HHS Public Access

Author manuscript

Alcohol. Author manuscript; available in PMC 2020 November 01.

Published in final edited form as:

Alcohol. 2019 November ; 80: 139–148. doi:10.1016/j.alcohol.2018.09.001.

Mesenchymal Stem Cell Treatment Attenuates Liver and Lung Inflammation after Ethanol Intoxication and Burn Injury

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Abstract

Cutaneous burn injury is one of the most devastating injuries one can obtain with tissue damage extending beyond the skin wound to distal organs, including the gastrointestinal tract, liver, and lungs. Multiple organ failure is a leading cause of death after burn injury resulting in excessive systemic and localized inflammation directly contributing to end organ damage. We postulated that the gut-liver-lung inflammatory axis underscores multiple organ failure in the context of burn injury and is hyper-activated when ethanol intoxication precedes burn. Mesenchymal stem cells (MSCs) are regenerative and anti-inflammatory and MSC treatment has been shown to be beneficial in several immune disorders and injury models. Our objective was to determine whether intravenous infusion of exogenous bone marrow-derived MSCs could reduce post-burn and intoxication pulmonary, hepatic, and systemic inflammation. Vehicle or ethanol (1.6 g/kg) treated mice were subjected to sham or 15% total body surface area scald burn. One hour post-injury, mice were given 5×10^5 CFSE-labeled MSCs or phosphate buffered saline intravenously (i.v.) and euthanized 24h later. We assessed circulating biomarkers of inflammation and liver damage, measured cytokine and chemokine production and quantified apoptosis in lung and liver tissue. Compared to intoxicated and burned mice, those treated with MSCs had less cellularity, limited apoptosis, and a slight reduction in the pro-inflammatory cytokine interleukin-6 (IL-6) and the neutrophil chemokine, KC (CXCL1) in lung tissue. MSCs treatment had more dramatic anti-inflammatory effects on systemic and hepatic inflammation, as serum IL-6 levels were diminished by 43%, *il6* and *kc* expression in liver tissue were markedly reduced, as were biomarkers of liver damage, aspartate transaminase (AST) and alanine transaminase (AST), compared with

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The authors declare no conflicts of interest.

intoxicated and burned mice. Taken together, our results suggest intravenous MSCs treatment can diminish systemic inflammation, lessen hepatic damage, and decrease liver and lung apoptosis and inflammation, indicating MSCs as a novel therapy for restoring homeostasis of multiple organ systems in intoxicated burn patients.

Keywords

alcohol; burn injury; lung; liver; apoptosis; mesenchymal stem cells

Introduction

In the United States, people are typically binge drinkers rather than chronic alcoholics [1]. Binge drinking, defined by the number of drinks consumed within a 2 hour window (4 for women, 5 for men) or a blood alcohol concentration of 0.08%, is a common drinking pattern among trauma patients, including those with burn injuries [2, 3]. In fact, nearly half all burn patients are intoxicated at the time of injury [4, 5]. Intoxication at the time of burn injury results in adverse effects in multiple major organ systems, including intestinal, hepatic and pulmonary damage [6-8]. As a result, respiratory failure is one of the leading causes of death after burn [9]. This combined insult results in longer hospital stays and greater fluid resuscitation and mechanical ventilation requirements, leading to increased risk for pulmonary complications, infections, and mortality [4, 10, 11]. Using a murine model of ethanol intoxication followed by a moderate size burn injury, our laboratory and others demonstrated that ethanol intoxication exacerbates inflammatory responses in multiple organ systems, including the lungs, liver, and gut after burn, similar to clinical observations.

Our laboratory has proposed a working model in which the gut-liver-lung axis is a major contributing factor underlying multi-organ failure after burn injury [12]. We and others have shown that burn injury reduces intestinal epithelial cell barrier integrity which is exacerbated when ethanol intoxication precedes injury [13-15]. After the combined insult, we found that bacteria and bacterial products, including lipopolysaccharide (LPS), are released and translocated to distal sites, including mesenteric lymph nodes [14]. The degree of intestinal damage is directly proportional to bacterial overgrowth in the intestine after injury [15]. LPS and other bacterial products trigger the hepatic acute phase and inflammatory response, contributing to the high levels of circulating interleukin-6 (IL-6) observed after burn in both preclinical models and burn patients [12, 16, 17] [18-20]. Elevated plasma levels of IL-6 in burn patients have been correlated with increased morbidity and mortality [21] and with sepsis severity [22]. These excessive circulating levels of IL-6 then contribute to pulmonary inflammation, as IL-6 knockout mice and mice treated with anti-IL-6 antibody had reduced pulmonary inflammation and leukocyte infiltration after intoxication and burn [23]. After the combined insult, the lungs display characteristics of acute respiratory distress syndrome, including alveolar wall thickening, neutrophil accumulation and heightened IL-6 levels, relative to either intoxication or burn alone [12, 23-27]. The amplification of pulmonary inflammation parallels impaired respiratory parameters and reduced survival [28]. Additionally, abnormal breathing patterns are correlated with increased neutrophil quantity and impaired lung function, and are exacerbated by intoxication [27, 28]. Taken together,

these studies suggest that managing the hyperactivation of the gut-liver-lung inflammatory axis after remote injury will likely limit morbidity and mortality in intoxicated burn patients.

Bone marrow and tissue resident mesenchymal stem cells (MSCs) have the ability to suppress immune cell responses and have been beneficial in clinical trials for treatment of various inflammatory disorders and injury models, including Crohn's disease [29], graft-versus-host disease [30, 31], ischemia [32], pulmonary fibrosis [33, 34], liver fibrosis/cirrhosis [35, 36], sepsis (reviewed in [37]), and ARDS [38] and in preclinical animal models, including a porcine model of acute lung injury [39], an ovine model of bacterial pneumonia [40], and in rat models of LPS endotoxemia and burn injury [41]. MSCs modulation of acute inflammation is partially due to their ability to influence macrophage phenotype [42]. MSC-macrophage interaction and the release of paracrine soluble factors by MSCs reduce lung inflammation and polarize macrophages into an anti-inflammatory phenotype [42-46]. Resident alveolar macrophages modulate pulmonary inflammation during both the onset and resolution stages of the inflammatory response [47-49]. Hence, factors that elicit an anti-inflammatory alveolar macrophage phenotype may therefore promote pulmonary homeostatic restoration after injury. The ability of MSCs to diminish lung inflammation through direct and indirect mechanisms suggests MSCs would be particularly advantageous in attenuating the excessive pulmonary inflammation observed after intoxication and injury.

The purpose of this study was to determine whether the intravenous infusion of exogenous bone marrow-derived MSCs could restore homeostasis of the liver-lung inflammatory axis by diminishing tissue damage and restoring hepatic and pulmonary homeostasis. Since intoxication at the time of injury results in greater pulmonary complications and mortality rates than burn injury alone [28], we chose to examine the effect of MSC treatment in intoxicated, burn-injured mice. *In vitro*-expanded MSCs were given intravenously 1 hour after injury. We assessed circulating biomarkers of hepatic damage and inflammation, measured cytokine/chemokine production, and quantified apoptosis in lung and liver tissue. Our results suggest intravenous MSCs treatment can attenuate systemic inflammation, reduce hepatic damage, and diminish liver and lung apoptosis and inflammation, establishing MSCs as a novel therapeutic agent for restoring the liver-lung axis to homeostasis in intoxicated burn patients.

Materials and Methods

Mice

Male (C57BL/6) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used at 8-10 weeks old. Mice were housed in sterile micro-isolator cages under specific pathogen-free conditions in the Loyola University Medical Center Comparative Medicine facility for a minimum of 1 week prior to experimentation. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee. Mice weighing between 22 to 27 g were used in these studies.

Mesenchymal stem cell culture

Gibco® Mouse (C57BL/6) bone marrow-derived MSCs (Life Technologies, Grand Island, NY) were expanded *in vitro* per manufacturer protocol. Briefly, MSC growth medium consisted of 10% FBS and 10mg/ml gentamicin in MEMα Medium with GlutaMAX™-I, ribonucleosides and deoxyribonucleosides. MSCs were plated at a density of 5,000 cells per cm^2 in T75 flasks and incubated at 37°C, 5% CO_2 and 90% humidity. Medium was changed every 2 days until cultures were 70-80% confluent. MSCs were detached from flasks using pre-warmed trypsin, fluorescently labeled with carboxyfluoresceindiacetate, succinimidyl ester (CFSE) using the manufacturer protocol (Life Technologies), and resuspended in sterile Dulbecco's phosphate buffered saline (PBS) [50]. Passage 4 cells were used in all experiments.

Murine model of binge ethanol and burn injury

A murine model of single dose binge ethanol intoxication and burn injury was employed using oral gavage, as described previously [13, 51, 52]. Animals were given 400 μl of 10% (v/v) ethanol solution (1.6 g/kg) or water control by gavage at a dose designed to elevate the blood alcohol concentration to 150 mg/dL at 30 min after ethanol exposure [53]. Thirty minutes following the ethanol exposure, the mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) and their dorsum shaved. The mice were placed in a plastic template exposing 15% of the total body surface area and subjected to a scald injury in a 92-95°C water bath or a sham injury in room-temperature water [51]. The scald injury results in an insensate, full-thickness burn [54]. The mice were then resuscitated with 1.0 ml saline and allowed to recover on warming pads. All experiments were performed between 8 and 9 am to avoid confounding factors related to circadian rhythms. One hour after injury, intoxicated and burn-injured animals either received an intravenous tail vein injection of 5×10^5 CFSE-labeled MSCs or PBS as a control in 200ul [55]. Animals were euthanized at 24 h. MSC treatment did not alter mouse weight (data not shown).

Flow cytometry analysis of $CFSE^+$ mesenchymal stem cells

The upper left lung lobe was removed and cut into small pieces with a razor blade. The lung tissue was then transferred to a C-tube (Miltenyi Biotec, Auburn, CA) and processed using digestion buffer containing 1mg/ml of Collagenase D and 0.1 mg/ml DNase I (Roche, Indianapolis, IN) in HBSS and a GentleMACS dissociator (Miltenyi Biotec), according to manufacturer's instructions [28]. The homogenates were then filtered through 70 um nylon cell strainers to obtain a single cell suspension. Red blood cells were lysed using ACK lysis buffer (Life Technologies). Cells were counted using trypan blue to exclude dead cells. To assess mesenchymal stem cells, 1×10^6 lung cells were first incubated with anti-CD16/32 (clone 93, eBioscience, San Diego, CA) to block unspecific binding to the Fcy II/III receptor. Cells were then incubated with rat anti-mouse F4/80 APC (clone BM8, eBioscience), CD11b eFluor 450 (clone M1/70, eBioscience), CD11c APC-eFluor 780 (clone N418, eBioscience), and Siglec-F PE-CF594 (clone E50-2440, BD Biosciences, San Jose, CA). Antibody incubation was carried out for 30 minutes at 4°C. Cells were washed and fixed as described [56, 57]. Samples were run on a BD Fortessa cytometer (BD

Biosciences). Data analysis was performed using Flow Jo FCS analysis software (Tree Star Inc., Ashland, OR).

Histopathologic examination of the lungs

The upper right lobe of the lung was inflated with 10% formalin and fixed overnight, as described previously [26], embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Sections were evaluated using light microscopy (Zeiss AxioVert, Zeiss, Thorndale, CA) and histology photographs were taken at 400x magnification.

Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) staining

Lung and liver tissue were fixed in 10% formalin overnight, embedded in paraffin, and sectioned, as described [26]. Click-it® Plus *in situ* terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) Alexa Fluor 488 assay was performed, according to manufacturer guidelines (Life Technologies). Lung and liver sections were evaluated using fluorescent microscopy. $TUNEL_+$ cells were counted in a blinded fashion in 10 individual high power fields. Two independent experiments were performed, n=7-12 per group, total. Data are presented as the average number of TUNEL+ cells per 200x field ± SEM.

Cytokine and chemokine analysis of lung homogenates and serum

The right middle lung lobe was snap-frozen in liquid nitrogen and then homogenized in 1 ml of BioPlex cell lysis buffer according to manufacturer's protocol (BioRad, Hercules, CA). The homogenates were filtered and analyzed for cytokine (IL-6) and chemokine (KC) production using an enzyme-linked immunosorbent assay (ELISA). The results were normalized to total protein using the BioRad protein assay (BioRad) [23, 25]. Serum aliquots were used to measure IL-6 by ELISA (BD Biosciences). Results from three independent experiments were pooled and are presented as mean cytokine level per milligram of protein (pg/mg total protein) for homogenates or pg/milliliter for serum. n= 10-14 per group

Quantitative RT-PCR of liver gene expression

RNA was extracted from liver tissue using the RNeasy Mini Kit (Qiagen, Germantown, MD) and converted to cDNA using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA), following manufacturer guidelines. Quantitative RT-PCR was performed using TaqMan Gene Expression Assays (ThermoFisher Scientific, Waltham, MA). Results were analyzed using the ΔΔCt algorithm[14] with *GAPDH* as the endogenous control. Data are presented as mean fold change ± SEM relative to sham-injured, vehicle treated controls. n = 2-6 per group. Representative data from two independent experiments are shown.

Serum aminotransferase measurements

Blood was collected via cardiac puncture and the serum was isolated and stored at −80°C. Serum aliquots were used to measure liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using a DRI-CHEM 7000 (HESKA, Loveland, CO).

Representative data from three independent experiments are shown. n=3-4 per group, per experiment. Data are presented as mean unit/liter (U/L) ± SEM.

Statistical analysis

Statistical comparisons were made between the sham vehicle, burn ethanol + PBS, and burn ethanol + MSC treatment groups. One-way analysis of variance (ANOVA) was used with Tukey's post-hoc test and values were considered statistically significant when $p < 0.05$. Data is reported as mean values ± the standard error of the mean (SEM). Data is representative of two independent experiments, unless otherwise stated. N = 3-6 animals per group in each experiment.

Results

Mesenchymal stem cells are present the lungs 24 hours after administration

MSCs were labeled with CFSE and administered to mice an hour after burn injury. At 24 hours, $CFSE^+$ MSCs were identified in the lungs of intoxicated and burn injured mice using flow cytometry. Lung cells were negatively selected for alveolar macrophage/eosinophil marker Siglec-F and granulocyte/neutrophil marker CD11b (data not shown), and analyzed for $CFSE^+$ cells compared to macrophage/monocyte marker F4/80 (Fig. 1). Additionally, $CFSE^+$ cells were negative for dendritic cell marker CD11c (data not shown). Our results indicated there were approximately 100 $CFSE^+$ MSCs recovered per 3.5×10^5 total lung cells. Lack of Siglec-F, CD11b, F4/80 and CD11c expression supports the identification of individual $CFSE^+$ cells and not CFSE+ cells engulfed by macrophage/monocytes, granulocytes, or dendritic cells. These data confirm that MSCs are in a position to attenuate pulmonary inflammation by their ability to localize in the lungs and remain there at least 24 hours after infusion.

Mesenchymal stem cell treatment improves lung histopathology after burn and intoxication

We previously demonstrated that ethanol intoxication prior to burn leads to increased neutrophil infiltration and cellular edema [23-26, 28]. Here, we performed similar histochemical analyses of sectioned lung tissue and observed that ethanol intoxication prior to burn injury results in increased cellularity and pulmonary congestion in the distal airway at 24 hours, which was reduced by MSC administration (Fig. 2). No changes in liver histology were observed at this time point (data not shown).

Mesenchymal stem cell treatment decreases pulmonary cytokine and chemokine levels

Since we observed lower lung cellularity with MSC treatment, we next examined levels of the pulmonary neutrophil chemoattractant, CXCL1, and the pro-inflammatory cytokine, IL-6. Consistent with previous studies [24-26, 28], there was an 83% rise in CXCL1 in lung tissue of intoxicated mice subjected to burn injury, in comparison to control animals (Fig. 3A) ($p<0.05$). MSC treatment marginally limited CXCL1 levels observed in intoxicated, burned mice by 11% (Fig. 3A). We observed a similar pattern with IL-6, as levels were increased by 78% after intoxication and burn, and slightly reduced by 13% after MSC treatment, though not statistically significant (Fig. 3B). These data support our observation

that MSC treatment reduces leukocyte infiltration into lung tissue, though only modestly reducing pulmonary inflammation.

Mesenchymal stem cell treatment lowers post-burn and intoxication serum levels of IL-6 and aminotransferases

Dramatic increases in circulating IL-6 levels have been detected with intoxication and injury, relative to either insult alone [18] and a high serum level of IL-6 in burn injured patients has been correlated with morbidity and mortality [21]. Here, we also found that intoxication and burn injury elevated serum levels of this cytokine from 81.1 ± 57.9 pg/ml in control mice to 709.9 ±123.7 pg/ml in intoxicated burned mice ($p<0.05$ compared to sham vehicle), and that treating intoxicated, burned animals with MSCs reduced serum IL-6 by 43% to 404.1 ± 108.0 (Table 1). In addition, we also measured serum biomarkers of hepatic damage, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), since the liver is a primary source of circulating IL-6 after intoxication and injury [58]. Consistent with previous reports [12, 13, 17], intoxication and burn injury elevated serum ALT to 217.0 ± 38.2 U/L ($p < 0.05$) and serum AST to 1035.8 ± 209.0 U/L ($p < 0.05$), relative to control animal AST (24.3 ± 2.9 U/L) and ALT (145.3 ± 88.2 U/L) levels. Though not statistically significant compared to intoxication and injury, MSC treatment after intoxication and injury trended towards a moderate reduction in ALT levels by 20% to 174.0 ± 50.3 U/L and AST levels by 21% to 815.5 ± 229.2 U/L, to levels not statistically different from controls (Table 1).

Mesenchymal stem cell treatment attenuates hepatic il6 and cxcl1 expression

Since biomarkers of liver damage were moderately reduced by MSC treatment after burn injury and ethanol intoxication, we next measured gene expression levels of the pro-inflammatory immunomodulators, *cxcl1* and *il6,* using quantitative RTPCR. Compared to sham-injured, vehicle-treated controls, we observed a dramatic 29-fold increase in liver *cxcl1* expression after burn and intoxication ($p<0.05$ compared to sham vehicle). However, when intoxicated and injured mice were treated with MSCs, *cxcl1* did not increase as dramatically, as expression was 17-fold above controls ($p<0.05$ compared to sham vehicle; $p<0.05$ compared to burned, intoxicated mice) (Fig. 4A). Similarly, there was a 4.7-fold increase in *il6* expression when intoxication preceded burn ($p<0.05$ compared to control), which was significantly reduced to 2.1-fold above control levels ($p<0.05$ compared to burn, ethanol treated mice) (Fig. 4B).

Mesenchymal stem cell treatment diminishes liver and lung apoptosis

Cutaneous burn injury causes distal organ damage often leading to cell death. We previously reported alveolar macrophage apoptosis in our mouse model of 15% TBSA burn injury and ethanol intoxication [27] and others reported pulmonary microvascular endothelial cell apoptosis in a 30% TBSA rat burn model [59]. In addition, mitochondrial dysfunction, endoplasmic reticulum stress, and hepatocyte apoptosis have been observed in a rat 60%TBSA burn model [60]. Therefore, we next examined cellular apoptosis in liver and lung tissue sections to determine if MSC treatment could limit post-burn and intoxication-driven programmed cell death. Using TUNEL immunofluorescence staining, we quantified the number of apoptotic cells in lung tissue. As previously reported by our laboratory [27]

intoxication prior to burn elevates the number of TUNEL+ cells. In this study, animals given ethanol prior to burn had 11.5-fold more apoptotic cells (4.1 ± 1.1 TUNEL+ cells per 200x field) compared to vehicle-treated, sham-injured mice (0.3 ± 0.1 TUNEL+ cells per 200x field) ($p<0.05$). MSC treatment after intoxication and burn lowers the number of apoptotic cells to 2.9-fold above sham (1.3 ± 0.5 TUNEL+ cells per 200x field), which was not statistically different from other groups. (Fig. 5 A-D) To determine if stem cell treatment decreased liver apoptosis in our mouse ethanol intoxication and burn injury model, we performed TUNEL immunofluorescence staining in liver tissue sections. Intoxication prior to burn injury yielded in a 3.7-fold increase in the number of apoptotic liver cells (4.8 ± 0.6 TUNEL+ cells per 200x field), compared to controls (1.0 ± 0.2 TUNEL+ cells per 200x field) ($p<0.05$). MSC treatment after intoxication and burn reduced the number of TUNEL+ cells to 2.8 ± 0.6 per 200x field, which was statistically lower than the number of TUNEL+ cells observed in intoxicated, injured mice without MSC treatment ($p<0.05$) but not different when compared to controls. (Fig. 6 A-D) Together, these results demonstrate MSC treatment diminishes liver and lung apoptosis when ethanol intoxication precedes burn.

Discussion

The data presented herein demonstrate that intravenous administration of exogenous MSCs can moderate systemic, lung, and liver inflammation in the context of burn injury with antecedent ethanol intoxication. We observed reduced cellularity, pro-inflammatory cytokine and chemokine production, and apoptosis in the lungs of MSC-treated animals. In addition, circulating biomarkers of liver damage (AST and ALT) were diminished after treatment, which corresponded with lower hepatic apoptosis and inflammatory gene transcription, including the neutrophil chemokine, KC (CXCL1) and IL-6, a key pro-inflammatory cytokine driving multiple organ damage after intoxication and injury.

MSCs anti-inflammatory properties are derived from several mechanisms. Mathias et al. demonstrated that short-term localization of exogenous human MSCs (hMSCs) within the lungs was sufficient to inhibit allergic inflammation. Through clodronate liposome depletion of alveolar macrophages it was established that the anti-inflammatory effect of hMSCs was dependent on the indirect role of the alveolar macrophage in interleukin-10 (IL-10) production [61]. hMSCs can also attenuate allergic inflammation and improve lung function through transforming growth factor β1 (TGFβ1) signaling and promoting an anti-inflammatory alveolar macrophage phenotype [62]. Due to their large size, several studies suggest MSCs become trapped within the pulmonary vasculature with only a fraction of these cells passing through and localizing in other organs [55, 63, 64]. The resulting reduction in capillary flow allows MSCs to secrete anti-inflammatory mediators, including tumor necrosis factor-α induced protein 6, into the lung niche, as well as into the bloodstream, to reduce local inflammation and at the primary site of injury [65]. MSCs secretion of anti-inflammatory mediators, such as IL-10 and TGFβ1, can also down-regulate IL-6 production in alveolar macrophages [62]. While we did not see increased lung IL-10 after MSC treatment in intoxicated, burn-injured mice, we did see a reduction in localized and systemic pro-inflammatory cytokines. It is possible that the secretion of anti-inflammatory mediators by MSCs into the circulation diminished inflammation at the burn site, which in turn, lowers IL-6 release from the wound bed, thereby decreasing systemic

IL-6 levels and inflammation. Additionally, MSCs entrapment within the lung capillary bed may induce an anti-inflammatory alveolar macrophage phenotype and help to restore lung homeostasis.

MSCs also elicit anti-inflammatory effects through activation of multiple signaling cascades, inhibition of apoptosis, and enhancement of cellular proliferation. Bone marrow derived MSCs restored renal, hepatic, and pulmonary parameters in a LPS-induced rat sepsis model by attenuation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator or transcription-3 (Stat3) and p38-mitogen-activated protein kinases (MAPK) signaling pathways [66]. These signaling pathways have all been implicated as important regulators of inflammatory responses in our mouse model of burn and intoxication. We have previously demonstrated that blockade of p38-MAPK signaling via pharmacologic inhibition (SB203580) reduced hepatic damage, pulmonary inflammation, and alveolar wall thickening after ethanol and burn [17] and that attenuation of IL-6 signaling, through anti-IL-6 antibody and in IL-6 knockout mice, limited pulmonary STAT3 phosphorylation after the combined insult [23]. In addition, we showed that Toll-like receptor (TLR)-4 activation, an upstream activator of NF-κB nuclear translocation, is critical for pulmonary inflammation after intoxication and burn injury [25]. MSCs have also been shown to reduce Kupffer cell apoptosis in a mouse transplant model [67] and lung apoptosis in a LPS-induced rat acute lung injury model. [68], and decrease acute-liver injury related apoptosis in a D-galactosamine and LPS-injury rat model [69]. MSCs directly promote tissue repair in animal models of acute liver failure by inducing renal-tubule epithelial cell proliferation [70]. In this study, we observed a reduction in both liver and lung apoptosis after MSC treatment in mice given ethanol prior to burn.

Several studies have used experimental MSC therapy in burn injury, though most utilized either topical application or injection of MSCs near the site of injury [41, 71-73]. Xue et al. demonstrated the plasticity of MSCs to differentiate into tissue-specific cells and to promote accelerated wound healing, while others have shown an increase in neoangiogenesis and a decrease in cellular infiltration at the wound site [73, 74]. Yagi et al. investigated the therapeutic effectiveness of intramuscular MSC transplantation in rats given a 30% TBSA burn injury, and observed reduced inflammatory cell infiltration, cell death, and inflammatory cytokine levels in multiple organs, including kidney, liver, and lungs, 48 hours after injury [41]. Recently, Oh et al. performed an *in vivo* mouse experiment where they tracked fluorescently labeled MSCs injected intravenously to the burn injury site using bioluminescence imaging. MSCs appeared localized in the lungs at 24 hours and in the burn lesion after 4 days. A similar bioluminescent MSC trafficking approach showed that MSCs are able to localize to bone fracture injury sites and promote healing but that a large percentage of MSCs are present in the lungs 1 day after transfer and localize to the bone fracture site by 3 to 14 days after administration [75-77]. Of note, intoxication at the time of bone fracture did not inhibit the localization of MSCs to the site of injury [78], suggesting intoxication does not directly affect exogenous MSCs migration through the vascular system. In the current study, we observed MSCs in the lungs of intoxicated burned mice 24 hours after injection, but histological examination of the liver and skin burn margin did not reveal $CFSE^+$ cells in either of these organs (data not shown).

Burn injury increases capillary permeability and causes tissue edema, underscoring the need for fluid resuscitation [79]. Intoxicated burn patients require more fluids compared to those who did not consume alcohol [4]. Our laboratory reported that intoxication heightens post-burn dehydration, even after fluid resuscitation, while also causing a shift in fluid compartments, resulting in greater ischemic end-organ damage [80]. The increase in capillary permeability and loss of fluid from the vascular space may highly influence the fate of infused MSCs. The large size of MSCs may not only trap them within the lung capillary bed, but also retain them within constrained vasculature of intoxicated and burn-injured mice. It is also possible the migration aptitude of the Gibco® MSCs differs from freshly isolated primary bone marrow-derived MSCs. However, published studies have confirmed the immunosuppressive ability of this cell line [81]. Overall, we can conclude that even in the presence of acute dehydration and ischemia, a portion of MSCs are able to localize to the lungs and potentially help mediate pulmonary inflammation and apoptosis, as well as liver and systemic IL-6 and biomarkers of liver damage.

Taken together, these data demonstrate that intravenous infusion of MSCs after burn and ethanol intoxication attenuates circulating, hepatic, and lung inflammation, and further supports the central role of the gut-liver-lung axis in controlling the cytokine storm after injury. Future studies to determine the effectiveness of MSCs in reducing multiple organ failure and sepsis after burn are warranted.

Acknowledgements

Research in this publication was supported by NIH R01 GM115257 (EJK), R21 AA023193 (EJK), F31 AA022566 (JAS) and the Marian and Ralph C. Falk Medical Research Trust. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors would like to thank Mary Brown and Jessica Palmer for help with animal procedures and tissue processing, Patricia Simms for technical assistance in experiments involving flow cytometry, and Dr. John Callaci for assistance with mesenchymal stem cells.

Research funding: NIH R01 GM115257 (EJK), R21 AA023193 (EJK), F31 AA022566 (JAS), and the Marian and Ralph C Falk Medical Research Trust.

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Highlights

- Burn injury and ethanol intoxication leads to excessive inflammation
- Mesenchymal stem cell treatment (MSC) attenuates inflammation after burn and intoxication
- Circulating biomarkers of liver damage after burn and intoxication are decreased by MSCs
- MSC treatment reduces lung and liver inflammation and apoptosis after injury

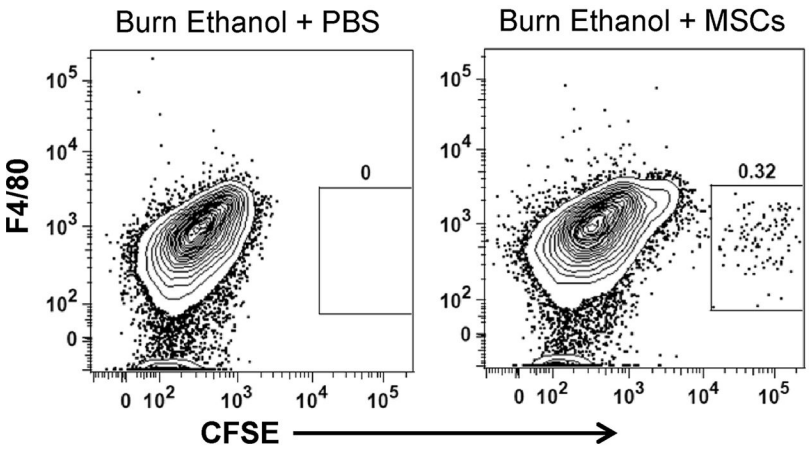

Fig 1.

Flow cytometry of MSCs in dissociated lung tissue. Representative flow cytometry plots of each treatment group. $CFSE^+$ MSCs were identified in dissociated lung tissue from intoxicated and injured mice at 24 h using flow cytometry. Total dissociated lung cells were negatively selected for Siglec-F and CD11b (data not shown), followed by $F4/80^-$ $CFSE^+$ cells (box) in burn ethanol + PBS and burn ethanol + MSCs treatment groups.

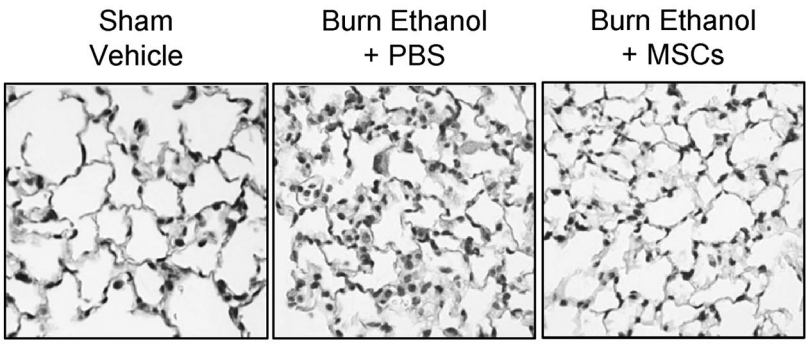

Fig 2.

Histological assessment of pulmonary inflammation. Lungs were sectioned and stained with H&E and assessed for cellular infiltration. Representative sections from each treatment group are shown at 400x.

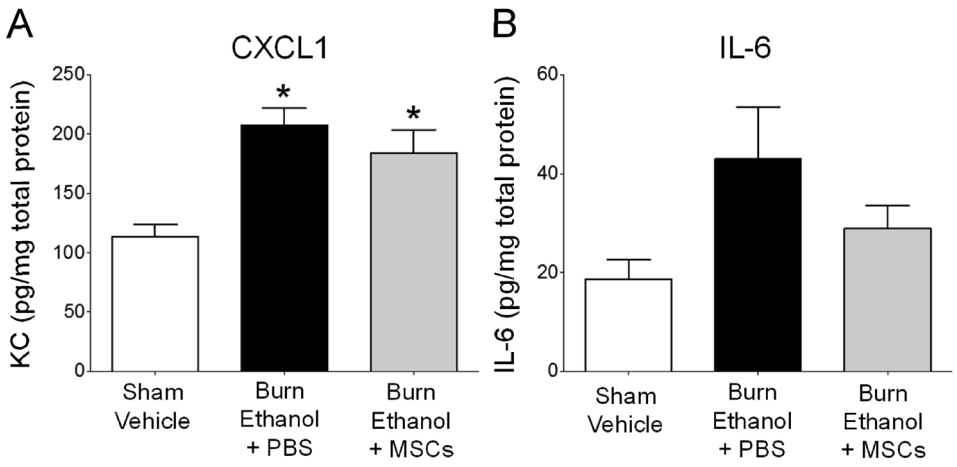

Fig 3.

Pulmonary neutrophil and chemokine levels. Lung homogenates were analyzed for levels of A) CXCL1 (KC) and B) IL-6. *$p<0.05$ versus sham vehicle by One Way ANOVA with Tukey's post multiple comparison post test. Data are presented as mean pg/mg protein ± SEM. Data combined from 2 independent experiments.

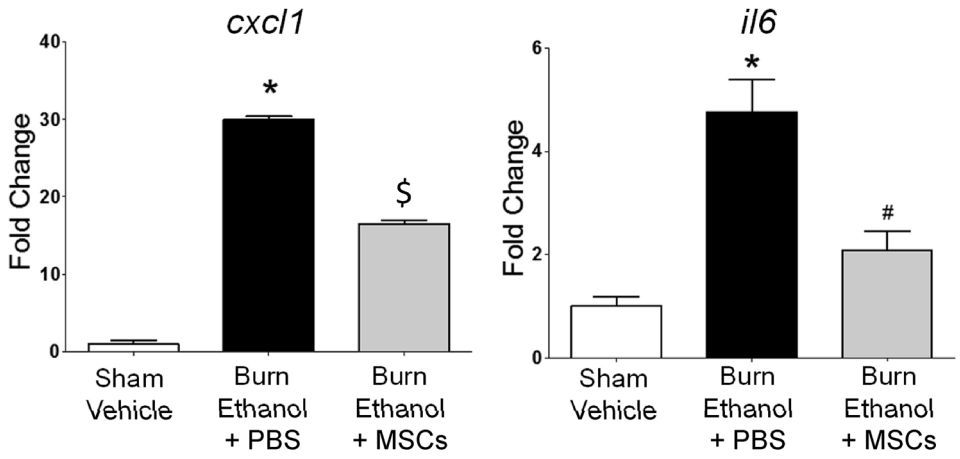

Fig. 4.

Gene expression levels of liver cytokines and chemokines. Quantitative RTPCR was performed to measure expression levels of A) *cxcl1* and B) *il6* in liver tissue. Results were analyzed using the ΔΔCt algorithm[14] with *GAPDH* as the endogenous control. Data are representative of two independent experiments and presented as mean fold change ± SEM relative to sham-injured, vehicle treated controls. n = 2-6 per group; *$p<0.05$ compared to sham vehicle, $^{\#}p<0.05$ compared to burn ethanol + PBS, and $^{\$}p<0.05$ compared to all other groups by One-way ANOVA with Tukey's multiple comparison test.

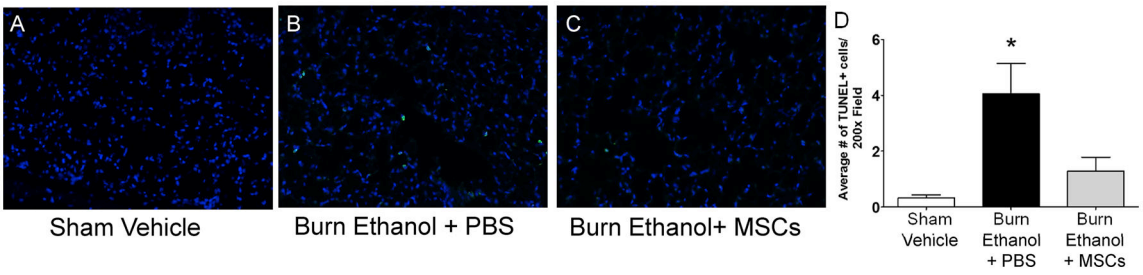

Fig. 5.

Quantification of lung apoptosis. Representative images of TUNEL+ cells in A) sham vehicle, B) burn ethanol + PBS, and C) burn ethanol + MSC treated mice are shown, where green indicates TUNEL+ cells and blue indicates DAPI nuclear stain. D) Quantification of TUNEL+ cells. Representative data from two independent experiments are shown, n=4-6 per group, per experiment. Data are presented as the average number of TUNEL+ cells in one 200x high power field. *$p<0.05$ compared sham-injured, vehicle treated controls, by One-way ANOVA with Tukey's multiple comparison post-test.

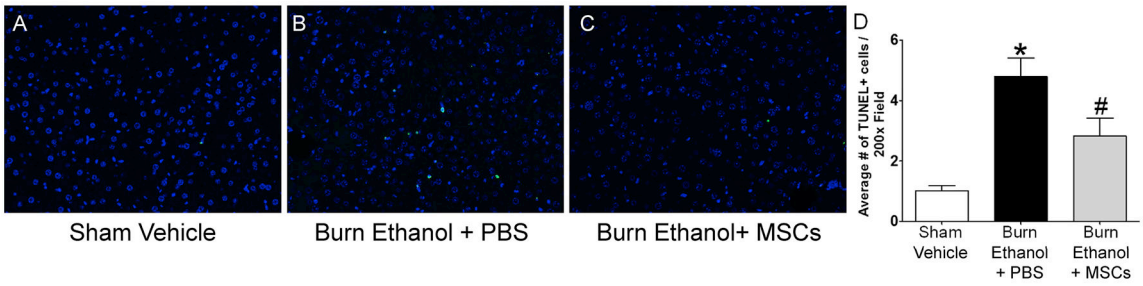

Fig. 6.

Quantification of liver apoptosis. Representative images of TUNEL+ cells in A) sham vehicle, B) burn ethanol + PBS, and C) burn ethanol + MSC treated mice are shown, where green indicates TUNEL+ cells and blue indicates DAPI nuclear stain. D) Quantification of TUNEL+ cells. Data from two independent experiments were combined, n=7-12 per group. Data are presented as the average number of TUNEL+ cells in one 200x high power field. *$p<0.05$ compared sham-injured, vehicle treated controls, # $p<0.05$ compared burn-injured, intoxicated mice by One-way ANOVA with Tukey's multiple comparison post-test.

Table 1:

Serum cytokine and aminotransferase levels

	Sham Vehicle	Burn Ethanol + PBS	BBurn Ethanol + MSCs
Serum			
IL-6	81.1 ± 57.9	709.9 ± 123.7 *	404.1 ± 108.0
ALT	24.3 ± 2.9	217.0 ± 38.2 *	174.0 ± 50.3
AST	145.3 ± 88.2	1035.8 ± 209.0 *	815.5 ± 229.2

Serum cytokine and aminotransferase levels. Serum was analyzed for IL-6, AST, and ALT levels. Serum IL-6 levels are presented as mean picograms/ milliliter ± SEM. AST and ALT are presented as the mean units/liter ± SEM.

* $p<0.05$ versus sham vehicle by One-way ANOVA with Tukey's multiple comparison post-test