Early loss of bone marrow hematopoietic stem cells drives regeneration failure in cirrhosis

Nidhi Nautiyal
Amity University

Deepanshu Maheshwari
Institute of Liver and Biliary Sciences (ILBS)

Dhananjay Kumar
Institute of Liver and Biliary Sciences (ILBS)

E Pranshu Rao
All India Institute of Medical Sciences

Dinesh Mani Tripathi
Institute of Liver and Biliary Sciences (ILBS)

Sujata Mohanty
All India Institute of Medical Sciences

Prakash Baligar
Amity University

Anupama Kumari
Institute of Liver and Biliary Sciences (ILBS)

Chhagan Bihari
Institute of Liver and Biliary Sciences (ILBS)

Subhrajit Biswas
Amity University

Rakhi Maiwall
Institute of Liver and Biliary Sciences (ILBS)

Shiv Kumar Sarin
Institute of Liver and Biliary Sciences (ILBS)

Anupam Kumar

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Abstract

Liver failure is failure of regeneration. Underlying cause of regeneration failure in cirrhosis is not well-defined. Bone marrow stem cells (BMSC) and their progeny play a central role in tissue repair and regeneration and are defective in patients with chronic liver failure. Whether BM failure is cause or consequence of liver failure in cirrhosis is not known. In this study we aim to decipher the underlying relation between BM failure and regeneration failure in cirrhosis. Using murine model of chronic liver injury we showed that loss of BMSC occurs prior to failure of liver regeneration and onset of non-acute decompensation. We further showed, infusion of healthy-BM in cirrhotic-BM dampens the inflammation, increase glycolysis and induce the repopulation of native LT-HSCs. Restoring LT-HSCs reserve in cirrhotic animals restore liver macrophage number and function, accelerate regression of fibrosis, enhanced liver regeneration and delay the onset of non-acute decompensation. It improved liver clearance of immune complex, dampens neutrophil-mediated inflammation and shifted energy metabolism from glycolysis to OXPHOS. Therefore, early loss of BMSC reserve compromise innate immune function of liver and drive the regeneration failure in cirrhosis. We also provide the proof-of-concept that rejuvenating BM-HSC reserve as putative therapeutic approach to prevent regeneration failure in cirrhosis.

Introduction

Chronic liver injury is a slowly progressive disease that often progresses to cirrhosis and slowly leads to portal hypertension (PH) and hepatic decompensation\(^1\)–\(^3\). The underlying pathomechanisms of the transition from compensated to decompensated cirrhosis are an area of active investigation. Emerging evidence from the pathophysiology of cirrhosis highlights the potential role of portal hypertension\(^4\), aggravated systemic inflammation\(^5\),\(^6\), increased intestinal bacterial translocation\(^7\)–\(^11\) and regeneration failure\(^12\)–\(^14\) in this transition. The mechanisms underlying this poor resolution of liver injury/infection and regeneration failure in cirrhosis is not well defined.

Liver macrophage together with recruited BM myeloid cells plays a central role in effective clearance of liver injury and infection required for liver regeneration\(^15\). This increased demand of myeloid cells in response to injury and infection is primarily supported by the demand-adapted myeloid biased BM hematopoiesis\(^16\),\(^17\). Patients with liver cirrhosis show broad defects in bone marrow stem cells (BMSC)\(^18\)–\(^21\), and their progeny\(^22\) is required for adequate clearance of injury and infection\(^9\),\(^23\). Indeed, syngeneic BM cell therapy has shown significant improvement in the regression of fibrosis and regeneration in preclinical studies\(^24\). Nevertheless, the clinical application of autologous BM hematopoietic stem cell (HSCs) transplantation resulted in mixed responses\(^25\),\(^26\), with transient improvements in outcome, liver histology and regeneration in cirrhotic patients with preserved HSCs reserve\(^27\). This further highlights the association between defective BMSC reserve and poor resolution of injury and regeneration in cirrhosis. The underlying relationship between defects in BMSC reserve, liver repair, and regeneration failure during chronic liver injury is unknown.
We address whether the loss of BMSC reserve is the cause or consequence of the liver failure by examining the kinetic changes in BMSCs during the progression of injury and regeneration in a progressive model of decompensated cirrhosis. Given the crucial role of BMSCs and their progeny in tissue repair and regeneration, we also evaluated the potential therapeutic benefits of restoring BM-HSCs reserve in promoting the resolution of liver injury and regeneration in cirrhosis.

**Results**

**Loss of BM-HSCs reserve precedes regeneration failure and the onset of decompensated cirrhosis.**

To assess the temporal changes in liver injury, regeneration, and BMSC population, C57BL/6 mice were subjected to increasing doses of i.p. administration of carbon tetrachloride (CCL4) until week-15, time when ascites becomes evident (a feature of decompensated cirrhosis). Samples were collected post weeks-3, 6, 10, and 15 of CCL4 doses (Fig. 1A). Biochemical analysis showed a progressive increase in AST and ALT from control to week-15 (Figure S1A), suggesting progressive liver injury. Between weeks-10 and 15, a significant rise in total bilirubin and ammonia was observed (Fig. 1B), and ascitic fluid was detected with a serum-ascites albumin gradient (SAAG) greater than 1.1 g/dl (Fig. 1C), indicating the presence of decompensated cirrhosis. Portal pressure (PP) analysis revealed a 34.9% increase in PP by week-10 in the CCL4 group compared to controls, which further increased by 23.6% at week-15 (Fig. 1D). These findings suggest liver failure and the development of non-acute decompensation by week-15 of CCL4 chronic injury.

The gross anatomy of the dissected liver showed the change from a micronodular (week-10) to a macronodular surface (week-15) (Figure S1B). Histopathology of liver tissue showed the presence of inflammation, steatosis (Figure S1C), mild portal fibrosis at week-3, bridging fibrosis at week-6, and cirrhosis from weeks-10 to 15 (Fig. 1E and S1D). There was a progressive increase in fibrosis compared to controls, as evidenced by changes in MT, SR, collagen-1, and α-SMA-positive areas until week-10. However, from weeks-10 to 15, the extent of fibrosis was comparable (Fig. 1E and S1D). Interestingly, we observed a significant increase in TUNEL+ hepatocytes at week-15 however it remained confined to immune cells near the fibrotic septa at weeks-3, 6, and 10 (Fig. 1F and S2). Further analysis of PCNA+ hepatocytes showed a progressive increase in hepatic regeneration until week-10. However, from week-10 to 15, there was a significant reduction in the number of PCNA+ hepatocytes (Fig. 1G). This suggested that while the progressive increase in liver fibrosis accounts for the development of cirrhosis, increased hepatocyte death with regeneration failure underlies the transition from compensated to decompensated cirrhosis during chronic liver injury.

To explore the kinetic change in BM reserve, we investigated the changes in BM histology and distribution of hematopoietic stem and progenitor cells (HSPCs) in the same group of animals. In control animals, while BM histology was preserved till week-15, animals with CCL4 showed a progressive increase in the loss of bone trabeculae and fat accumulation from week-6 onwards (Figure S3A). FACS analysis of BM mononuclear cells (BM-MNCs) showed a progressive increase in HSPCs, defined as LSK, with the
progression of chronic liver injury compared to an age-matched healthy control (Fig. 1H-I and S4). Further enumeration of the LSK sub-population, including long-term HSCs (LT-HSCs), short-term HSCs (ST-HSC), and multipotent progenitors (MPPs), showed a progressive increase in MPPs and a decrease in LT-HSCs from week six onward in animals with chronic injury as compared to controls (Fig. 1H-I). No significant difference was detected for the ST-HSCs population (Figure S3B).

Interestingly, between week-6 and 10, there was more than a 60% reduction in LT-HSCs with the expansion of MPPs in CCl4 animals (Fig. 1I). This suggested that an increase in the LSK pool after six-weeks of chronic injury was contributed by the expansion of MPPs at the expense of LT-HSCs. This highlighted the disturbed balance between HSCs self-renewal and their differentiation, which is required to maintain demand-driven hematopoiesis in response to injury or infection\textsuperscript{16}. Further, to check whether the observed loss of LT-HSCs in these animals is due to liver injury or CCL4 intoxication of BM, we compare the change in BM LT-HSCs in thioacetamide-induced chronic liver injury (Figure S3C). Like CCL4-induced chronic liver injury, animals with thioacetamide injury had significantly reduced BM LT-HSCs compared to controls (Figure S3D).

BM mesenchymal stem cells (MSC) plays a crucial role in maintaining HSC self-renewal\textsuperscript{28,29}. To investigate the underlying cause of LT-HSCs loss, we analyzed the change in number of Nestin + MSCs and CFU-F colonies as readout MSCs. As depicted in Fig. 1J-K and S3e-f, both the Nestin + MSCs and CFU-F colonies were comparable to the control until week-3. However, from week-3 to week-6, while the number of Nestin + cells significantly increased, there was a marked reduction in CFU-F, suggesting the loss of MSC self-renewal capacity during the period of chronic liver injury. Subsequently, at week-6, both the number of Nestin + cells and their CFU-F colonies exhibited a significant decrease, coinciding with the progression of chronic liver injury. Overall, our findings demonstrate a sequential loss of BM-MSCs (starting at week-3), followed by LT-HSCs (beginning at week-6) during the progression of chronic liver injury. This ultimately leads to regeneration failure (between weeks 10–15) and the development of decompensated cirrhosis.

**Intra-BM infusion of syngeneic healthy-BMC induces repopulation of native LT-HSC**

An exogenous infusion of healthy BMC has been shown to induce functional recovery in BM dysfunction\textsuperscript{30}, and it is routinely used in clinics to manage various hematological dysfunctions\textsuperscript{31}. Hence, we next explore whether exogenous administration of BMC can restore the LT-HSC in cirrhotics. As immune function in cirrhotic animals is already compromised, and they show poor LT-HSC reserve, we first identify the appropriate route: intravenous (IV) or directly to the BM cavity by intra-femoral (IF) injection for maximum cell delivery without any preparative regimen in cirrhotic animals (Figure S5A). Compared to IV, the IF group showed significantly higher recruitment of injected cells in BM and liver at 24H post-infusion, followed by D11 where infused cells cleared from both BM and the liver in the IV group. In contrast, the IF group showed decreased ROI (Figure S5B). Without a preparative regimen, IF showed better and more
transient recruitment of donor BM-MNCs in the cirrhotic recipient's BM; therefore, IF was selected for cell administration.

Next, post 12-weeks of CCl4 injury, the animals were divided into three groups: Group 1 received healthy BM-MNCs (hBMT), Group 2 received cirrhotic BM-MNCs (cBMT), and Group-3 was the vehicle control. The hBMT and cBMT cells were administered via IF injection. CCl4 administration was stopped to allow natural recovery. Animals were sacrificed at 24H, D11, and D21 (Fig. 2a and S6a). In flow cytometry analysis, the percentage of GFP + donor cells was comparable between cBMT and hBMT animals at 24H post-infusion; however, more than 75% of both healthy and cirrhotic donor cells get cleared by D11 and become undetectable by D21 (Fig. 2B). In the further enumeration of total BM cells, while the LSK population significantly decreased in animals with hBMT compared to cBMT and control (Fig. 2C), animals with hBMT showed a significant increase in LT-HSC as compared to control or cBMT at 24H, which further increased and reached the level of healthy animals by D11 (Fig. 2D). In hBMT, out of the total LT-HSC population, only 0.5% at 24H and 1.2% at D11 were GFP + LT-HSC (Fig. 2D), suggesting an increase in BM LT-HSCs post-therapy contributed by increase in native LT-HSCs. Further CFU-F analysis of total BM-MNCs showed a significant increase in CFU-F at 24H and D11 in hBMT animals compared to controls (Fig. 2E and S7A-B). Unlike LT-HSC, CFU-F colonies in animals with hBMT showed more than 50% GFP + cells at 24H, which decreased to 20% by D11 (Figure S7B), suggesting the transient establishment of an MSC supportive niche in animals with hBMT, which might have contributed to increasing the repopulation of native LT-HSC post-BMT.

To gain insight into the mechanisms underlying the increased repopulation of native LT-HSCs following infusion of hBM-MNCs, we compared the change in the global protein profile of sorted native HSPCs (GFP(-)-LSK) from 24H to D11 in both control (with natural recovery) and hBMT animals (Figure S8A). Differentially expressed proteins (DEP, log2FC > 1, p0.05; Figure S8B) of HSPCs from 24H to D11 in the hBMT group showed a significant increase in proteins associated with NAD (binding and activity) and glycolysis and a decrease in neutrophil degranulation, apoptosis, and cell cycle (Fig. 2F). In contrast, the control groups exhibited a significant increase in proteins related to neutrophil degranulation, inflammation, and bacterial response and a decrease in proteins associated with PI3K-AKT signaling, cell cycle, and negative regulation of apoptosis (Fig. 2G). These findings collectively suggested the transient establishment of a supportive MSC niche with a suppressed inflammatory response and elevated glycolysis in HSCs could facilitate the self-renewal of endogenous LT-HSCs following hBMT.

**Restoring BM LT-HSC reserve accelerates regression of fibrosis and regeneration.**

Next, determine whether restoring BM LT-HSC is associated with the native liver repair or regeneration in cirrhosis. We compared the alterations in liver injury and regeneration between the control (with low levels of BM-LTHSCs) and hBMT (with enhanced BM-LTHSCs) groups during the natural recovery process at 24H and D11 post-therapy (as illustrated in Fig. 2B). At 24H post-BMT, animals showed a significant increase in AST and ALT as compared to controls; however, at D11, animals showed a significant reduction in AST, ALT, and total bilirubin, where controls did not show much of the change (Fig. 3A-B).
Histology of liver tissue of hBMT group showed increased necroinflammation compared to control at 24H (Fig. 3C). Further fibrosis (MT, SR, and SMA) was comparable at 24H in both groups; however, at D11, hBMT animals showed a significant reduction in fibrosis as compared to controls (Fig. 3D and S9A-B), suggesting increased regression of fibrosis with hBMT. After observing the fibrosis regression, we investigated the proliferation of hepatocytes. We found a noteworthy increase in the count of PCNA+ hepatocytes on D11 following BMT compared to the control group. However, it was comparable at 24H (Fig. 3E). In contrast to hBMT, the animals subjected to cBMT not only failed to induce the repopulation of endogenous LT-HSCs but also exhibited low resolution of fibrosis and higher levels of hepatocyte death at D11 compared to the control and hBMT groups (as illustrated in Figure S6B). Interestingly, animals with cBMT also showed acute tubular necrosis (ATN) and pulmonary inflammation (Figure S6C). Altogether, this suggests that restoration of BMSC reserve accelerates regression of fibrosis and regeneration in cirrhosis animals.

**Restoring BM LT-HSC reserve augments innate immune function in cirrhosis**

To further understand the underlying cause of increased regression of fibrosis and regeneration in BMT, we analyzed the change in the global protein profile of control and BMT animals’ livers at 24H and D11 post-BMT. Global proteomic analysis of liver tissue identified 187 (109-upregulated and 78-downregulated) DEP (log2FC > 1.5, p < 0.05) at 24H and 186 (73-Up and 113-downregulated) at D11 in BMT animals compared to control (Figure S10). In comparison to control, hBMT animals liver showed a significant increase in proteins associated with cell cycle, autophagy, immune clearance, oxidative phosphorylation (OXPHOS), and innate immune system and a decrease in proteins associated with negative regulation of cell cycle, glucose metabolism and glycolysis, neutrophil, and humoral immune response mediated inflammation and protein degradation at 24H (Fig. 4A). Further at D11 BMT animals showed an increase in proteins associated with translation, OXPHOS, mitochondrial energy metabolism; and decrease in proteins associated with lipid and cholesterol biosynthesis and inflammation compared to controls (Fig. 4B), overall, the proteomic data suggested that restoring BM-LT-HSC reserve helps to augment innate immune function, promote liver regeneration, and restore mitochondrial energy metabolism in cirrhotic liver.

The innate immune system of the liver, particularly the liver macrophage, plays a central role in the clearance of liver injury and infection. Increased bacterial translocation and endotoxemia due to gut dysfunction and poor liver clearance are the hallmark of cirrhosis\(^9\). Our proteomic data showed increased innate immune function and immune cell clearance in animals with BMT. Hence, we investigated the changes in the distribution and phagocytosis of liver macrophages and plasma endotoxin levels in the progression and regression of chronic liver injury with or without hBMT. During cirrhosis, the number and phagocytic function of F4/80+ liver macrophages reduced at week-3, followed by an increase at week-6, where F4/80+ cells were confined to portal fibrotic septa. Later, both the number and phagocytosis of F4/80+ cells declined from week-6 to 10 and after that (Fig. 4C-E). In contrast to this, plasma endotoxin
level showed up and down expression from week-3 to week-10, with an increase at week-15 (Fig. 4F). Change in plasma endotoxin level was inversely correlated ($r = -0.895; p = 0.039$) with liver macrophage number and phagocytosis (Fig. 4G), supporting liver innate immune dysfunction and systemic endotoxemia in the progression of chronic liver injury. During regression after stopping the CCl4, while there was a significant reduction in F4/80 + macrophage in controls, it was comparable in animals with BMT from 24H to D11 post-BMT (Fig. 4H). Compared to the control, hBMT animals showed a significant increase in the phagocytosis of F4/80 + liver macrophage at 24H, further increasing at D11. However, it remained unchanged in control between 24H and D11 (Fig. 4I). Altogether these data showed that restoring BMLT-HSCs in cirrhosis augments the innate immune function of the liver, which may further contribute to accelerated regression of fibrosis and regeneration in cirrhotic animals.

**Restoring BM LT-HSC reserve ameliorates progression to decompensated cirrhosis.**

We observed a significant increase in the regression of fibrosis and regeneration with the restoration of BMSC reserve. During chronic liver injury, we demonstrated that the loss of BM LT-HSC occurs before regeneration failure and the development of decompensated cirrhosis. Hence, we hypothesized that restoring BM LT-HSC by intra-BM infusion of healthy BM cells may prevent the progression to decompensated cirrhosis. To check this, post 12-weeks of CCl4-induced chronic liver injury, animals were divided into two groups, Group-1 received an intra-BM infusion of healthy BM-MNCs, and Group-2 was vehicle control. CCl4 treatment was maintained in both groups, and animals were followed up to develop ascites till day 18 post-BMT. All the animals were sacrificed on day 18 post-therapy (Fig. 5A).

During this follow-up, ascites became prominent in control as compared to BMT (Fig. 5B). All the animals sacrificed with ascites showed the presence of ascitic fluid with SAAG of > 1.1 g/dl (Fig. 5C). In comparison to control, hBMT showed a significant reduction in ALT and ammonia at D18 post-therapy (Fig. 5D). Histopathology of liver tissue showed a significant reduction in MT, SR, and α-SMA positive area in hBMT compared to controls (Fig. 5E and S11). Animals with BMT also showed a significant reduction of TUNEL + hepatocytes (Fig. 5F) and an increase in the number of PCNA + hepatocytes (Fig. 5G) and F4/80 + Kupfer cells (Fig. 5H) compared to controls, suggesting decreased liver injury and increased hepatocyte regeneration post-hBMT in cirrhotic animals. We also observed a significant increase in serum creatinine in controls, suggesting renal dysfunction (Fig. 5I). Histology of kidney sections showed the presence of ATN with increased TUNEL + renal tubular epithelial cells in control compared to BMT (Figure S12A). Unlike hBMT, control animals also showed the presence of pulmonary fibrosis (Figure S12B). This suggested that intra-BM infusion of healthy BM-MNCs ameliorates the progression of liver injury, increases regeneration, and prevents the development of decompensated cirrhosis.

**Discussion**

Demand-adapted hematopoiesis in response to injury and infection plays a central role in the resolution of damage and repair$^{16,17}$. In patients with advanced liver cirrhosis, BM-HSC reserve is severely...
compromised and shows poor resolution of injury and infection. The underlying relationship between the exhaustion of BMSC reserve and liver repair and regeneration failure in cirrhosis is unknown.

This study shows that loss of BM LT-HSC reserve precedes regeneration failure and the development of decompensated cirrhosis during disease progression. We further demonstrated that rejuvenating BM LT-HSC reserve accelerates fibrosis regression, potentiates hepatic regeneration, and prevents the progression of non-acute decompensation.

Progressive increases in liver fibrosis and portal-hypertension with aberrant hepatocyte regeneration are associated with architectural disruption and the development of cirrhosis\textsuperscript{32,33}. Further, an increase in portal-hypertension and systemic inflammation due to increased liver injury and gut dysbiosis are thought to be associated with the development of decompensated cirrhosis \textsuperscript{6,34}. We observed a progressive increase in fibrosis and hepatocyte regeneration until the compensatory stage (week-10). Further, in the transition from compensated to decompensated cirrhosis, while fibrosis was comparable, there was an increase in PP with a marked increase in hepatocyte death and a decrease in hepatocyte regeneration (from weeks 10–15), highlighting the potential contribution of increased liver injury and regeneration failure in the transition from compensated to decompensated cirrhosis in this model similar to human cirrhosis\textsuperscript{6,34}. In further analysis of BM, we observed a progressive increase in the number of HSPCs and BM-LSK with the progression of chronic liver injury, indicating the demand adapted hematopoiesis. The balance between HSC self-renewal and their differentiation are required to maintain demand-driven hematopoiesis in response to injury or infection\textsuperscript{16}. In the initial stage of liver injury, this balance is maintained; however, during the transition from fibrosis (week-6) to cirrhosis (week-10), it gets disrupted, and a further increase in the LSK pool is mainly contributed by the expansion of MPPs at the expense of LT-HSCs. BM-MSC plays an essential role in the maintenance of HSC self-renewal\textsuperscript{28,29}, and we observed a significant loss of MSC colonies from week three onward before the loss of LT-HSCs. Earlier, we have shown that cellular and functional exhaustion of BM-MSCs is associated with loss of BM-HSCs and osteodystrophy in cirrhotic patients\textsuperscript{19,21}. Hence, the prior loss of BM-MSCs may contribute to the loss of LT-HSCs during injury. Interestingly, we observe that loss of BM LT-HSCs precedes regeneration failure and the onset of decompensated cirrhosis. Further restoring BM LT-HSCs accelerates fibrosis regression, decreases hepatocyte death, potentiates regeneration, and prevents the transition to decompensated cirrhosis. Hence, the loss of BM LT-HSCs accounts for increased hepatocyte injury and regeneration failure in the transition to decompensated cirrhosis.

Restoring BM LT-HSCs reserve showed a significant increase in proteins associated with immune clearance, innate immune system, and decreased neutrophil-mediated inflammation in the liver. In disease progression, we observed a significant reduction in the number and phagocytic function of liver macrophages with an increase in systemic endotoxemia from week-6, similar to loss of BM LT-HSCs. Further, during natural regression after stopping the CCl4, there was a significant reduction in the liver macrophage pool. Restoring BM LT-HSCs reserve prevents the loss of the liver macrophage pool and augments its phagocytic function. As innate immune cells depend entirely on BM-HSPCs to meet their
increased demand in response to injury and infection\textsuperscript{16,28}, loss of BM-LTHCs reserve may account for compromised liver macrophage response in the transition from fibrosis-to-cirrhosis and thereafter. Indeed, recruitment of BM-HSCs to the liver has been shown to augment fibrosis regression and regeneration in animals and humans by improving innate immune function\textsuperscript{22–24}. Myeloid cells such as neutrophils, monocytes, and monocyte-derived macrophages play a central role in the clearance of cellular debris and gut bacteria/bacterial product\textsuperscript{35–37}. Increased intestinal bacterial translocation due to gut dysfunction and poor liver clearance is the hallmark of cirrhosis, particularly in the transition to decompensated cirrhosis\textsuperscript{7,11}. Endotoxemia and infection have been shown to hamper liver regeneration and promote hepatocyte injury in cirrhotic animals with acute decompensation\textsuperscript{12,13}. Our data showed that during chronic liver injury, loss of both liver macrophage pool and BM LT-HSCs starts before increasing liver injury and regeneration failure. Restoring BM LT-SHCs increase cell cycle and hepatocyte regeneration, restoring liver macrophage number and their phagocytic function, suggesting the potential link between liver macrophage dysfunction and regeneration failure in decompensated cirrhosis. Indeed, exogenous administration of macrophage has been shown to improve liver clearance and promote liver regeneration in chronic and acute\textsuperscript{15} liver injury.

In cirrhosis, mitochondrial dysfunction leads to a shift in hepatocyte energy metabolism from oxidative phosphorylation (OXPHOS) to glycolysis\textsuperscript{38}. Our data have shown that with the restoration of innate immune function, there is a shift from glycolysis to OXPHOS, restoration of metabolic function in the liver along with restoration of BM reserve. Based on these observations, we propose that loss of BM-HSCs reserve contributes to defects in the innate immune function of the liver during injury. This may result in increased hepatocyte death and regeneration failure due to compromised liver clearance of invading gut-derived infections during the transition from compensated to decompensated cirrhosis.

Exogenous infusion of hBM cells has been shown to induce functional recovery in BM dysfunction\textsuperscript{33} and is routinely used in clinics to manage various hematological dysfunctions\textsuperscript{32}. BM-HSC therapy in pre-clinical studies showed significant improvement in the regression of fibrosis and regeneration\textsuperscript{11,18–24}. In contrast, therapeutic use of autologous BM-HSC showed mixed response with transient improvement in outcome and liver histology in cirrhotic patients with preserved HSC reserve\textsuperscript{23}. This further highlights the potential role of BM-HSC reserve in resolving liver injury and regeneration. Whether exogenous administration of HSC can restore the BM-HSC reserve in cirrhosis is unknown. Our data showed that direct infusion of unfractionated hBM-MNCs to cirrhotic BM induces the repopulation of native LT-HSC by dampening the inflammatory and bacterial response and enhancing HSCs glycolysis. This further augments the innate immune function of the liver and enhances the regression of fibrosis and regeneration.

In contrast, infusion of cirrhotic unfractionated whole BM cells failed to increase the BM LT-HSCs number. It even worsens fibrosis, consistent with the previous report\textsuperscript{39}. Hence, restoring/rejuvenating BM LT-HSC by intra-BM infusion of exogenous hBM-MNCS can serve as a better alternative approach than autologous liver-directed BM cells therapy in the management of cirrhosis.
In the current study though the donor BM cells did not engraft, and the increase in LT-HSCs is mainly contributed by the repopulation of native LT-HSCs, which reduces the risk of graft versus host response, further study with MHC mismatched donor is required to establish the safety of allogenic BM cells infusion for future clinical translation. Also, in this study, we have used a heterogeneous population of BM-MNCs for infusion, which didn't answer the potential cell type responsible for the induction of native LT-HSCs repopulation in the recipient. Further investigation is required to elucidate the underlying mechanism and establish future clinical protocols.

In summary, the current study documents that loss of BMSC reserve accounts for the poor resolution of liver injury and regeneration failure during chronic liver injury and provides evidence that approaches like allogenichBM cells infusion to BM can alleviate this problem. It will be intriguing for future work to fine-tune further and develop potential interventional strategies to treat and prevent loss of BMSC reserve in chronic liver injury to potentiate regression of fibrosis and regeneration in cirrhosis.

**Materials and methods**

**Ethics Statement:** The animal studies were conducted in accordance with the guidelines by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Govt. of India and were approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Liver and Biliary Sciences, New Delhi, India with approval number IAEC/ILBS/17/02. All investigations were conducted following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) protocols and the principles outlined in the Basel Declaration (http://www.basel.declaration.org), which encompass the 3R principle.

**Disease Model:** Wild-type C57Bl/6 mice (6–8 weeks, male) were obtained from breeding facility at CCM, ILBS and housed in a clean, temperature-controlled environment with a 12H light and dark cycle, provided with free access to a regular laboratory chow diet and water. Chronic liver injury induced by intraperitoneal (i.p.) injections of carbon tetrachloride (CCL4, Central Drug House, Delhi, India) in olive oil (HiMedia Pvt. Ltd., India) started at a dose of (0.1–0.5) ml/kg of body weight and were given twice weekly for 15 weeks. Mice were sacrificed post week-3/6/10/15 to know the fibrosis level during chronic liver injury. The animals were studied for the progression of hepatic decompensation.

**Method of Euthanasia:** Animals were sacrificed at the given time point or the end of the study by a cocktail of ketamine (50mg/ml), xylazine (20mg/ml) and saline (0.9% V/V) by i.p injection (0.1ml/20gm of mice).

**Cell therapy:** After completing the 11th week of chronic CCl4 treatment, mice (N=120) were randomly divided into different groups based on the experimental design. Set 1: To study mode of cell therapy, mice were divided into three groups: group-1 received IF-BM infusion, group-2 received intra-venous (IV) BM infusion, and group-3 served as the vehicle control; mice were sacrificed at 24H, D11 and D21 of post-cells infusion. Set 2: To study the effectiveness of cell therapy in restoring native BM-HSC reserve and impact on liver repair and regeneration. Group-1 received cells from syngeneic healthy C57Bl/6-GFP,
Group-2 received IF-BM cells from syngeneic cirrhotic (10-weeks of CCl4 injury) C57Bl/6-GFP while group-3 was vehicle control. Set 3: To study the infused BM cells’ ability to ameliorate the progression of decompensated cirrhosis. Cells were isolated from Femurs and tibias and labeled with DIR (#D12731) dye for ex-vivo imaging. Approximately, 4x10^6 cells were infused per mice intra-femorally.

**Cells preparation:** Femurs and tibias were removed from 6-8 weeks aged healthy or cirrhotic C57BL/6-Tg(UBC-GFP)30Scha/J. BM cells were extracted from both the bones and a single cell suspension was prepared by passing through a 75µm filter. The cells further labeled with DIR (#D12731) dye for ex-vivo imaging. Approximately, 4X10^6 cells were infused per mice.

**Ex-vivo Imaging:** DIR, 1, 1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (#D12731, Invitrogen, Life Technologies, USA), used to tag cells at 10µM concentration for 15minutes at room temperature and washed with 1XPBS. These GFP+ DIR tagged cells were used to see the migration of donor’s cells. At every time point (24H, D11 and D21) mice were euthanized. Imaging was done using AllMS IVIS facility, New Delhi, India.

**Blood Serum Biochemistry:** At the time of sacrifice, mice were deeply anaesthetized with the cocktail of ketamine (50mg/ml), xylazine (20mg/ml) and saline (0.9% V/V) by i.p injection (0.1ml/20gm of mice). Blood was collected via retro-orbital puncture, in a blood collecting tube and centrifuged for 15minutes at 3000 rpm to collect serum. The serum levels for liver and kidney injury were determined by the biochemical analyzer at Institutional facility.

**Flow Cytometry Analysis:** Cells were analyzed based on surface markers: LSK (LIN-/c-Kit+/SCA-1+); LT-HSC (LIN-/c-Kit+/SCA-1+/FLT3-/CD34-); ST-HSC (LIN-/c-Kit+/SCA-1+/FLT3-/CD34+); and MPPs (LIN-/c-Kit+/SCA-1+/FLT+/CD34+) for HSCs. For MSCs: TER119-/CD45-/CD31-/NESTIN+. Liver kupffer cells were analyzed based on F4/80+ surface marker and checked for phagocytosis (Cayman Phagocytosis assay kit as per the manufacturer protocol).

**Cells staining:** Cells were incubated with an antibody cocktail for 40 minutes at 4°C, washed with 1XPBS and acquired using FACS Verse, and analyzed using FlowJo (Treestar Inc., V10) for the FCS file.

**Colony Forming Unit-Fibroblasts (CFU-F):** The CFU-F assay involved culturing 2X10^6 bone marrow cells per well in a six-well plate in triplicates. On Day 11, the cells were washed, fixed (4% paraformaldehyde, 20 minutes), and stained with 0.5% crystal violet for 15-20 minutes before being counted.

**Hemodynamic assessment:** Animals were anesthetized with isoflurane inhalation for assessment of portal pressure; the ileocolic vein was cannulated with PE-10 catheters connected to a pressure transducer (Edwards Life Sciences, Irvine, CA), and the pressure transducers were connected to a PowerLab (4SP) linked to a computer using the Chart version 5.0.1 for Windows software (AD Instruments, Australia). The temperature of the animals was maintained at 37±0.5°C. Hemodynamic data were collected after 8–10 minutes of the stabilization period.
**Histopathology and Immunohistochemistry (IHC):** Liver and femur (decalcified using 14% EDTA at 4°C for 3-4 days) tissues were fixed, processed using paraffin block techniques, and stained with H&E, Sirius red, and MT. IHC was performed to study liver regeneration and fibrosis. Paraffin-embedded sections were stained with primary antibodies (PCNA, Collagen I, α-SMA, F4/80, Nestin+). DAB substrate and streptavidin-horseradish peroxidase were used for visualization. TUNEL assay was performed using in-situ Cell Death Detection Kit (Roche #11684795910) as per the manufacturer's protocol.

**Endotoxin Assay:** The "Pierce Chromogenic Endotoxin Quant Kit" (ThermoFisher#A39553) is used as per the manufacturer's protocol to analyze the endotoxin level in mouse samples.

**Mass spectrometry:**

Sample preparation: Proteins were isolated from liver tissue (approximately 150mg) and BM sorted LSK cells (approximately 1 x 10^5) using RIPA buffer (with Proteinase K). Proteins were estimated by Bradford's method. 50µg of protein sample was used for digestion and reduced with 5mM TCEP and further alkylated with 50mM iodoacetamide and then digested with Trypsin (1:50, Trypsin/lysate ratio) for 16h at 37°C. Digests were cleaned using a C18 silica cartridge to remove the salt and dried using a speed vac. The dried pellet was resuspended in bufferA (2% acetonitrile, 0.1% formic acid).

**Mass Spectrometric Analysis of Peptide Mixtures:** All the experiments were performed using EASY-nLC 1200 system (Thermo Fisher Scientific) coupled to Thermo Fisher-QExactive plus equipped with nano electrospray ion source. 1µg was loaded on C18 column 50cm, 3.0µm Easy-spray column (ThermoFisher Scientific). Peptides were eluted with a 0–40% gradient of bufferB (80% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min) and injected for MS analysis. LC gradients were run for 60 minutes. MS1 spectra were acquired in the Orbitrap at 70k resolution. Dynamic exclusion was employed for 10 s excluding all charge states for a given precursor. MS2 spectra were acquired at 17500 resolutions. (Protein isolation and proteomics run/analysis was done by Valerian Chem Private Limited, New Delhi, Delhi 110066, India).

**Data Processing:** All the samples once processed, were subjected to mass spectrometry run. Raw files containing mass/charge values were generated for each of the samples. Further these raw files were analysed through Thermo Proteome Discoverer (v2.2) against mouse proteome database. For Sequest and Amanda (ML algorithms used to map peptides identified by mass spectrometer against the protein database) search, the precursor and fragment mass tolerance were set at 10 ppm and 0.02 Da, respectively. The protease used to generate peptides, i.e. enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") along with maximum missed cleavages value of two. Carbamidomethyl on cysteine as fixed modification and oxidation of methionine were considered as Variable modifications for database search. Both peptide spectrum match and protein false discovery rate were set to 0.01 FDR. PD output is generally in the form of a matrix that contains accession IDs and abundance values for each of the identified proteins per sample, along with relevant
annotation for each identified protein. Peptides and proteins sheet are exported individually and then are used for downstream analysis.

**Statistical analysis:** Differential Expression Analysis: Raw abundance values and accession IDs are extracted from within the matrix. This matrix is used for differential expression analysis for the identification of significant proteins. For Differential Expression Analysis between different groups for liver and BM samples, the data was first transformed on log2 scale and filtered on the basis of valid values (features which were quantified in 70% of the samples). The remaining values after filtering were imputed based on normal distribution and then normalized using MBQN. Once the data is normalized, t-test analysis is performed that provides pValues and logFC (log2 Fold Change) values. The proteins that have pvalues<0.05 are considered significant. Volcano plot were generated using log10 pValues and log2 Fold change values.

All sections (liver, lungs, kidney and spleen were evaluated in a blind manner by the pathologist and were examined using EVOS@FL light microscopy. Micrographs quantified using ImageJ (FJI, 1.47V) in three non-overlapping random fields and dot plots were made using R-studio (RStudio, PBC, 4.2.1). Data are presented as mean ± standard error of the mean. Two-tailed Student’s t and Mann-Whitney U tests were used to analyze parametric and nonparametric data, respectively using Prism (Graph-Pad Software, Inc, 6.01) unless otherwise stated.

**Declarations**

**Authors' Contribution:** Conceptualization: NN, RM, SKS, AK; Methodology: NN, DM, DK, REP, DMT, SM, PB, SB, RM; Investigation: NN, DM; Pathology: NN, CB; Mice strain: AP; Visualization: NN, AK; Funding acquisition: SKS, AK; project administration: SKS, AK; supervision: AK, SKS; Writing: original draft: NN, AK; Writing: review and editing: NN, SKS, AK.

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**Ethical approval:** This study was approved by the Institutional Animal Ethics Committee (IAEC) under the approval code: IAEC/ILBS/17/02 for all animal care and experimental procedures.

**Declaration of Conflict of Interest:** No authors have any conflicts of interest.

**Data availability statement:** The data that support the findings of this study are available upon reasonable request from the corresponding author.

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References


Figures
Figure 1

Loss of BM-HSC reserve precedes regeneration failure and the onset of decompensated cirrhosis. (A) Schematic representation of a progressive mouse model of chronic liver injury. (B-C) The blood biochemical levels of (B) total bilirubin and ammonia at each time point, and (C) serum ascites level measured as SAAG level post week-15 of CCl4 injury (N = 5). (D) The hemodynamic assessment measured between groups of mice at week-10 and week-15, and compared with the control mice (N = 5).
(E) The liver sections were stained with Sirius red and quantitate for progressive change in fibrosis between the groups. (F) The Representative immunofluorescent staining images of TUNEL+ hepatocytes (GFP+) and its quantitative analysis (20X). (G) The micrographs showing the immunohistochemistry for hepatocyte proliferation (PCNA staining; 20X) at every stage of CCL4 injury with their quantitative analysis. (H) The flow cytometry gating of LSK (LIN- SCA-1+ c-Kit+) and LSK sub-population based on CD34+/ and Flt3+/ cell surface markers. (I) The line graphs showing the change in percentage of LSK and LSK sub populations (LT-HSC, ST-HSC, MPPs) for CCl4 groups (N=5 to 10). (J-K) The MSC were analyzed based on their (J) stemness to form colonies (CFU-F) per million of BM-MNCs and (K) percentage change based on cell surface marker for ter119-CD45-CD31- Nestin+ at each stage of chronic liver injury (N = 5). Images were taken with an EVOS@FL microscope and quantified using ImageJ. Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 2

**Intra-BM infusion of syngeneic healthy BM cells induces repopulation of native LT-HSC.** (A) Schematic representation of intra-femoral (IF) infusion of healthy BM cells and points of sacrifice. (B) The bar graph showing the engraftment of the donor (hBMT and cBMT) GFP+ cells in recipient BM cells (N = 8 to 10). (C) The percentage change in LSK population was compared between hBMT, cBMT and controls (vehicle control) mice (N = 5 to 8). (D) The percentage change in LT-HSC at 24H and D11 post-BMT of recipients' BM cells, while the green bar showing the donor-derived LT-HSC% in recipients' BM LT-HSCs (N = 5). (E) The stemness of MSC was compared for CFU-F per million BM-MSCs at 24H and D11 post-BMT (N = 5 to 8). (F-G) Bubble or dot plot showing the expression of up-regulated and down-regulated pathways of BM-sorted LSK cells from BMT and a control set of mice based on biological processes (BP), cellular...
components (CC), Reactome pathways (RP), and KEGG pathways; and compared between 24H vs. D11 in both groups (N = 3). Size corresponds to counts, and color shows value. The data were compared based on -1<log2FC>1, P < 0.05 for their significant expression. Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 3
Restoring BM LT-HSC reserve accelerates regression of fibrosis and regeneration. (A-B) The liver injury was compared based on (A) AST, ALT, and (B) total bilirubin between the control and BMT groups at 24H and D11 post-BMT. (C) H&E staining of liver tissue at 24H showed inflammation and necrosis between the control and BMT groups. (D) Sirius red staining showing reduced fibrosis levels from 24H to D11 between the controls and BMT groups. (E) Hepatocyte proliferation was checked based on PCNA+ staining on liver sections at 24H and D11 and compared at 20X blindly. Images were taken in an EVOS@FL microscope and quantified using ImageJ. Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 4

Restoring BM LT-HSC reserve augments innate immune function in cirrhosis. (A-B) The bubble or dot plot showing the expression of up-regulated and down-regulated pathways of BMT and control group proteins isolated from liver tissue; and compared at (A) 24H and (B) D11 post-BMT (N = 5). The data were compared based on -1<log2FC>1, p < 0.05 for their significant expression. (C-D) F4/80+ staining for liver kupffer cells is shown by (C) IHC and (D) FACS staining (N = 5). (E) Liver kupffer cells were compared for...
their phagocytosis based on their phagocytic activity at every time of CCL4 injury (N = 5). (F) At every time point of chronic injury, mice were compared for endotoxin level and compared with the control set of mice (N = 5). (G) The level of endotoxemia was correlated with the F4/80+ cells. (H) Representative IHC stained for F4/80+ cells post-BMT and compared between groups (control and BMT) at 24H and D11. (I) These F4/80+ kupffer cells were compared for their phagocytic activity post-BMT (N=5). Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 5

Restoring BM LT-HSC reserve ameliorates progression to decompensated cirrhosis. (A) The schematic representation of hBMT during the progression of hepatic decompensation. (B) The Kaplan Meier graph showing the development of ascites during the CCl4 administration. (C) The bar graphs showing the biochemical analysis of the SAAG of controls at D18 post-BMT (N = 5). (D) The blood biochemical test was done to compare the levels of AST, ALT, total bilirubin and ammonia post hBMT. (E) The micrographs representing the fibrosis regression through Sirius red staining (4X) in BMT and control mice, and a bar graph showing their quantitative analysis at D18 post-BMT. (F-G) The micrograph (20X) and a bar graph showing the (F) TUNEL+ and (G) PCNA+ hepatocytes and their quantitative analysis at D18 post-BMT of treated groups in comparison to controls. (H) The F4/80+ staining done at D18 post-BMT (20X) between the groups. (I) the creatinine levels were compared to test the secondary organ damage. Images were taken in an EVOS@FL microscope and quantified using ImageJ. Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Supplementary Files

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- Supplementarydata.docx