, respectively.

Although all the G-CSF-treated groups demonstrated an increased presence of endogenous OCs by immunohistochemistry or double immunofluorescence ($CK19^+$ or $GFP^-/CK19^+$, respectively), the 12-day G-CSF treatment produced the highest numbers of OCs ($p < 0.01$, Figs. 4C–D and 5A, D). Similarly, increased numbers of BM-derived mature hepatocytes ($GFP^+/panCK^+$) were measured in hepatic sections of G-CSF-treated mice but only the 12-day G-CSF treatment significantly induced this type of cells in the liver ($p = 0.007$, Figs. 5C–D).

In contrast, Plerixafor-alone mobilization did not induce either OC proliferation or the generation of BM-derived mature hepatocytes (Figs. 4D and 5D). Liver-committed BM stem cells ($GFP^+/CK19^+$cells) could be rarely detected in liver sections, irrespectively of the mobilization type (p = ns, Figs. 5B, D).

Despite the increased presence of BM-derived mature hepatocytes and endogenous OCs in the livers of the 12-day G-CSF treatment mice, the overall frequency of these subpopulations in the liver remained very low (0.7% and 0.3%, respectively: Fig. 5D).

Discussion

Cell-based therapies as a means to repopulate the damaged liver, currently represent the most promising alternative option to liver transplantation. BM has been suggested as a source of liver repopulating cells and hematopoietic, mesenchymal and endothelial progenitor cells have been proposed as candidate cells for liver regeneration [33]. Among these BM resident candidates, HSCs can be forced to circulate in large amounts or/and be non-invasively harvested after G-CSF-mobilization that causes their egress from BM to peripheral blood [21,34]. The

Table 1
Circulating LK cells and fibrosis evaluation.

	Normal	CCl_4 (n = 30)	6 days GCSF (n = 23)	12 days GCSF (n = 16)	Plerixafor (n = 24)	GCSF + Plerixafor (n = 22)	p Value
LK cells	150 ± 23	624 ± 160	¥2213 ± 465	¥3595 ± 942	¥2412 ± 46	#5014 ± 1223	$p < 0.001$
Fibrotic stage	0	3.1 ± 0.1	*1.4 ± 0.2	*0.7 ± 0.2	*1.5 ± 0.2	*1.3 ± 0.2	$p < 0.001$
a-SMA^+ cells	0	63.3 ± 7.2	*18.2 ± 2.3	*16.2 ± 2.4	*30.6 ± 5.3	*21.9 ± 4.6	$p < 0.001$
SGPT (U/l)	*12.9 ± 1.8	59.4 ± 10.3	*19.3 ± 6.2	*19.2 ± 2.9	*32.2 ± 9.6	*20.8 ± 2.7	$p < 0.001$

Data are presented as mean ± SEM, $p < 0.001$ *: *vs* CCl_4-treated animals, ¥: *vs* normal, CCl_4-treated animals, #: *vs* normal, CCl_4-treated, Plerixafor, 6 days-G-CSF, by Tukey's test.

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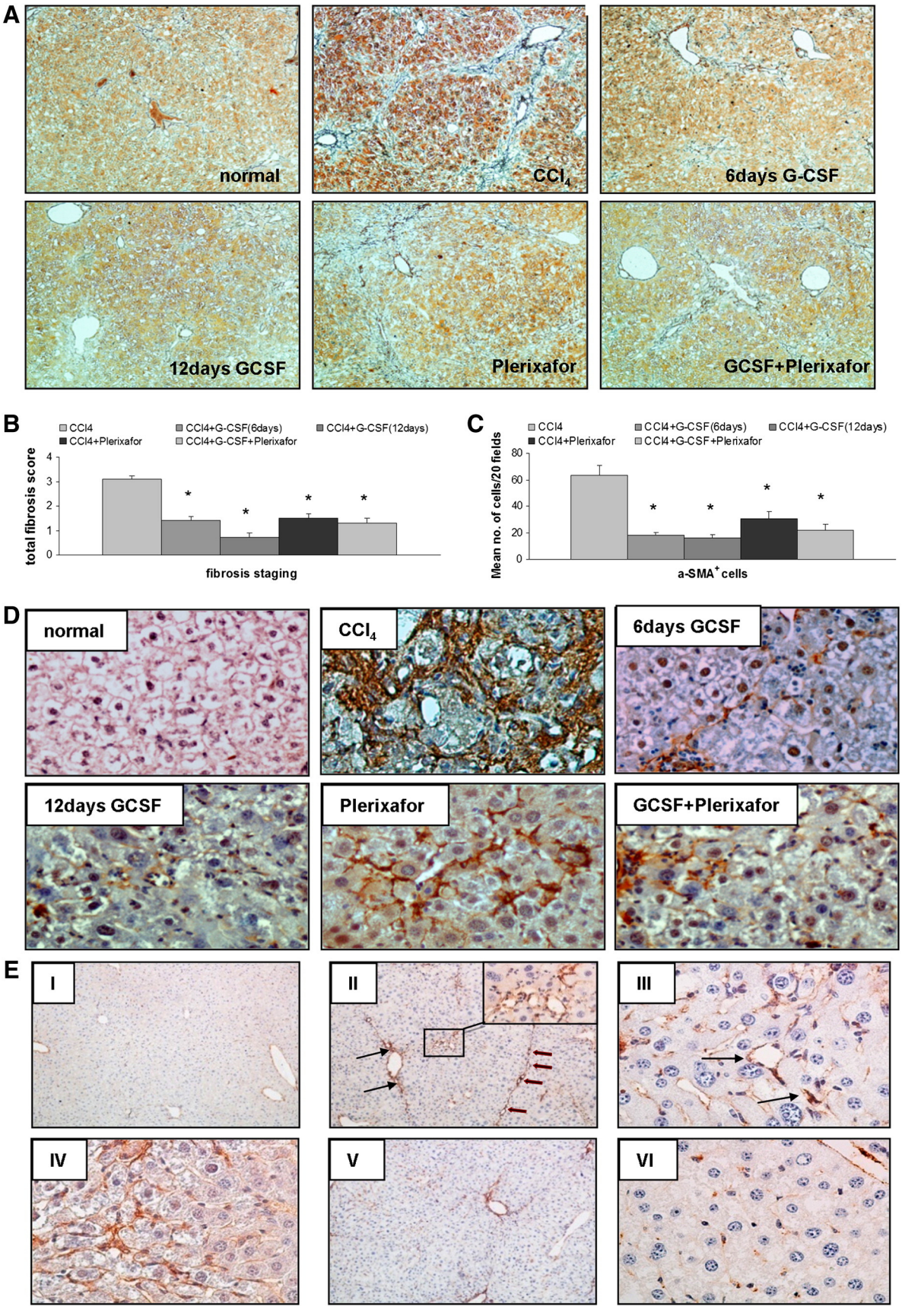

Fig. 2. Evaluation of liver injury. (A–B) Gomori staining (magnification × 100) and fibrosis staging. (C–D) Quantitation and immunohistochemistry of a-SMA^{+} cells (magnification × 200). Experiments were performed three times (n = 4–5 animals/group/experiment). Bars show the mean ± SEM, $^{*}p < 0.001$ *vs* CCl_4 by Tukey's test. (E: I) Limited FVIII expression in normal liver endothelium. (E: II, III) "Arterialization" of small vein branches in portal tracts and fibrous septa (II: long and short arrows, respectively). "Capillarization" of hepatic sinusoids (II: insert, III: arrows), (E: IV) stimulated myofibroblasts in perisinusoidal areas. (E: V–VI) Limited FVIII expression in liver sections of Plerixafor-treated mice.

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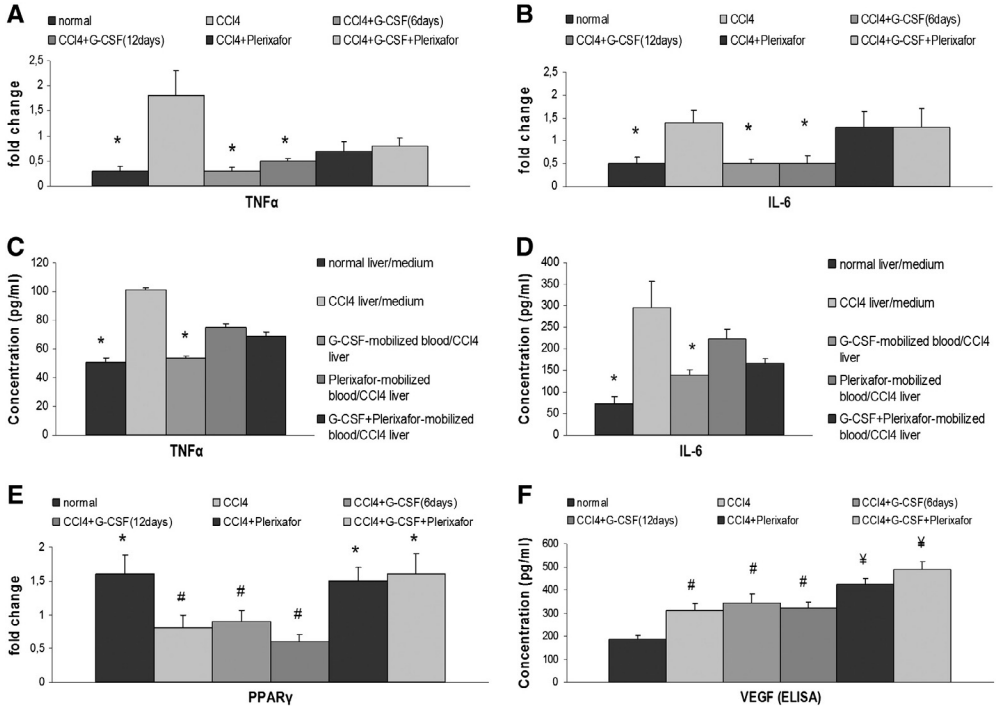

Fig. 3. Hepatic TNFα, IL-6, PPAR-γ and VEGF expression. (A) TNFα and (B) IL-6 hepatic gene expression assessed by qRT-PCR and expressed as mean fold change compared to CCl_4-only treated group, $^*p = 0.004$, $^*p = 0.009$ respectively, by Tukey's test. (C) TNFα and (D) IL-6 expression measured by cytometric bead assay in culture supernatants, $^*p < 0.001$, $^*p = 0.003$ respectively, by Tukey's test. (E) PPAR-γ hepatic expression assessed by qRT-PCR and (F) VEGF expression measured by ELISA ($p < 0.001$, #: *vs* normal, *: *vs* CCl_4, ¥: *vs* normal & CCl_4, by Tukey's test).

relatively easy access to large quantities of HSCs potentially makes them ideally suited as liver repopulating cells.

HSCs have been shown to participate in hepatic proliferation and repair after injury [5,6,11–13], although the true contribution of BM stem cells to liver regeneration has been questioned [15,16,25]. Recent evidence suggests that, rather than transdifferentiation of HSCs to tissue-specific stem cells or fusion of donor with host cells, replenishment of damaged tissues occurs through activation of endogenous progenitors and repair mechanisms mediated by paracrine secretion of soluble factors by BM cells [17,26,27].

G-CSF, as a means of forced circulation of large numbers of HSCs, has been extensively investigated for its hepatic regenerative effect, both in animal models of liver injury [24,25,28] as well as in clinical trials [35–37]. We have previously shown that G-CSF accelerates the recovery process and improves survival in an acute liver injury model and that boost infusions of G-CSF-mobilized HSCs result in lasting amelioration of alcoholic cirrhosis in a pilot clinical study [25,35].

The novel agent, Plerixafor, induces mobilization by reducing the binding and chemotaxis of HSCs to the BM stroma through reversible inhibition of the SDF-1a/CXCR4 axis [38]. The combination of G-CSF + Plerixafor has been demonstrated to be highly synergistic in mobilizing HSCs, resulting in superior $CD34^+$ cell yields over single agent mobilization [30]. The liver repopulating potential, however, of Plerixafor- or G-CSF + Plerixafor-mobilization is largely unexplored [31].

In the present study, our aim was to comparatively explore the role of all currently available mobilization strategies in facilitating liver fibrosis regression as well as the mechanisms and cell subpopulations by which the repair potential is mediated.

All modes of mobilization resulted in improved survival, restoration of SGPT at almost normal levels and significant reduction of liver fibrosis as evidenced by the decreased total fibrosis score and diminished myofibroblasts in liver sections of mobilized mice. As expected, the combination of G-CSF + Plerixafor yielded significantly higher numbers of circulating HSCs over other groups, however, the degree of fibrosis reduction was similar among all mobilized groups. The latter suggested that the magnitude of fibrosis amelioration is not proportional to the efficacy of mobilization and that other mechanisms, non HSC-mediated, may also contribute to liver regeneration.

Irrespectively of the numbers of circulating HSCs, the liver homing potential of cells mobilized by G-CSF-alone, Plerixafor-alone or G-CSF + Plerixafor may differ, ultimately resulting in varying contribution of mobilized cells to liver regeneration. It has been shown that both hepatic activated stellate cells [39] and endothelial sinusoidal cells [40] produce SDF-1, thus regulating the migration of $CXCR4^+$ cells to the liver. All types of G-CSF treatment increased the presence of BM-derived hepatocytes (GFP^+/panCK$^+$ cells) as compared to the livers of CCl_4-only mice, however, this effect reached statistical significance only in the 12-day G-CSF treatment. The high levels of SDF-1 produced by the increased numbers of a-SMA^+ cells in the CCl_4-injured liver, potentially create a chemotactic motility of the G-CSF-mobilized HSCs towards the liver as it was demonstrated in our study by the increased migration of G-CSF-mobilized Lin^- cells towards the CCl_4-injured liver of mobilized animals.

The overall low frequency of BM-derived hepatocytes in liver sections of G-CSF-treated animals however, was highly disproportionate to the degree of fibrolysis, thus implying that BM-derived hepatocytes do not, in themselves, functionally rescue recipients. Our speculation is that either the, homed to the liver, G-CSF-mobilized HSCs trigger *in situ* repair mechanisms by paracrine signaling or G-CSF *per se* directly stimulates liver cell proliferation, exerting a predominantly hepatotrophic effect. Indeed, the endogenous oval cells (GFP^-/$CK19^+$ cells), which might have given rise to new hepatocytes, were also increased in liver sections of G-CSF-treated mice and significantly after the 12-day G-CSF treatment. The extended *vs* the standard 6-day G-CSF treatment, not only didn't seem to deplete BM stem cells but it further

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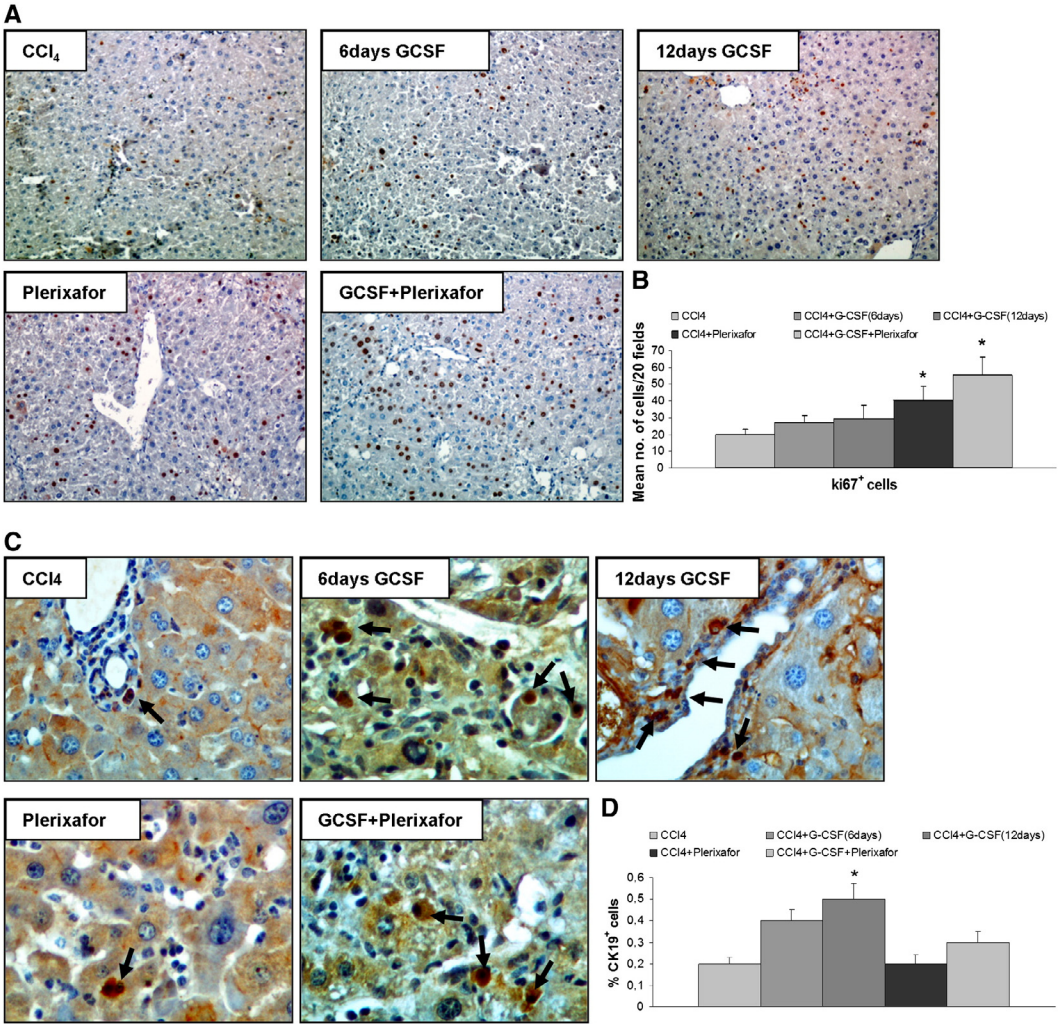

Fig. 4. Mitotic activity and oval cell frequency in liver sections. (A) Immunohistochemistry for Ki-67 (magnification × 100). (B) Hepatocyte proliferation index, $^{*}p = 0.008$ *vs* CCl_4 by Tukey's test. (C–D) CK19 immunohistochemistry (magnification × 200) and quantitation of OCs (percentage of $CK19^{+}$ cells), $^{*}p = 0.001$ *vs* CCl_4 by Tukey's test. Experiments were performed three times (n = 4–5 animals/group/experiment). Bars show the mean ± SEM.

increased HSC yields in the periphery, whereas importantly, the 12-day G-CSF treatment provided maximum contribution to liver restoration reaching the lowest fibrosis score and inducing the highest numbers of endogenous OCs. Resolution of fibrosis after G-CSF treatment was accompanied by *in situ* downregulation of proinflammatory cytokines.

It has been suggested that the pharmacological blockade of the SDF-1/CXCR4 interaction by Plerixafor inhibits the homing of extrahepatic cells expressing the CXCR4 to the liver [40,41]. Indeed, in our study, the contribution of BM-derived hepatocytes as well as OCs to liver restoration in Plerixafor-mobilized mice did not differ from the control mice, probably reflecting the decreased homing ability of Plerixafor-mobilized HSCs to the liver. This was supported in our study by the significantly reduced spontaneous migration of Plerixafor- or G-CSF + Plerixafor-mobilized Lin^{-} cells, towards the CCl_4-injured and subjected to mobilization livers, as compared to G-CSF-mobilized Lin^{-} cells. Because of the reduced access of Plerixafor-mobilized HSCs to the liver, one could hypothesize that mobilization with Plerixafor should not benefit the injured liver, if the case is that paracrine signaling from homed to the liver HSCs induces endogenous repair programs. In contrast, liver fibrosis regressed after Plerixafor-alone mobilization, indirectly suggesting that mechanisms independent of the recruitment of HSCs to the liver mediated the amelioration of fibrosis.

The Peroxisome Proliferator-Activated Receptors are a group of nuclear receptor isoforms (PPAR-α, PPAR-γ, PPAR-δ). PPAR-γ plays a key role in stellate cell-mediated fibrogenesis being markedly downregulated during stellate cell activation [42,43], whereas stimulation of the receptor with its ligands reverses the fibrogenic process and triggers a phenotype switching from the activated cell phenotype to a quiescent hepatic stellate cell phenotype [44–46]. Moreover, PPAR-γ or PPAR-γ activators have also been involved in mechanisms regulating angiogenesis by inducing the expression of angiogenic factors such as VEGF and its receptors in myofibroblasts, thus initiating angiogenic responses at the site of tissue injury [47–49].

Plerixafor in our study, upregulated the expression of both liver PPAR-γ and VEGF. PPAR-γ upregulation induced an anti-fibrotic process probably through the reversal of the activation and reduction of proliferation of hepatic stellate cells thus contributing to fibrosis amelioration seen in the Plerixafor-treated animals. It is not clear from our study, whether Plerixafor directly increased VEGF or this was a secondary effect mediated through the PPAR-γ signaling pathway. Although the role of VEGF in promoting or inhibiting fibrosis remains arguable [50–53], the Plerixafor-induced hepatic VEGF expression was associated, in our study, with restoration of sinusoidal "capillarization" and significant reduction of perisinusoidal myofibroblasts and of Factor VIII expression in sinusoidal endothelial cells. In addition, the livers of

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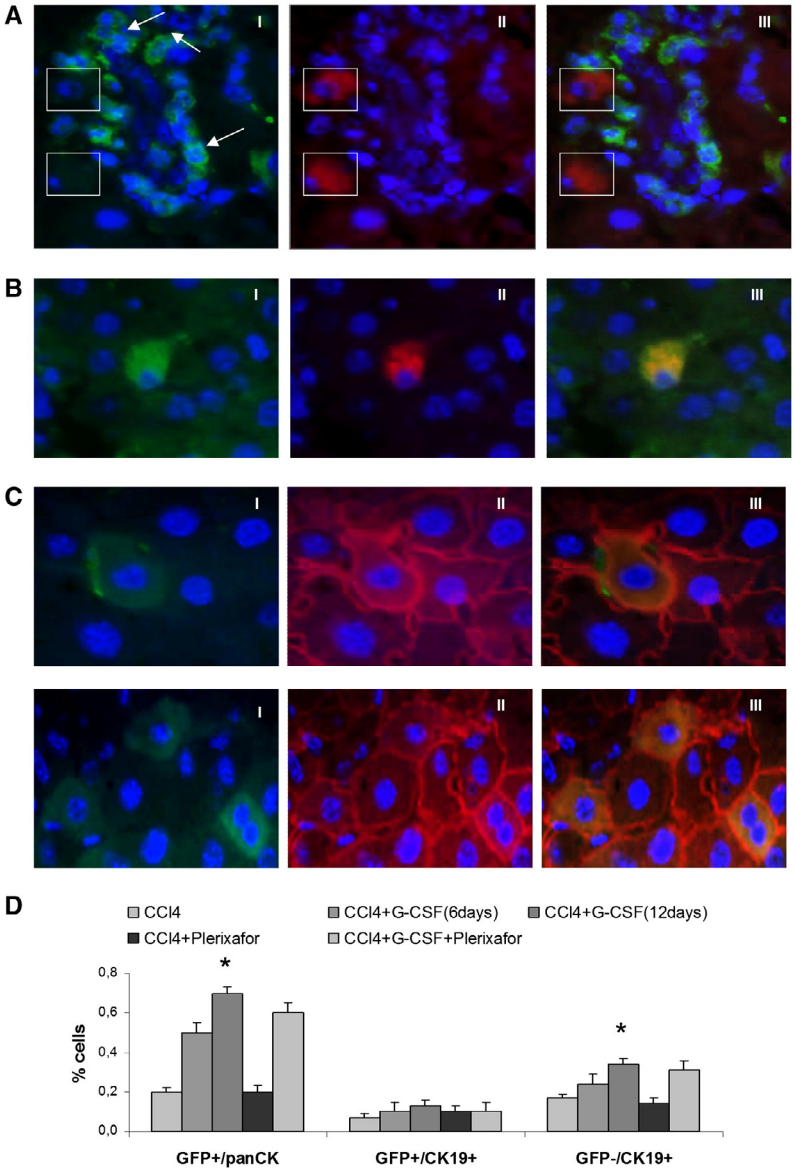

Fig. 5. Contribution of BM cells to liver recovery. (A) Double immunofluorescence for identification of endogenous OCs ($GFP^-/CK19^+$), (I) GFP^+ (green: FITC-labeled): BM-derived cells (arrows); GFP^-: non BM-derived cells (squares), (II) $CK19^+$ (red: TRITC-labeled): OCs (squares), (III) merged images, endogenous OCs: $GFP^-/CK19^+$ (squares, magnification × 400). (B) Double immunofluorescence for identification of liver committed BM-derived cells ($GFP^+/CK19^+$) (I) GFP^+ BM-derived cell, (II) $CK19^+$ OC, (III) merged image, fusion orange double staining (magnification × 400). (C) Double immunofluorescence for GFP/panCK, (I): GFP^+ cells of BM origin, (II): cytoplasmic and membranous red staining for panCK in hepatocytes, (III) co-expression of GFP and panCK identifies BM-derived mature hepatocytes by orange fusion staining. (D) % contribution of BM-derived mature hepatocytes ($GFP^+/panCK^+$, $^*p = 0.007$ *vs* CCl_4), endogenous OCs ($GFP^-/CK19^+$, $^*p = 0.01$ *vs* CCl_4) and liver committed BM-derived cells ($GFP^+/CK19^+$, p = ns) to liver reconstitution. Experiments were performed three times (n = 4–5 animals/group/experiment). Bars show the mean ± SEM.

Plerixafor or Plerixafor + G-CSF-treated mice displayed the highest mitogenic activity, probably reflecting an increased *in situ* proliferation of endothelial cells, triggered by the increased VEGF hepatic expression.

Conclusions

There is accumulating evidence over the years that G-CSF exerts a clear antifibrotic effect. Our data show that not only G-CSF, but also the novel mobilizing modalities Plerixafor and G-CSF + Plerixafor, ameliorate chronic liver damage through different mechanisms. The greater anti-fibrotic potential is shown by an extended G-CSF treatment; it is not clear however, whether the liver repair mechanisms are triggered, but not ultimately mediated, by the HSCs that home to the injured liver or/and by a hepatotrophic effect of G-CSF *per se*. A direct trophic effect to the liver probably underlies the liver healing potential of Plerixafor, as its anti-fibrotic effect seems to be non-HSC-dependent. Further studies are needed to possibly dissect the paracrine or "by stander" effect of mobilized HSCs to liver cell progeny from a direct, "trophic" effect of mobilizing agents to the liver.

Authorship

Yannaki E. and Tsolaki E. conceived and designed the experiments. Tsolaki E., Gounari E., and Siotou E. performed the experiments. Tsolaki E., Athanasiou E., Gounari E., and Zogas N. acquired the data. Yannaki E. and Tsolaki E. analyzed and interpreted the data. Yiangou M. and Anagnostopoulos A. provided reagents and analytical tools and interpreted the data. Yannaki E. and Tsolaki E. wrote the paper. All the authors reviewed and finally approved the article.

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Conflict of interest

The authors declare that they do not have anything to disclose regarding financial conflict of interest with respect to this manuscript.

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