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Activated and nonactivated MSCs increase survival in humanized mice after acute liver injury through alcohol binging

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Abstract

The ability of the liver to regenerate after injury makes it an ideal organ to study for potential therapeutic interventions. Mesenchymal stem cells (MSCs) possess self-renewal and differentiation properties, as well as anti-inflammatory properties that make them an ideal candidate for therapy of acute liver injury. The primary aim of this study is to evaluate the potential for reversal of hepatic injury using human umbilical cord–derived MSCs. Secondary aims include comparison of various methods of administration as well as comparison of activated versus nonactivated human umbilical cord stem cells. To induce liver injury, humanized mice were fed high-cholesterol high-fat liquid diet with alcohol binge drinking. Mice were then treated with either umbilical cord MSCs, activated umbilical cord MSCs, or a placebo and followed for survival. Blood samples were obtained at the end of the binge drinking and at the time of death to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Histology of all mouse livers was reported at time of death. Activated MSCs that were injected intravenously, intraperitoneally, or both routes had superior survival compared with nonactivated MSCs and with placebo-treated mice. AST and ALT levels were elevated in all mice before treatment and improved in the mice treated with stem cells. *Conclusion:* Activated stem cells resulted in marked improvement in survival and in recovery of hepatic chemistries. Activated umbilical cord MSCs should be considered an important area of investigation in acute liver injury.

Juan Carlos Hernandez and Da-Wei Yeh contributed equally to this work.

INTRODUCTION

Alcohol-associated liver disease (ALD) is a spectrum of disease that includes steatosis (fatty liver), steato-hepatitis, and in severe cases, fibrosis and/or cirrhosis. Hepatic steatosis develops acutely in most individuals who consume even moderate amounts of alcohol. Alcoholic hepatitis is distinct from chronic liver disease in that it can occur with or without long-term drinking. The histopathologic findings seen in alcoholic hepatitis include steatosis, hepatocyte ballooning, Mallory Denk bodies, and lobular inflammation, including a prominent component of neutrophils.[1]

The clinical course is variable with the possibility of reversal with abstinence but is associated with a significant mortality rate of 15%–40% with progression to cirrhosis in a significant number of survivors. Treatment options are limited and include cessation of alcohol, supportive care, and in some cases steroids and/or pentoxifylline. There have been attempts to use support devices to provide a form of "hepatic dialysis"; however, this demonstrated only a small, nonsignificant benefit to this study subgroup.[2] Liver transplantation has proven successful, but has limited application due to strict selection criteria as well as availability of donor organs.[3,4]

The ability to widely differentiate makes mesenchymal stem cells (MSCs) an important area of research for the treatment of patients with liver disease. The liver's regenerative abilities are well known, and the potential for stem cells to contribute to and catalyze this regenerative process offers the possibility of ameliorating morbidity and mortality due to organ failure following liver injury. There are several animal models and human studies that use various types of MSCs to ameliorate different types of liver injury in both acute and chronic liver disease.[5-15]

MSCs derived from mouse bone marrow injected intraperitoneally demonstrated reduced liver steatosis, oxidative stress, and inflammatory cell infiltration.[13] Additional studies have demonstrated improvement in hepatic chemistries including alanine transaminase (ALT), aspartate transaminase (AST), and steatosis, and had an anti-inflammatory effect.[16,17] Levine et al. summarized stem cell therapy in people impacted by ALD, with most studies including a small study population.[18] The results are varied with some showing a decrease in hepatic chemistries and Model for End-Stage Liver Disease and Child-Pugh scores.[19] The use of pluripotent stem cells has been discussed with patients who cannot receive a liver transplant.[20] Given the delicate time balance between regeneration and fatal complications, activated MSCs may offer an advantage in promoting regeneration and improving outcomes in acute liver failure caused by alcoholic hepatitis.

Clearly, alternative treatments are required for patients with advanced chronic and acute liver failure in the face of organ shortages and contraindications to liver transplantation. To investigate the impact on survival in an acute liver injury model, acute alcoholic liver injury was induced in mice prepared with humanized livers to test the hypothesis that human umbilical cord MSCs (hUCMSCs) can improve survival compared with placebo. Furthermore, the use of activated hUCMSCs was evaluated to determine whether activation offered a benefit in this model of acute alcoholic hepatitis.

MATERIALS AND METHODS

Animals and animal experimental design

Our lab generated *Fah−/−*, *Rag2−/−*, and *Il2rgc−/−* (FRG) by crossbreeding of *Fah−/−* mice (RIKEN) and *Rag2−/−*; *Il2rgc−/−* mice on a C57Bl/6J background (Jackson Laboratory). These FRG mice are different from the commercially available strain. Detailed methods for generating our FRG KO mice are detailed in the Supporting Materials and Methods. Institutional IACUC approval was obtained. All research was conducted according to the ethical rules and regulations of our institution.

Human umbilical cord MSC culture

hUC-MSCs under informed consent were isolated from the perivascular Wharton's jelly region of the human umbilical cord and were provided by RoosterBio Inc. (Frederick, MD; RoosterVial-hUC-XF manufactured and sold by RoosterBio and supported by licensed technology from Tissue Regeneration Therapeutics Inc. core technology and patent family: US 8,790,923; US 8,278,102; US 7,547,546; US 9,611,456; US 9,611,456; US 8,481,311; US 9,611,456). The purchased hUC-MSC vials were fully characterized according to the International Society for Cell and Gene Therapy's minimal criteria[21] and performed by RoosterBio. RoosterBio performed additional tests for hUC-MSC characterizations for the expression of surface markers by flow cytometry, trilineage mesoderm differentiation potential (adipocytes, osteocytes, and chondrocytes), Indoleamine 2,3-dioxygenase activity, sterility, endotoxin, and mycoplasma test (data not shown). The hUC-MSCs were cultured and harvested following RoosterBio manufacturing protocols. A total of 1.5 million to 2 million hUC-MSCs were plated in a T225 vented flask (Corning or Thermo Fisher Scientific) in 25 mL of RoosterBio complete medium RoosterNourish-MSC-XF and cultured for 48 hours and incubated at 37°C with 5% CO_2.

Activation of hUC-MSCs

At 36–38 hours after initial culture of hUC-MSCs, PrimeGenUS Inc. triple activation solution (Santa Ana, CA; proprietary triple activation solution by PrimegenUS

and supported by exclusive licensed technology patent family: US 8,685,728; US 9,301,979; US 10,046,011; US 10,898,523; US20210213067) consisting of human TNF-a, human interferon gamma, and IL-17 (all from PeproTech, Inc.) was added to each T225 flask with hUC-MSCs at final concentration of 2 ng/mL for each cytokine.[22] Each hUC-MSC flask as allowed to culture with added activation media for an additional 10–12 hours in at 37°C with 5% CO_2 incubator.

Cohort 1

Fifty humanized mice were fed modified high-fat Lieber-DeCarli liquid diet (Bioserv, NJ, USA) with alcohol (3.5% wt/vol) and high-fat or isocaloric dextrin for 4 weeks. To induce prolonged liver damage, mice were given ethanol binges by oral gavage at a dosage 4 g/kg ethanol as a 53% ethanol solution in water twice a week for 4 weeks along with the high-fat chow diet. Thirty-one died before treatment following attempts to obtain imaging with computerized tomography ± ultrasound elastography (data not shown). It was then determined to discontinue imaging and preserve the remaining 19 mice for the first set of experiments. The remaining 19 humanized mice at age 104 days were randomized to complete the cohort 1 studies, which included 10 males and 9 females. Mice were randomly assigned to placebo (phosphate buffered saline [PBS]) (n = 5) or 1 million nonactivated MSCs (n = 14) 3 times in the first week and 2 times each week for the next 2 weeks (8 times during 3 weeks) (Figure 1A). Of the 14 mice treated with MSCs, 8 received the MSC intravenously and intraperitoneally, and 6 received intraperitoneally only. There were 7 males in the treated group of 14, and 2 males in the placebo group of five. The male mice were evenly distributed to both the treated and placebo groups. The mice were then followed until death, and surviving mice were followed until 93 days following onset of treatment, at which time they were euthanized.

Cohort 2

For the second set of experiments, 33 mice were prepared in the same manner. However, given the high rate of death after 4 weeks of binge drinking, they underwent binge alcohol preparation for 3 weeks and were then divided into five groups:

Group 1: 5 mice received nonactivated MSCs intravenously and intraperitoneally (60% male)

Group 2: 7 mice received intravenous and intraperitoneal vehicle (PBS) (57% male)

**Group 3**: 7 mice received activated MSCs intraperitoneally (43% male)

Group 4: 7 mice received activated MSCs intravenously (43% male)

**Group 5**: 7 mice received activated MSCs intravenously and intraperitoneally (57% male)

Mice were administered either 1 million MSCs or PBS 3 times during the first week and once each week for an additional 2 weeks (Figure 2A). The mice were then followed until death, and surviving mice were followed until 25 days following onset of treatment, at which time they were killed.

Histologic analysis

After euthanasia, liver tissue was fixed with neutral buffered 10% formalin and processed for histological evaluation. The degree of steatosis, necrosis, as well as fibrosis was quantified by blinded specimen analysis by a single pathologist on representative hematoxylin and eosin (HE)–stained section examination. Steatosis was graded on a four-tier score (0–3) with 0 being <5%
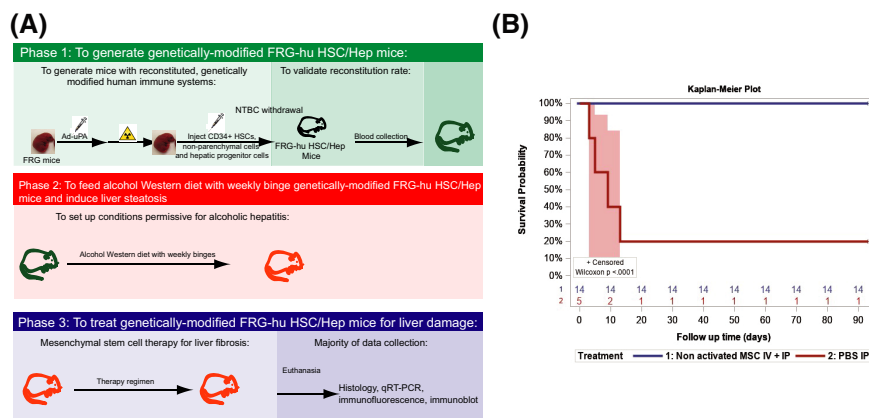
FIGURE 1 Mesenchymal stem cell (MSC) treatment rescued mortality of humanized *Fah−/−*, *Rag2−/−*, and *Il2rgc−/−* (FRG) mice with alcoholic hepatitis. (A) Schematic Illustration showing relevant time points for ASH1 cohort. (B) Mice treated with nonactivated MSCs had significantly better survival that mice treated with phosphate-buffered saline (PBS) (Wilcoxon *p* < 0.0001). HSC, hematopoietic stem cell; Hep, Hepatocytes; IP, intraperitoneally; IV, intravenously; qRT-PCR, quantitative real-time polymerase chain reaction

(A)

(B)
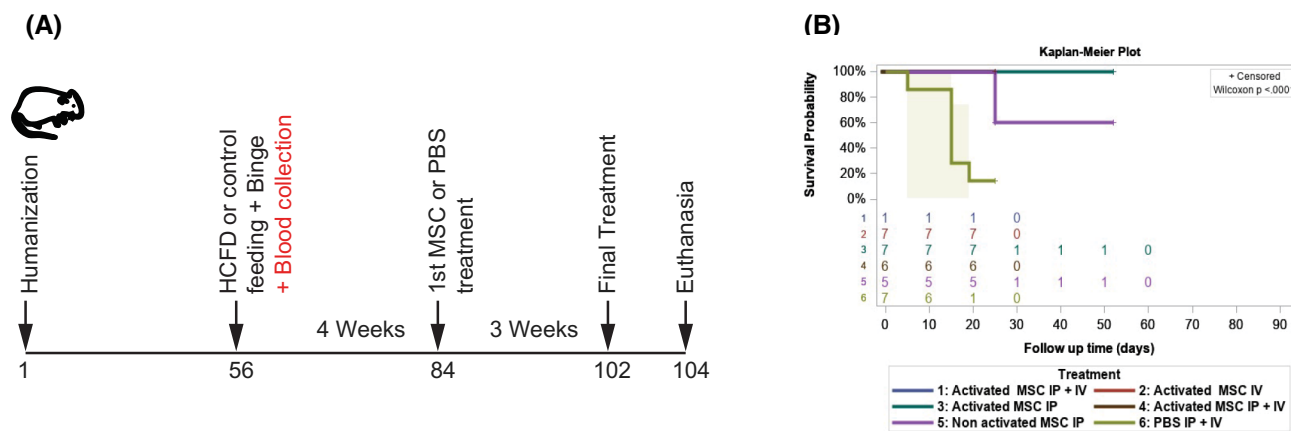
FIGURE 2 MSC treatment rescued mortality of humanized FRG mice with alcoholic hepatitis. (A) Schematic showing relevant time points for ASH2 cohort. (B) Mice treated with activated MSCs via any route had significantly better survival than mice treated with nonactivated MSCs or PBS alone (Wilxcoxon *p* < 0.0001). Group activated MSC IP + IV, activated MSC IV, activated MSC IP, and activated MSC IP + IV overlapped, and all had the same 100% survival line. HFCD, high-fat chow diet. *= *p* < 0.05.

steatosis, 1 being 5%–33%, 2 being 34%–66%, and 3 being >66%. Necrosis was also graded on a four-tier score (0–3) with 0 being <5% necrosis, 1 being 5%–10%, 2 being ≤20%, and 3 being >21%.

Statistical design

Demographics were reported as median and interquartile range for continuous variables and frequency and percentage for categorical variables. Wilcoxon sum rank test or Kruskal-Wallis test was used for continuous variables between group comparisons when appropriate. Wilcoxon signed-rank test was used for AST/ALT change between day 1 and last day. Chi-square or Fisher's exact test was used for categorical variables between group comparisons when appropriate. Kaplan-Meier curve with log-rank test or Wilcoxon test were used for survival data between groups, when appropriate. For *post hoc* comparison between the treatment group and PBS, Bonferroni adjustment was used for continuous and categorical data, and Sidak adjustment for survival data, to account for multiple comparisons. Significance level is set as 0.05, two-sided. All analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

MSC treatment prolonged survival in humanized FRG mice with alcoholic hepatitis (cohorts 1 and 2)

Table S1 provides the details of the mice pertaining to sex, treatment, survival, AST and ALT levels, and histology. However, in various treatment groups there was a nonsignificant improvement in survival for male mice compared with female mice (Figure S1). Furthermore, there was no

statistically significant difference of AST or ALT levels at baseline among the different treatment groups.

For cohort 1, four of the five control mice died on days 3, 5, 9, and 13 following randomization and first treatment. All 14 mice that were treated with nonactivated MSCs, regardless of route of administration, survived and were sacrificed 93 days after randomization.
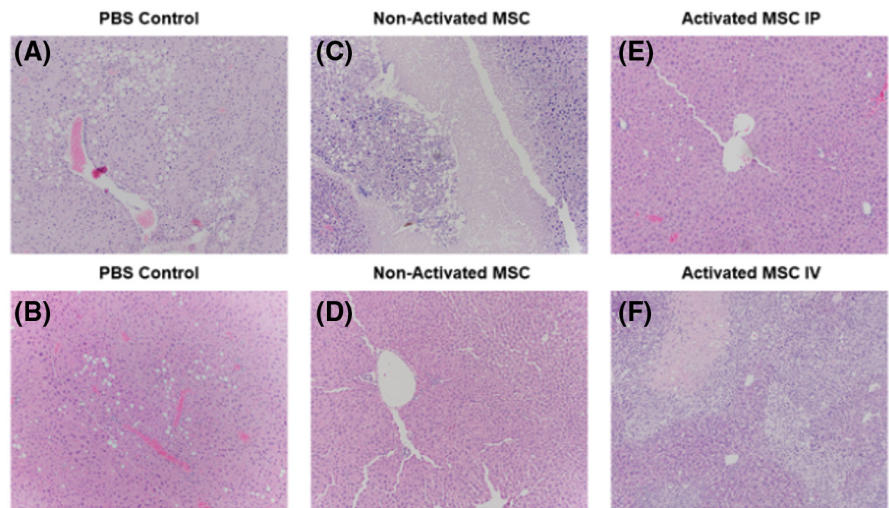
After 4 weeks, all 14 MSC-treated mice (100%) survived compared with only one of five (20%, SEM = 0.18) surviving in the PBS control group (*p* < 0.0001) (Figure 1B).

Pathology revealed a varying degree of steatosis, in which only six animals had between 5% and 10% necrosis. No fibrosis was found on HE-stained sections. All PBS control mice (n = 5) had some degree of steatosis, and three had some degree of lobular inflammation. Of the 14 mice treated with MSCs, 11 had no steatosis and 3 had no steatosis and only minimal inflammation. Of note, the nonactivated MSC–treated mice were killed over 2 months after the last injection. Consequently, this may have been too late to see the damage, as the surviving mouse would likely have histologic recovery at that point. This observation was considered in the next cohort.

Surviving mice from cohort 2 were killed 25 days after the first treatment. In the nonactivated MSC–treated group (n = 5), two died 4 days after randomization, and this group had a survival rate of 60%. For the PBS-treated mice (n = 7), six of the seven (86%) died on days 5–19 after randomization and first treatment, and only 14% survived. Overall, 100% of the 21 mice treated with activated MSCs survived.

For cohort 2, the activated MSC group had better survival than both the PBS and nonactivated MSC groups. This further corroborates the role of MSCs in survival in this animal model as well as indicates that activated MSCs may have better outcomes (*p* < 0.0001) (Figure 2B). The comparison of the various routes of

injection of activated MSCs indicated that intraperitoneal and/or intravenous injection can be used for the MSC treatment.

In the placebo group, three of seven mice showed no significant pathologic changes, three showed steatosis, and two showed 5%–10% necrosis. Of the seven mice that received activated MSCs (intraperitoneally and intravenously), five had +1 steatosis. No necrosis or significant inflammation was seen in any of the mice. Of the seven mice that had activated MSCs intraperitoneally, six had steatosis and one had no significant findings. No necrosis or significant inflammation was seen in any of these mice.

Of the even mice which had activated MSCs intravenously, five had steatosis, two had no significant findings, and one had necrosis. Of the five mice treated with nonactivated stem cells, two had steatosis, three had no significant findings, and one had necrosis. No fibrosis was found in any of the groups on HE stains. Histologic examination showed no significant difference among all of the groups. Except for two mice, all animals that died demonstrated some degree of steatosis and or necrosis (Figure 3A–F).

AST/ALT levels are decreased in FRG mice after MSC treatment

AST and ALT were examined at onset of treatment and at death, including those that were killed. All mice had elevated enzymes at the time of randomization, indicating liver damage. One hundred percent of PBS-treated mice, including the one surviving mouse, had elevated enzymes at death. All mice that received nonactivated or activated cells, including those that died (two with nonactivated cells), demonstrated a significant decrease in the enzymes at time of death. The most pronounced decreases were seen in the mice that received MSCs ($p < 0.0001$) (Figure 4A,B).

To determine the significance of the elevated AST and ALT, a control group of mice was fed isocaloric dextrin-maltose by oral gavage twice a week for 4 weeks without alcohol binging. These mice underwent blood sampling for AST and ALT at the same timepoint as the mice that underwent alcohol binging. ALT and AST levels ranged between 7 U/L and 16 U/L, compared with the elevated labs for the study mice.

Presence of MSC lineage marker vimentin validates human MSCs in liver

To examine the location of human MSCs, we stained PBS, nonactivated, and activated MSCs with a human-specific MSC lineage marker: Vimentin.[23] Immunohistochemistry revealed Vimentin expression in only activated MSC–treated mice (Figure 5A). To further analyze Vimentin expression, we isolated RNA from all three mouse groups and performed quantitative polymerase chain reaction (PCR). Quantitative PCR revealed a statistically significant increase of Vimentin expression in activated MSC–treated groups (n = 3) compared with PBS controls (n = 3) (Figure 5B). These results validate that MSCs found in the livers of the activated MSC group were in fact human.

KI67 and myeloperoxidase complementary DNA levels show the importance of activated MSCs

Ki-67, a liver regeneration marker, has been previously shown to be elevated in patients with alcohol liver.[24] Myeloperoxidase (MPO), a neutrophil marker, has also been shown to be elevated in alcohol-treated mice.[25] To examine the efficacy of MSCs, we isolated RNA from all three groups of treated mice and performed

(A)

(B)
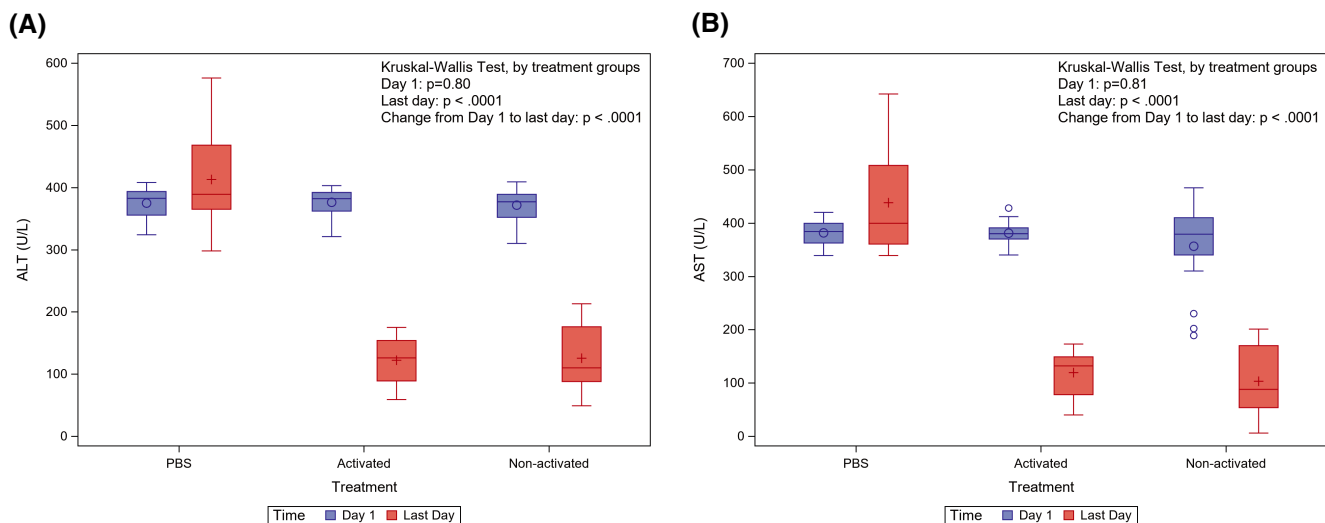
FIGURE 4 Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels from cohort 2. AST and ALT were drawn at onset of treatment and at death, including those that were killed. (A) *Post hoc* analysis with Bonferroni adjustment for last-day ALT: activated vs. PBS: <0.0001; nonactivated vs. PBS: <0.0001. (B) *Post hoc* analysis with Bonferroni adjustment for last-day AST: activated vs. PBS < 0.0001: nonactivated vs. PBS: <0.0001. *Post hoc* analysis with Bonferroni adjustment for change of AST: activated vs. PBS: <0.0001; nonactivated vs. PBS: <0.0001

quantitative PCR (Table S2). Ki-67 expression was significantly elevated in activated MSCs compared with PBS control (Figure 5C). Conversely, MPO levels were significantly decreased in MSC-treated mice (Figure 5D). Collectively, these data sets show the importance of activated MSCs in alleviating alcohol-induced liver injury in these mice.

Activated MSC-treated mice retained human serum albumin levels after treatment

To examine the quantity of functional hepatocytes in the liver after treatment, we isolated RNA from all three groups and measured human albumin levels relative to mice. Before alcohol liver injury, we showed human mitochondria DNA levels of our humanized FRG mice to be between 60% and 70% (Figure S2). Quantitative PCR analysis revealed a significantly higher human albumin level relative to mouse in activated MSC–treated mice (Figure 5E). These data indicate the importance of activated MSCs in alleviating liver injury in our humanized mouse model.

Receptor-interacting protein kinase 3 (RIPK3) immunofluorescence shows ability of MSCs to inhibit necroptosis pathway

Receptor-interacting protein kinase (RIPK3) has been previously shown to be an important molecule in

regulating necroptosis.[26] To determine whether our MSC-treated mice express RIPK3, we stained paraffin-embedded PBS, nonactivated, and activated MSC liver tissue. Confocal microscopy revealed elevated levels of RIPK3 in PBS-treated mice compared with MSC-treated groups (Figure 6A). The immunoreactive score of confocal images showed significantly lower RIPK3 levels in the activated MSC group compared with PBS control group (Figure 6B). To confirm the expression of RIPK3 at the protein level, we performed western blot analysis using protein lysates from the livers of activated and PBS treated groups. Our results revealed a decrease of RIPK3 levels in the activated MSC group compared with the PBS control (Figure 6C). Thus, our RIPK3 studies indicate that the activated MSCs inhibited necroptosis in this mouse group.

B cell lymphoma 2 (BCL2) is expressed in activated MSC–treated mice

B cell lymphoma 2 (BCL-2) has been well studied as an anti-apoptotic molecule that is involved in necroptosis and pyroptosis pathways.[27] To determine whether BCL-2 is expressed in our alcohol-binged FRG mice, we isolated protein lysates and performed a western blot analysis. Our results revealed BCL-2 expression in activated MSC–treated mice (Figure 6D). Although BCL-2 was slightly present in the nonactivated MSC–treated group, there was no expression in the PBS-treated mice. These results indicate the importance of activated MSC treatment in alleviating liver injury.
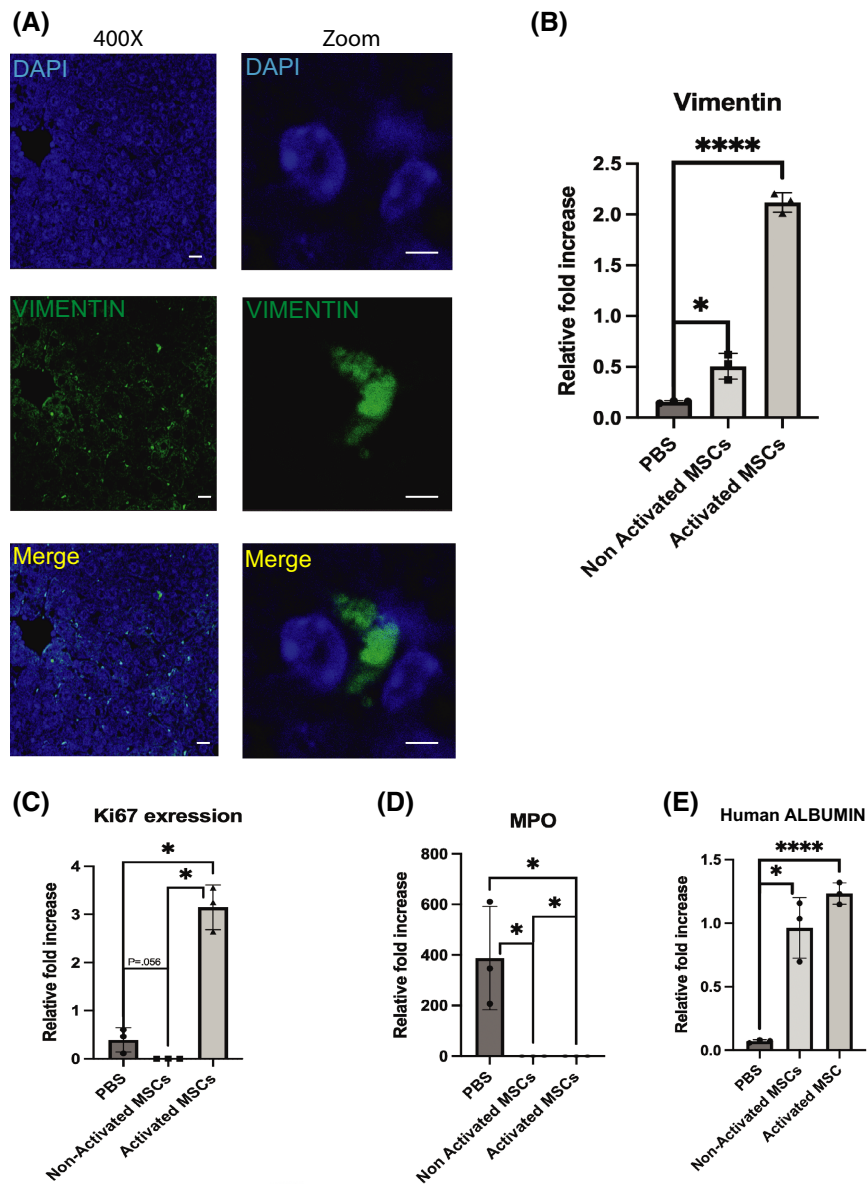
FIGURE 5 Vimentin validates the location of human MSCs in the liver. (A) Vimentin (human-specific) immunohistochemistry (IHC) shows minimal expression in only the activated MSC–treated group. Scale bar = 10 μm. (B) Quantitative PCR shows a significant 2-fold increase in Vimentin MSC marker compared with PBS control group. (C) Ki-67 marker was significantly elevated in activated MSCs compared with PBS and nonactivated MSCs. (D) Myeloperoxidase (MPO) expression marker was significantly decreased in both activated and nonactivated MSCs compared with PBS-treated group. (E) Activated MSCs expressed significantly lower human serum albumin levels compared with PBS control. DAPI, 4′,6-diamidino-2-phenylindole. *= $p < 0.05$, **: $P < 0.01$, ****: $p < 0.0001$.

BCL-2 promoter is induced after the addition of MSC conditioning media

We next performed luciferase reporter assays targeting signal transducer and activator of transcription 3 (STAT3) and cyclic adenosine monophosphate response element-binding protein (CREB1) in BCL-2 promoter, as BCL-2 has been shown to inhibit necroptosis and pyroptosis.[27] Specifically, Huh7 cells were transfected with various BCL-2 promoter constructs and stimulated with either Plasmalyte or MSC conditioning media. Our results showed that MSC conditioning media turned on BCL-2 expression in the BCL-2 construct with STAT3 and CREB1 deletions (Figure 6E). Interestingly, other BCL-2 promoter constructs showed minimal relative luciferase activity in both groups. This could be because of the AML-1 (acute myeloid leukemia 1) binding site (−1473 upstream from TSS), which has previously shown to be a repressor of BCL-2 expression.[28]
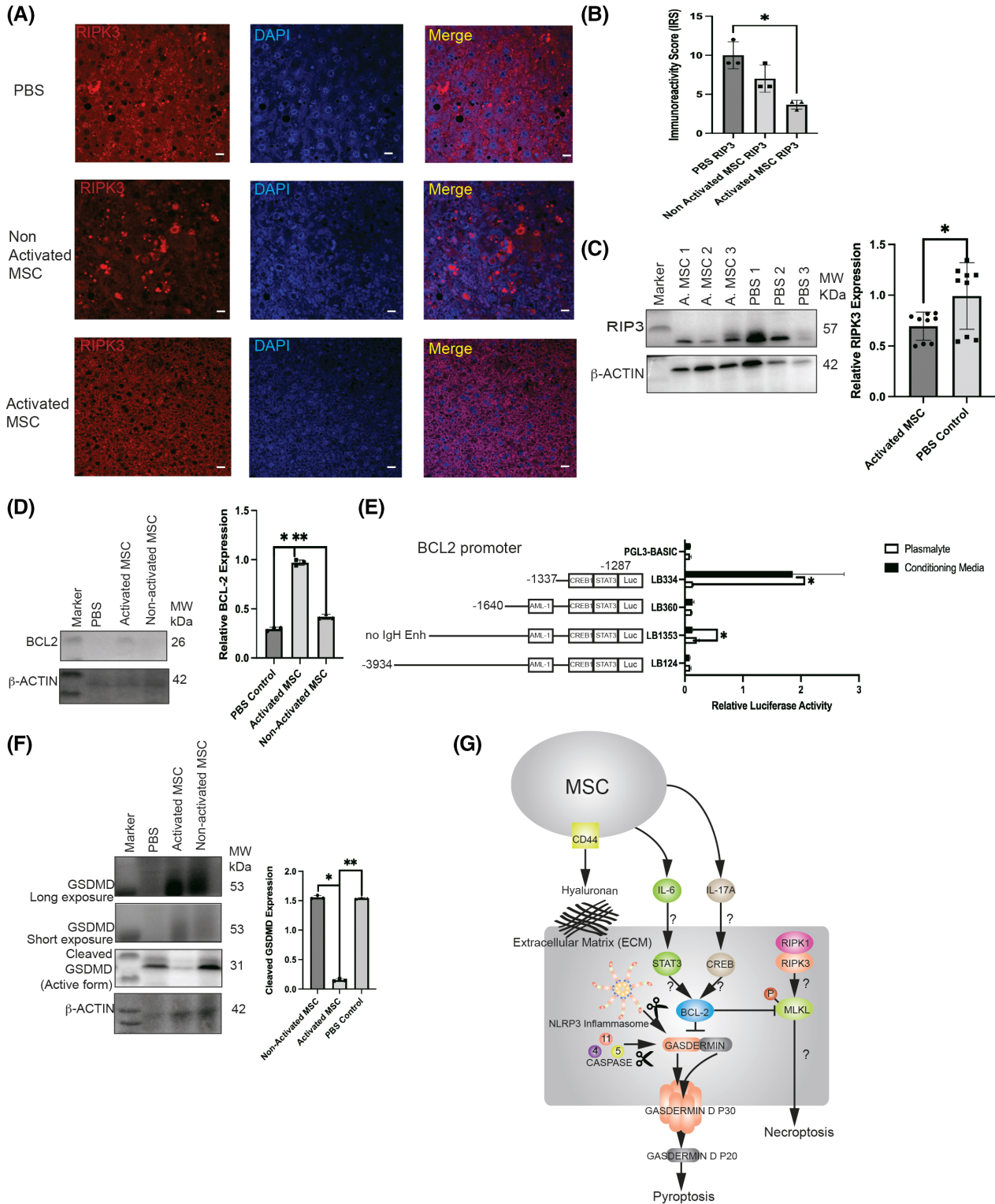
FIGURE 6 Receptor-interacting protein kinase (RIPK3) IHC provides insights about the necroptosis pathway. (A) Confocal fluorescent images showing DAPI (blue) and RIPK3 (red) expression in PBS, nonactivated MSCs, and activated MSCs. Scale bar = 10 μm. (B) Immunoreactive score (IRS) reveals significant decrease in RIPK3 expression in activated MSC tissue. Three representative paraffin-embedded liver tissues were stained for each group. (C) Western blot showing RIP3 expression in activated MSC–treated and PBS-treated mice groups. RIP3 expression is decreased in the activated MSC group compared with the PBS control group. (D) Western blot showing expression of B cell lymphoma 2 (BCL-2). Activated MSC–treated group shows highest expression of BCL-2 compared with PBS and nonactivated MSC groups. (E) BCL-2 promoter is regulated by signal transducer and activator of transcription 3 (Stat3) and cyclic adenosine monophosphate response element-binding protein (CREB1) binding sites. (F) Western blot showing a reduced cleaved Gasdermin D (GSDMD) expression in activated MSC–treated mice. Cleaved GSDMD was highly expressed in both nonactivated and PBS-treated mice. (G) Proposed hypothetical mechanism. *: $p < 0.05$, **: $P = < 0.01$, ***: $p < 0.001$.

Cleaved gasdermin D levels highlight the importance of activated MSCs in alleviating liver injury

We next examined Gasdermin D (GSDMD) levels in PBS-treated, nonactivated MSC–treated, and activated MSC–treated groups. GSDMD has been shown to be an important inflammatory response molecule.[29] Western blot showed a reduced expression of cleaved GSDMD in the activated MSC–treated group compared with both nonactivated MSC and PBS control groups (Figure 6F). This result shows he importance of activated MSCs in alleviating liver injury in our treated mice.

Transduction of sh-CD44 reduces MSCs' ability to travel to the liver

CD44 has been previously shown to be involved in cell trafficking by binding to its ligand hyaluronan.[30] To track the location of where MSCs go after alcohol-induced liver injury, we transduced activated MSCs with sh-CD44 lentivirus. Bioluminescence imaging revealed a higher number of cells present at the liver in sh-scrambled injected mice. In contrast, sh-CD44 injected mice had a lower amount of luciferase expression (Figure 7A,B). These images show that CD44 has an impact on MSC traveling to the liver after alcohol-induced liver injury.
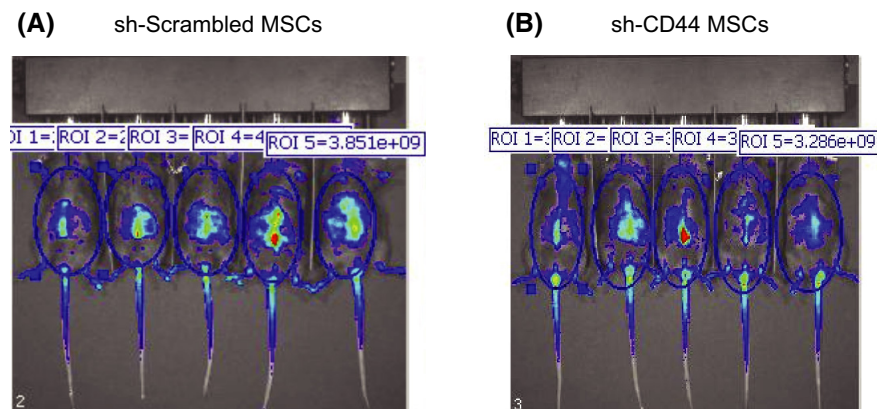
DISCUSSION

MSCs have the potential to differentiate into various types of cells, migrate to injured sites, and exhibit anti-inflammatory properties.[31-33] When tissue damage or injury occurs in the body, MSCs will migrate to the site of injury.[34,35] Once the MSCs reach this injury site, they interact with various inflammatory cells and different types of stromal cells to start the regeneration process and repair the damaged area.[36,37] Previous studies have shown that MSCs secrete different types of growth factors, cytokines, and adhesion molecules that affect the damaged tissue area and therefore maintain a positive paracrine effect on the tissue repair process.[38] Other studies have shown that MSCs can produce many different growth factors such as vascular endothelial growth factor, hepatocyte growth factor, epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor 1, and IL-6.[39-42] Most of these cytokine factors are up-regulated by the activation of NF-κB, from the exposure of pro-inflammatory stimuli such as TNF-α, IFN-γ, IL-1β, lipopolysaccharide, and hypoxia.[43]

Several studies propose that MSCs are not spontaneously immunosuppressive but that they require activation for the up-regulation of their immunomodulatory properties. The most important activating or priming factors of MSCs are IFN-γ, TNF-α, IL-17, and IL-1β.[44-46] After MSC activation from these three pro-inflammatory cytokines, these growth cytokine factors are up-regulated to promote tissue regeneration and repair by the recruitment or stimulation of tissue progenitor cells, fibroblasts, and endothelial cells in the damaged tissue area or by production anti-inflammatory cytokines.[47,48] These activated MSCs can function to inhibit the proliferation of T helper and cytotoxic T cells through various pathways. The initiation of the anti-inflammatory response is triggered by the activation of T helper type 2 cells and regulatory T cell differentiation.[49] IL-6 can inhibit the maturation of immature dendritic cells and inhibition of T-cell activation by the reduction in the expression of co-stimulatory molecules CD40, CD80 and CD86, by suppression of proinflammatory cytokines and up-regulation of anti-inflammatory cytokines like IL-10.[50]

In previous studies (Figure S3), using our proprietary method, our activated MSCs were able to highly express IL-6 *in vitro*. We propose that the increase

(A) sh-Scrambled MSCs   **(B)** sh-CD44 MSCs

ROI 1= ROI 2= ROI 3= ROI 4=4 ROI 5=3.851e+09   ROI 1= ROI 2= ROI 3= ROI 4=3 ROI 5=3.286e+09

FIGURE 7 Bioluminescence imaging shows a reduction of MSC after short hairpin CD44 (sh-CD44) transduction. (A) Images of mice after sh-scrambled activated MSC IP injection. (B) Images of mice after sh-CD44 transduced activated MSCs. Images show lower amount of Luciferase compared with sh-scrambled. ROI, region of interest

production of IL-6 in our activated MSCs could be responsible in modulating the inflammatory conditions in the acute alcoholic liver injury model, to increase survival, prevent apoptosis, and pyrolysis. In future studies, we would like to analyze all of the potential pro-inflammatory and anti-inflammatory cytokines in our treatment groups to better understand and propose potential anti-inflammatory, anti-apoptosis pathways, and mechanisms to explain why our activated MSCs increased survival in our model.

Acute alcoholic hepatitis differs from chronic liver disease in many aspects, most importantly in the potential for reversibility. Therefore, the humanized mouse liver injured with alcohol binging presented the ideal model to test bot nonactivated and activated umbilical cord cells for the potential of increasing survival and affecting the course of the liver injury. Our first alcoholic hepatitis cohort had two groups (PBS control and nonactivated MSC treatment). The non-activated MSC–treated mice all survived, while the PBS-treated control group had a 20% survival rate. Statistical significance showed $p < 0.0001$. In the second cohort, activated MSC–treated mice had 100% survival, nonactivated MSC–treated mice had 60% survival, and, like cohort 1, the PBS control group had 14% survival.

Analysis of hepatic chemistries before and after treatment with PBS or cells revealed a significant improvement in the animals receiving MSCs compared with those receiving PBS. Furthermore, those receiving activated cells demonstrated more marked improvement compared with nonactivated cells. In cohort 1, there were varying degrees of steatosis in the PBS-treated mice, whereas there were no significant findings in the 14 surviving mice treated with MSCs. We hypothesized that the lack of findings in the surviving mice is likely due to the prolonged time of observation, allowing healing of the liver and corroborated by the marked decrease in hepatic chemistries. In cohort 2, the animals were killed 2 days following the final treatment, and most of the mice showed varying degrees of steatosis as well as other signs of injury. In addition, as the liver pathology did not explain the differences in survival, we can hypothesize that the alcohol may have had a more systematic effect. Publicly available RNA-sequencing data sets have shown the importance of BCL-2 and CD44 in pyroptosis and necroptosis pathways.[51] Our findings with RIPK3, BCL-2, CD44, and GSDMD provide clues on whether activated MSCs alleviated liver injury inhibiting necroptosis and pyroptosis (Figure 6G). In the future we would like to perform chromatin immunoprecipitation/quantitative PCR and site-directed mutagenesis to see whether CREB1 and STAT3 do in fact turn on BCL-2 promoter.

The results in our two cohort experiments show promising results as a treatment to combat alcoholic hepatitis. Although our mice studies were limited in numbers, we look forward to a higher number of FRG mouse cohorts and eventually larger animal studies. It would be interesting to see how long-term high fat/cholesterol + alcohol binge feeding would fare with activated MSC treatment. In summary, activated MSC treatment is a strategic strategy to rescue the high mortality rate of patients with alcoholic hepatitis.

CONFLICT OF INTEREST
The study was fully funded by PrimeGenUS, INC. Joel Marh and Julia Kim are employed by PrimeGenUS INC, Linda Sher and Leonard Makowka have been consultants for PrimeGenUS, INC.

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REFERENCES
1. Philips CA, Augustine P, Yerol PK, Rajesh S, Mahadevan P. Severe alcoholic hepatitis: current perspectives. Hepat Med. 2019;8:97–108.
2. Thompson J, Jones N, Al-Khafaji A, Malik S, Reich D, Munoz S, et al.; VTI-208 Study Group. Extracorporeal cellular therapy (ELAD) in severe alcoholic hepatitis: a multinational, prospective, controlled, randomized trial. Liver Transpl. 2018;24:380–93.
3. Burra P, Bizzaro D, Forza G, Feltrin A, Volpe B, Ronzan A, et al. Severe acute alcoholic hepatitis: can we offer early liver transplantation? Minerva Gastroenterol Dietol. 2020;67:23–25.
4. Mellinger JL, Stine JG. Early liver transplantation for severe alcoholic hepatitis. Dig Dis Sci. 2020;65:1608–14.
5. Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Osaki M, et al. IFATS collection: in vivo therapeutic potential of human adipose tissue mesenchymal stem cells after transplantation into mice with liver injury. Stem Cells. 2008;26:2705–12.
6. Wang M, Zhang X, Xiong XI, Yang Z, Li P, Wang J, et al. Bone marrow mesenchymal stem cells reverse liver damage in a carbon tetrachloride-induced mouse model of chronic liver injury. In Vivo. 2016;30:187–93.

7. Sungkar T, Putra A, Lindarto D, Sembiring RJ. Intravenous umbilical cord-derived mesenchymal stem cells transplantation regulates hyaluronic acid and interleukin-10 secretion producing low-grade liver fibrosis in experimental rat. Med Arch. 2020;74:177–82.

8. El Baz H, Demerdash Z, Kamel M, Atta S, Salah F, Hassan S, et al. Transplant of hepatocytes, undifferentiated mesenchymal stem cells, and in vitro hepatocyte-differentiated mesenchymal stem cells in a chronic liver failure experimental model: a comparative study. Exp Clin Transplant. 2018;16:81–9.

9. El Baz H, Demerdash Z, Kamel M, Atta S, Salah F, Hassan S, et al. Potentials of differentiated human cord blood-derived unrestricted somatic stem cells in treatment of liver cirrhosis. Exp Clin Transplant. 2019;17:251–8.

10. Smets F, Dobbelaere D, McKiernan P, Dionisi-Vici C, Broué P, Jacquemin E, et al. Phase I/II trial of liver-derived mesenchymal stem cells in pediatric liver-based metabolic disorders: a prospective, open label, multicenter, partially randomized, safety study of one cycle of heterologous human adult liver-derived progenitor cells (HepaStem) in urea cycle disorders and Crigler-Najjar Syndrome patients. Transplantation. 2019;103:1903–15.

11. Hu C, Zhao L, Li L. Current understanding of adipose-derived mesenchymal stem cell-based therapies in liver diseases. Stem Cell Res Ther. 2019;10:199.

12. Zheng J, Lu T, Zhou C, Cai J, Zhang X, Liang J, et al. Extracellular vesicles derived from human umbilical cord mesenchymal stem cells protect liver ischemia/reperfusion injury by reducing CD154 expression on CD4+ T cells via CCT2. Adv Sci. 2020;7:1903746.

13. Lanthier N, Lin-Marq N, Rubbia-Brandt L, Clément S, Goossens N, Spahr L. Autologous bone marrow-derived cell transplantation in decompensated alcoholic liver disease: what is the impact on liver histology and gene expression patterns? Stem Cell Res Ther. 2017;8:88.

14. Xu WX, He HL, Pan SW, Chen YL, Zhang ML, Zhu S, et al. Combination treatments of plasma exchange and umbilical cord-derived mesenchymal stem cell transplantation for patients with hepatitis B virus-related acute-on-chronic liver failure: a clinical trial in China. Stem Cells Int. 2019;4:4130757.

15. Cao Y, Zhang B, Lin R, Wang Q, Wang J, Shen F. Mesenchymal stem cell transplantation for liver cell failure: a new direction and option. Gastroenterol Res Pract. 2018;4:9231710.

16. Wan YM, Li ZQ, Liu C, He YF, Wang MJ, Wu XN, et al. Mesenchymal stem cells reduce alcoholic hepatitis in mice via suppression of hepatic neutrophil and macrophage infiltration, and of oxidative stress. PLoS One. 2020;15:e0228889.

17. Wan YM, Li ZQ, Zhou Q, Liu C, Wang MJ, Wu HX, et al. Mesenchymal stem cells alleviate liver injury induced by chronic-binge ethanol feeding in mice via release of TSG6 and suppression of STAT3 activation. Stem Cell Res Ther. 2020;11:24.

18. Levine P, McDaniel K, Francis H, Kennedy L, Alpini G, Meng F. Molecular mechanisms of stem cell therapy in alcoholic liver disease. Dig Liver Dis. 2014;46:391–7.

19. Spahr L, Chalandon Y, Terraz S, Kindler V, Rubbia-Brandt L, Frossard JL, et al. Autologous bone marrow mononuclear cell transplantation in patients with decompensated alcoholic liver disease: a randomized controlled trial. PLoS One. 2013;8:e53719.

20. Tolosa L, Pareja E, Gómez-Lechón MJ. Clinical application of pluripotent stem cells: an alternative cell-based therapy for treating liver diseases? Transplantation. 2016;100:2548–57.

21. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315–7.

22. Han X, Yang Q, Lin L, Xu C, Zheng C, Chen X, et al. Interleukin-17 enhances immunosuppression by mesenchymal stem cells. Cell Death Differ. 2014;21:1758–68.

23. Yu H, Shin J, Yun H, Ryu CM, Lee S, Heo J, et al. A preclinical study of human embryonic stem cell-derived mesenchymal stem cells for treating detrusor underactivity by chronic bladder ischemia. Stem Cell Rev Rep. 2021;17:2139–52.

24. Lanthier N, Rubbia-Brandt L, Lin-Marq N, Clement S, Frossard JL, Goosens N, et al. Hepatic cell proliferation plays a pivotal role in the prognosis of alcohol hepatitis. J Hepatol. 2015;609–21.

25. Shen B, Feng H, Cheng J, Li Z, Jin M, Zhao L, et al. Geniposide alleviates non-alcohol fatty liver disease via regulating Nrf2/AMPK/mTOR signaling pathways. J Cell Mol Med. 2020;24:5097–108.

26. Roychowdhury S, McCullough R, Sanz-Garcia C, Saikia P, Alkhouri N, Matloob A, et al. Receptor interacting protein 3 protects mice from high-fat-diet induced liver injury. Hepatology. 2016 Nov;64:1518–33.

27. Shi C, Kehrl J. BCL-2 regulates pyroptosis and necroptosis by targeting BH3-like domains in GSDMD and MLKL. Cell Death Discovery. 2019;9:151.

28. Zhuang W, Cen J, Zhao Y, Chen Z. Epigenetic silencing of Bcl-2, CEBPA and p14(ARF) by the AML1-ETO oncoprotein contribute to growth arrest and differentiation block in the U037 cell line. Oncol Rep. 2013;30:185–92.

29. McDonald B, Kubes P. Interactions between CD44 and Hyaluronan in leukocyte trafficking. Front Immunol. 2015;6:68.

30. Wang C, Yuan W, Hu A, Lin J, Xia Z, Yang C, et al. Dexmedetomidine alleviated sepsis-induced myocardial ferroptosis and septic heart injury. Molecular Medicine Reports. 2020;22:175–84.

31. Bartolucci J, Verdugo FJ, González PL, Larrea RE, Abarzua E, Goset C, et al. Safety and efficacy of the intravenous infusion of umbilical cord mesenchymal stem cells in patients with heart failure: a phase 1/2 randomized controlled trial (RIMECARD Trial [Randomized Clinical Trial of Intravenous Infusion Umbilical Cord Mesenchymal Stem Cells on Cardiopathy]). Circ Res. 2017;121:1192–204.

32. Meng F, Xu R, Wang S, Xu Z, Zhang C, Li Y, et al. Human umbilical cord-derived mesenchymal stem cell therapy in patients with COVID-19: a phase 1 clinical trial. Signal Transduct Target Ther. 2020;5:172.

33. Couto PS, Shatirishvili G, Bersenev A, Verter F. First decade of clinical trials and published studies with mesenchymal stromal cells from umbilical cord tissue. Regen Med. 2019;14:309–19.

34. Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell. 2009;4:206–16.

35. Ullah M, Liu DD, Thakor AS. Mesenchymal stromal cell homing: mechanisms and strategies for improvement. iScience. 2019;31:421–38.

36. Guo Y, Yu Y, Hu S, Chen Y, Shen Z. The therapeutic potential of mesenchymal stem cells for cardiovascular diseases. Cell Death Dis. 2020;11:349.

37. Moreira A, Kahlenberg S, Hornsby P. Therapeutic potential of mesenchymal stem cells for diabetes. J Mol Endocrinol. 2017;59:R109–20.

38. Jiang W, Xu J. Immune modulation by mesenchymal stem cells. Cell Prolif. 2020;53:e12712.

39. Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, et al. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells. 2014;6:552–70.

40. Park CW, Kim KS, Bae S, Son HK, Myung PK, Hong HJ, et al. Cytokine secretion profiling of human mesenchymal stem cells by antibody array. Int J Stem Cells. 2009;2:59–68.

41. Shi Y, Su J, Roberts AI, Shou P, Rabson AB, Ren G. How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. 2012;33:136–43.

42. Deng Y, Zhang Y, Ye L, Zhang T, Cheng J, Chen G, et al. Umbilical cord-derived mesenchymal stem cells instruct monocytes towards an IL10-producing phenotype by secreting IL6 and HGF. Sci Rep. 2016;5:37566.

43. Saparov A, Ogay V, Nurgozhin T, Jumabay M, Chen WC. Preconditioning of human mesenchymal stem cells to enhance their regulation of the immune response. Stem Cells Int. 2016;2016:3924858.

44. Zachar L, Bačenková D, Rosocha J. Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment. J Inflamm Res. 2016;15:231–40.

45. Shi Y, Wang Y, Li Q, Liu K, Hou J, Shao C, et al. Immunoregulatory mechanisms of mesenchymal stem and stromal cells in inflammatory diseases. Nat Rev Nephrol. 2018;14:493–507.

46. Noronha NC, Mizukami A, Caliári-Oliveira C, Cominal JG, Rocha JLM, Covas DT, et al. Priming approaches to improve the efficacy of mesenchymal stromal cell-based therapies. Stem Cell Res Ther. 2019;10:131.

47. François M, Romieu-Mourez R, Li M, Galipeau J. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. Mol Ther. 2012;20:187–95.

48. Xie Q, Liu R, Jiang J, Peng J, Yang C, Zhang W, et al. What is the impact of human umbilical cord mesenchymal stem cell transplantation on clinical treatment? Stem Cell Res Ther. 2020;11:519.

49. Duffy MM, Ritter T, Ceredig R, Griffin MD. Mesenchymal stem cell effects on T-cell effector pathways. Stem Cell Res Ther. 2011;2:34.

50. Nasir GA, Mohsin S, Khan M, Shams S, Ali G, Khan SN, et al. Mesenchymal stem cells and Interleukin-6 attenuate liver fibrosis in mice. J Transl Med. 2013;11:78.

51. Affo S, Dominguez M, Lozano J, Sancho-Bru P, Rodrigo-Torres D, Morales-Ibanez O, et al. Transciptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis. Gut. 2013;62:452–60.

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