Impaired Respiratory Function and Heightened Pulmonary Inflammation in Episodic
Binge Ethanol Intoxication and Burn Injury.

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Abstract

Clinical data indicate that cutaneous burn injuries covering greater than ten percent total body surface area are associated with significant morbidity and mortality, where pulmonary complications, including acute respiratory distress syndrome (ARDS), contribute to nearly half of all patient deaths. Approximately 50% of burn patients are intoxicated at the time of hospital admission, which increases days on ventilators by three-fold, and doubles length of hospital admittance, compared to non-intoxicated burn patients. The most common drinking pattern in the United States is binge drinking, where one rapidly consumes alcoholic beverages (4 for women, 5 for men) in 2 hours and an estimated 38 million Americans binge drink, often several times per month. Experimental data demonstrate a single binge ethanol exposure prior to scald injury, impairs innate and adaptive immune responses, thereby enhancing infection susceptibility and amplifying pulmonary inflammation, neutrophil infiltration, and edema, and is associated with increased mortality. Since these characteristics are similar to those observed in ARDS burn patients, our study objective was to determine whether ethanol intoxication and burn injury and the subsequent pulmonary congestion affects physiological parameters of lung function using non-invasive and unrestrained plethysmography in a murine model system. Furthermore, to mirror young adult binge drinking patterns, and to determine the effect of multiple ethanol exposures on pulmonary inflammation, we utilized an episodic binge ethanol exposure regimen, where mice were exposed to ethanol for a total of 6 days (3 days ethanol, 4 days rest, 3 days ethanol) prior to burn injury. Our analyses demonstrate mice exposed to episodic binge ethanol and burn injury have higher mortality, increased pulmonary congestion and neutrophil infiltration, elevated neutrophil chemoattractants, and respiratory dysfunction, compared to burn or ethanol intoxication alone. Overall, our study identifies plethysmography as a useful tool for characterizing respiratory function in a murine burn model and for future identification of therapeutic compounds capable of restoring pulmonary functionality.

Keywords: *Lung, Alcohol, Burn, Plethysmography, Neutrophils, Cytokines*

Introduction

Burn injury is associated with significant morbidity and mortality, with greater than 10% of patients succumbing to their injuries when the burn size exceeds 10% of their total body surface area (ABA, 2014). Clinical data suggest that 42% of the mortality observed in burn patients is due to pulmonary complications (Achauer, Allyn, Furnas, & Bartlett, 1973; Phillips & Cope, 1962). Severe burn, even in the absence of inhalation injury, is a common predisposing factor for the development of Acute Respiratory Distress Syndrome (ARDS) (Liffner, Bak, Reske, & Sjoberg, 2005; Turnage et al., 2002). ARDS is associated with rigid lungs, hypoxemia, and bi-lateral infiltrates in chest radiographs (Ashbaugh, Bigelow, Petty, & Levine, 1967). Overall, respiratory dysfunction in burn patients is characterized by shallow breathing and fluid accumulation in the lung interstitium, leading to heightened vascular resistance and an increased effort to breathe (Achauer et al., 1973; Turnage et al., 2002). A net result of insufficient gas exchange underlies the large percentage of burn fatalities due to pulmonary complications.

Binge alcohol drinking is an increasingly prevalent activity, affecting an estimated 38 million adults in the United States (CDC, 2012). It is characterized by either the number of alcoholic drinks one consumes in 2 hours (4 for women, 5 for men) or by a blood alcohol concentration of 0.08%. Interestingly, approximately 50% of burn patients are under the influence of alcohol at the time of hospital admission (Grobmyer, Maniscalco, Purdue, & Hunt, 1996; Silver et al., 2008). Intoxicated burn patients have three times as many days on ventilators and an overall twice as long hospital stay compared to burn patients who were not intoxicated (Hadjizacharia et al., 2011; Silver et al., 2008). This leads to an increased risk of pulmonary complications that predisposes burn patients to multiple organ failure, with the lungs preceding all other organs, as well as a higher chance of mortality (Ciesla et al., 2005; Hollingsed, Saffle, Barton, Craft, & Morris, 1993). Notably, the vast majority of intoxicated burn patients are binge drinkers and not chronic dependent drinkers (Howland & Hingson, 1987; Schermer, 2006; Smith & Kraus, 1988). Experimental models of binge alcohol intoxication and burn injury have demonstrated alterations in innate and adaptive immunity that result in the marked immune dysfunction, greater susceptibility to infection, and amplified pulmonary inflammation (Bird, Morgan, Ramirez, Yong, & Kovacs, 2010; Bird, Zahs, et al., 2010; Choudhry et al., 2000; Faunce, Gregory, & Kovacs, 1997, 1998; Kawakami, Switzer, Herzog, & Meyer, 1991; Messingham, Faunce, & Kovacs, 2002; Murdoch, Brown,

Gamelli, & Kovacs, 2008; Murdoch, Karavitis, Deburghgraeve, Ramirez, & Kovacs, 2011; Patel, Faunce, Gregory, Duffner, & Kovacs, 1999). Previously, our laboratory established that in a mouse model of single-dose binge ethanol exposure and burn injury there is amplified neutrophil infiltration, alveolar wall thickening, and edema in the lungs when alcohol precedes burn injury (Bird, Morgan, et al., 2010; Bird, Zahs, et al., 2010; Chen et al., 2013; Chen et al., 2014; Patel et al., 1999). Since ARDS is associated with similar pulmonary characteristics in humans, including increased neutrophil infiltration, capillary permeability and pulmonary edema (Dancey et al., 1999; Liffner et al., 2005; Steinvall, Bak, & Sjoberg, 2008), the objective of our study was to determine whether intoxication and burn injury and the resulting histological pulmonary congestion affects physiological parameters of lung function using a murine model system.

In these studies, we used non-invasive and unrestrained plethysmography to examine the impact of pulmonary congestion caused by burn injury on breathing patterns and respiratory function (Irvin & Bates, 2003). The Center for Disease Control (CDC) has reported 1 in 6 adults binge drink at least 4 times a month. Additionally, weekend binge drinking is a pattern observed in many cultures (Lundqvist, Alling, Knoth, & Volk, 1995). To mirror this drinking pattern, our laboratory used a mouse model of episodic binge ethanol intoxication prior to burn injury (adapted from (Callaci et al., 2004), (Przybycien-Szymanska, Mott, & Pak, 2011; Przybycien-Szymanska, Rao, & Pak, 2010; Qin et al., 2014; Vaagenes et al., 2015)) to assess respiratory physiology and gain an understanding of the effect of intoxication on lung function after burn injury.

Overall, our analyses identify plethysmography as a useful tool for characterizing respiratory function in a murine model of ethanol intoxication and burn injury. Our studies demonstrate that episodic binge ethanol intoxication prior to burn causes increased pulmonary neutrophil infiltration. The timing of neutrophil accumulation in the lung parallels the heightened levels of lung neutrophil chemoattractants and is associated with respiratory dysfunction, which likely contributes to diminished survival rates.

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. 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.

Murine Model of Binge Ethanol and Burn Injury

A murine model of episodic binge ethanol intoxication and burn injury was employed using intraperitoneal injections as described previously (Faunce et al., 1997; Messingham, Fontanilla, Colantoni, Duffner, & Kovacs, 2000; Qin et al., 2014). Animals were given ethanol (1.2g/kg) or saline vehicle at a dose designed to elevate the blood alcohol concentration (BAC) to 150 mg/dL at 30 min after ethanol exposure (Murdoch et al., 2008). This dose of 150 μ l of 20% (v/v) ethanol solution or saline control was given daily for 3 days consecutively, mice were given 4 days without ethanol, and then given 3 additional daily ethanol doses. Thirty minutes following the final ethanol exposure, when the BAC was 150 mg/dl, the mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) and their dorsums 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 (Faunce et al., 1997). The scald injury results in an insensate, full-thickness burn (Faunce et al., 1999). 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. Animals were either euthanized at 24 hours or survival was measured out to 7 days post-injury.

Plethysmography

Pulmonary function was assessed at 24 hours post-injury by using barometric plethysmography (Buxco Research Systems). BAC levels had returned to baseline undetectable levels at this time point (Karavitis, Murdoch, Gomez, Ramirez, & Kovacs, 2008). Mice were placed in an unrestrained whole body barometric plethysmography

chamber and allowed to acclimate to the environment before lung function parameters (Chen et al., 2014) were recorded for 10 minutes on a breath-by-breath basis. Enhanced pause (Penh), breath frequency (f), tidal volume (TVb) and minute volume (MVb) were analyzed.

Histopathologic Examination of the Lungs

The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously (Patel et al., 1999), embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E). Sections were evaluated using light microscopy (Zeiss AxioVert, Zeiss, Thornsdale, CA) and histology photographs were taken at 1000x magnification. To measure pulmonary congestion, photographs were taken in a blinded fashion of 10 high power fields (400X) per animal and analyzed using the Java-based imaging program ImageJ (National Institutes of Health, Bethesda, MD). The images were converted to binary to differentiate lung tissue from air space and then analyzed for the percent area covered by lung tissue in each field of view as described previously (Chen et al., 2013). Neutrophils were counted in a blinded fashion in 10 high power fields (400X).

Measuring Neutrophil Counts by Flow Cytometry

The upper left lung lobe was removed and cut into small pieces with a razor. 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. The homogenates were then filtered through 70 μ m nylon cell strainers to obtain a single cell suspension. Red blood cells were lysed using ACK lysis buffer (Life Technologies, Grand Island, NY). Cells were counted using trypan blue to exclude dead cells. To assess neutrophil numbers, 1×10^6 lung cells were first incubated with anti-CD16/32 (clone 93, eBioscience, San Diego, CA) to block unspecific binding to the Fc γ II/III receptor. Cells were then immunostained with rat anti-mouse antibodies: CD45 e780 (clone 30-F11, eBioscience), CD11b e450 (clone M1/70, eBioscience), and Ly6G (Gr-1) PE Cy7-conjugated (clone RB6-8C5,

eBioscience). Antibody incubation was carried out for 30 minutes at 4°C. Cells were washed and fixed as described (Boehmer, Goral, Faunce, & Kovacs, 2004; Murdoch et al., 2011). Samples were run on a BD Fortessa cytometer (BD Biosciences, San Jose, CA). Data analysis was performed using Flow Jo FCS analysis software (Tree Star Inc., Ashland, OR).

Cytokine Analysis of Lung Homogenates

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 production using a BioPlex multiplex bead array. The results were normalized to total protein using the BioRad protein assay (BioRad) (Bird, Zahs, et al., 2010; Chen et al., 2013).

Statistical Analysis

Statistical comparisons were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol 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 $\pm$ the standard error of the mean (SEM). The Gehan-Breslow-Wilcoxon test was used to generate comparisons between burn groups in the survival study and Pearson's correlation test was used to generate the correlation coefficient between neutrophil numbers and Penh, breath frequency, tidal volume, and minute volume.

Results

Binge Ethanol Intoxication Decreases Survival after Burn Injury

Mice exposed to episodic binge ethanol intoxication and burn injury were monitored for survival out to 7 days post-injury. Both sham groups had 100% survival regardless of ethanol intoxication (Figure 1). Survival of burn-injured mice exposed to ethanol was significantly lower (48% survival) ($p < 0.05$) when compared to burn-injured mice not exposed to ethanol (84% survival) ($p < 0.05$). Observed mortality in the combined injury group occurred within 72 hours of injury, with 5% succumbing by 24 hours, 16% mortality between 24 and 48 hours and the majority of death, 31%, occurring

between 48 and 72 hours. This suggests there is a therapeutic window between time of injury and 24 hours to potentially prevent mortality when ethanol precedes burn injury.

Intoxication Reduces Lung Function in Mice Subjected to Burn Injury

Lung function was assessed 30 minutes prior to animals being euthanized at 24 hours post-injury. Enhanced pause (Penh), a measure of bronchoconstriction and airway resistance, revealed that animals that were intoxicated at the time of burn injury had an average Penh of 8, compared to sham groups with a Penh of less than 1 and burn injury alone with an average Penh of 3 (Figure 2A). When ethanol intoxication precedes burn injury, this corresponds with an 8-fold increase in airway resistance, compared to sham groups ($p < 0.05$), and a 2.5-fold increase, compared to burn injury alone ($p < 0.05$). Moreover, our data reveal that animals with a Penh of 7 or above are at a greater risk for mortality. The frequency of breaths per minute was also significantly decreased in both burn groups. Burn injury alone had a 39% decrease in the average number of breaths per minute compared to shams ($p < 0.05$), while intoxicated and burn-injured mice had a 59% decrease compared to shams ($p < 0.05$) and a 33% decrease compared to burn alone ($p < 0.05$) (Figure 2B). Additionally, tidal volume, the amount of air that is inhaled and exhaled in a single breath, was decreased in both burn groups compared to sham groups ($p < 0.05$) (Figure 2C). Minute volume, the amount of air inhaled and exhaled per minute, was also decreased in both burn groups (Figure 2D). Compared to shams, burn injury alone had a 60% decrease in the amount of air per minute ($p < 0.05$), while intoxicated and burn-injured mice had an 80% decrease ($p < 0.05$). Furthermore, combined injury had a 52% decrease compared to burn alone, though this did not reach statistical significance. These data indicate burn injury alone induces abnormal breathing patterns, however, when intoxication precedes burn injury, there is a dramatic increase in airway resistance and a pronounced reduction in lung function. Overall, intoxicated and burn injured animals have a shallow, slower breathing rate than burn injury alone, indicative of overall respiratory dysfunction.

Elevated Pulmonary Neutrophil Infiltration and Congestion after Intoxication and Burn Injury

Similar to the single-dose binge ethanol and burn injury results previously reported by our laboratory, (Bird, Morgan, et al., 2010; Bird, Zahs, et al., 2010; Chen et al., 2013; Patel et al., 1999) histochemical analyses of sectioned lung tissue demonstrated that episodic binge ethanol intoxication prior to burn injury results in increased cellularity and alveolar wall thickening 24 hours post-injury (Figure 3A-C). Neutrophils were counted by light microscopy as previously described (Patel et al., 1999). When intoxication preceded burn injury, there was a 10-fold increase compared to sham groups ($p < 0.05$) and an approximate 1.5-fold increase compared to burn alone (Figure 3B). Additionally, these neutrophils were localized to the interstitium and not the alveolar space. Pulmonary congestion was quantified using imaging software to measure the area of lung tissue in 10 high power fields per animal and is reported as a percentage of the entire field of view. Intoxicated and burn injured animals had a significant increase in tissue area compared to all other groups ($p < 0.05$), correlating to a decrease in air space and an increase in pulmonary congestion (Figure 3C). Notably, these changes were isolated to the distal airways. There were no apparent changes in the larger airways.

To further demonstrate that neutrophils were the key cell type contributing to the escalated cellularity, flow cytometry was performed. Flow cytometric analysis of cellular subsets in the lung revealed dramatically increased CD45⁺CD11b⁺Ly6G⁺ neutrophil cell counts in intoxicated and burn injured mice at 24 hours post-injury, compared to sham and isolated burn injury alone ($p < 0.05$) (Figures 4A and 4B). Mice exposed to the combined insult had a 7-fold increase ($p < 0.05$) in neutrophil numbers when compared to both sham groups and a 1.5-fold increase when compared to burn-injured animals without ethanol intoxication, confirming that neutrophils contribute to increased cellularity in the lungs of intoxicated and burn-injured mice.

It was also observed that the increase in the number of neutrophils corresponded to an increase in Penh and decreases in breath frequency, tidal volume and minute volume. Pearson's correlation coefficient was used to determine if there was a linear relationship between the number of infiltrating neutrophils and lung function measurements. We found a positive correlation between the number of neutrophils and

Penh ($r = 0.9597$) and negative correlations between neutrophil numbers and breath frequency ($r = -0.9866$), tidal volume ($r = -0.9777$) and minute volume ($r = -0.9784$). These data emphasize the potential use of plethysmography as an instrument to determine the effect of neutrophil accumulation in lung tissue on overall lung function.

Ethanol Intoxication Before Burn Injury Enhances Pulmonary KC and Macrophage Inflammatory Protein - 2 (MIP-2) Levels

Since we observed increased neutrophil infiltration, we next sought to examine levels of the neutrophil chemoattractants, KC and MIP-2. Similar to our single-dose binge ethanol and burn injury findings (Bird, Morgan, et al., 2010; Bird, Zahs, et al., 2010; Patel et al., 1999), the upregulation of neutrophil chemokines KC and MIP-2 highlight a mechanism behind the increase in pulmonary neutrophil accumulation in episodic binge ethanol exposed and burn-injured mice (Figure 4). Combined injured animals had an 11-fold more KC than both sham groups ($p < 0.05$) and 1.8-fold more KC than burn injury alone. Additionally, MIP-2 was significantly upregulated compared to all treatment groups, with a 6-fold increase in MIP-2 compared to sham groups ($p < 0.05$) and a 2.5-fold increase compared to burn injury alone ($p < 0.05$).

Discussion

Intoxication at the time of burn injury increases bacterial translocation from the intestines into the lymphatic system and bloodstream. This may induce heightened hepatic production of interleukin-6 (IL-6) that circulates to the vascular bed of the lungs and initiates pulmonary inflammation and congestion (Chen et al., 2014; Choudhry et al., 2004; Emanuele et al., 2009; Kavanaugh et al., 2005; Zahs, Bird, Ramirez, Choudhry, & Kovacs, 2013; Zahs et al., 2012). Prior to this study, it was not known how multiple doses of ethanol would affect burn-induced pulmonary inflammation. Ethanol intoxication alone has been shown to suppress immune responses in many organs, including the lungs (Gamble, Mason, & Nelson, 2006; Goral, Choudhry, & Kovacs, 2004; Happel & Nelson, 2005; Karavitis, Murdoch, Deburghgraeve, Ramirez, & Kovacs, 2012). Whether episodic ethanol intoxication before burn injury would yield heightened or diminished pulmonary inflammatory effects had yet to be determined. It was also not

known how intoxication prior to injury affects lungs function. Here, we have demonstrated that episodic binge ethanol prior to burn injury causes heightened pulmonary inflammatory responses and congestion, paralleling single-dose binge ethanol exposure. Importantly, we have found that these histologic inflammatory responses cause physiological derangements in intoxicated and burn-injured animals, including increased airway resistance and abnormal breathing patterns. These findings highlight an underlying mechanism contributing to the drastic mortality rate observed in mice exposed to both ethanol and burn injury.

Non-invasive, unrestrained whole-body plethysmography is a convenient method to evaluate normal breathing patterns in mice (Glaab, Taube, Braun, & Mitzner, 2007). Unlike invasive methods using a tracheotomy and mechanical ventilation to study plethysmography, non-invasive techniques do not require anesthesia or surgery. This significantly reduces stress and allows individual animals to be assessed multiple times, a useful feature for time course studies (J. H. Bates & Irvin, 2003; Glaab et al., 2007; Milton, Dickinson, Jenkin, & Lim, 2012). In non-invasive plethysmography, Penh is a dimensionless parameter that has been viewed as a measure of bronchoconstriction and airway resistance, though several studies claim Penh is an unreliable measure of respiratory mechanics (Adler, Cieslewicz, & Irvin, 2004; Hoymann, 2007). However, other studies suggest Penh should be used as a measure of overall lung function, highlighting changes in respiratory breathing patterns (J. Bates et al., 2004; Glaab et al., 2007; Verheijden, Henricks, Redegeld, Garssen, & Folkerts, 2014). Verheijden, et. al. recently compared non-invasive plethysmography (Penh) to invasive plethysmography (Resistance) in a severe model of allergic airway inflammation. Their data revealed an increase in both Penh and resistance with severe allergic airway inflammation. Notably, a Penh of 8 corresponded to a significant increase in resistance, compared to controls, suggesting that in severe models of inflammation, Penh is a reflective measure of resistance. Additionally, it was determined an increase in Penh paralleled an increase in bronchoalveolar lavage cells in the airways (Verheijden et al., 2014), furthering supporting our data that pulmonary congestion with intoxication and burn injury leads to functional lung complications.

We observed that ethanol intoxication at the time of injury leads to a longer pause between breaths (Penh), correlating with a decrease in the frequency of breaths per minute. With this data, one would expect this slower breathing pattern, an indicator

of increased lung resistance, would result in deeper breaths, represented by an increase in tidal volume. In contrast, we observed a decrease in tidal volume and minute volume, an indication that the mice are not able to compensate for the increase in lung resistance. This breathing pattern may mirror agonal breathing, which is characterized by infrequent, shallow breaths and has been observed in severely injured humans. This pattern is precipitated by hypoxemia regardless of arterial carbon dioxide levels or pH, which may be the case in our injured animals since agonal breathing in humans is typically viewed as the terminal stage of respiration, occurring immediately before death (Guntheroth & Kawabori, 1975). When the plethysmography measurements were taken at 24 hours in our experiments, majority of the animals appeared either moribund or lethargic in the plethysmography chamber. With 47% of death occurring between 24 and 72 hours, the breathing pattern we detected in mice at 24 hours strongly suggests it can be characterized as agonal breathing, indicating a mechanism behind the large percentage of mortality in the following 48 hours.

The location of the dorsal burn injury in this model could also restrict chest wall movement during both the inflammatory and the wound healing phases, promoting the observed decrease in breaths per minute, overall leading to decreased gas exchange. Humans with chest, back or torso circumferential burns have breathing patterns with rapid shallow breaths, not slow shallow breaths, as we have observed in our model (Achauer et al., 1973). These differences, however, may be attributed to the immediate care provided to human burn patients. Torso circumferential burns can limit respiration and require a chest escharotomy where incisions are made in the burn wound, releasing constricted and non-elastic damaged tissue, to restore chest movement. The mice in our model do not receive circumferential burns, removing the need for escharotomy in the model, but the injury location may still lead to an altered breathing pattern. Tidal breathing is usually interrupted every few minutes by a deep breath or yawn, which is needed to expand underinflated alveoli. Restricted chest wall movement can cause burn patients to lose this ability. Without this interruption in tidal volume, the alveoli eventually collapse in a process known as atelectasis, resulting in decreased blood flow, oxygen exchange and the ability of the lungs to expand (Achauer et al., 1973; McCutcheon, 1953). Our data demonstrate that the loss of lung compliance with burn injury, even in the absence of inhalation injury, is further decreased when intoxication precedes burn injury. The restricted chest movement and subsequent collapse of alveoli may also

explain the differences in breathing patterns in our mouse model, in comparison to human burn patients. However, the 3-fold increase in Penh with burn alone does not correspond to significantly increased mortality rates. This suggests ethanol, not the location of the burn, leads to impaired lung function and elevated mortality rates.

In conclusion, these data emphasize the importance of immediate burn care treatment within the few hours after injury, to stabilize breathing patterns and to maintain respiratory function, especially when patients are intoxicated at the time of injury. Non-invasive, unrestrained whole-body plethysmography will be a useful tool in future animal studies to help identify therapeutic reagents that can improve respiratory function in all burn patients.

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Figure 1. Survival of mice exposed to ethanol prior to burn injury. Mice were exposed to ethanol or saline as a control for a total of 6 days (3 days ethanol, 4 days rest, 3 days ethanol) prior to burn injury and monitored for survival out to 7 days post-injury. $p < 0.05$, burn ethanol compared to burn vehicle by Gehan-Breslow-Wilcoxon test. Data were combined from 2 independent experiments and are expressed as percent survival. $N = 4$ (sham groups), $n = 10-19$ (burn groups).

Figure 2. Effects of ethanol intoxication on post-burn lung function. Mice were placed in an unrestrained whole body barometric plethysmography chamber and lung function parameters were recorded for 10 minutes. Penh, $p < 0.05$ burn ethanol versus all groups. Breath frequency, $p < 0.05$ burn ethanol versus all groups, $p < 0.05$ burn vehicle versus sham groups. Tidal volume, $p < 0.05$ burn ethanol compared to sham groups, $p < 0.05$ burn vehicle compared to sham groups. Minute volume, $p < 0.05$ burn ethanol compared to sham groups, $p < 0.05$ burn vehicle compared to sham groups. Data were combined from 2 independent experiments. Data points shown as individual animals. $N = 6-11$ animals per group.

Figure 3. Histological assessment of the lungs. Lungs were sectioned and stained with H&E and assessed for cellular infiltration and alveolar wall thickening. A) Representative sections from each treatment group are shown at 1000x. B) Neutrophils were counted by light microscopy in H&E-stained lung sections 24 hours after intoxication and burn injury. Data are shown as the total number of neutrophils in 10 high power fields (400x). C) Quantification of pulmonary congestion 24 hours after injury. * $p < 0.05$ compared to sham groups; # $p < 0.05$ compared to all groups. Data are presented as mean values $\pm$ SEM. $N = 3-6$ animals per group.

Figure 4. Flow cytometry analysis of neutrophils in lung isolates. Lung tissue was obtained 24 hours post-injury from all treatment groups. Tissue was digested into a single cell suspension and cells were analyzed by flow cytometry. A) Representative gating for Ly-6G⁺CD11b⁺ neutrophils (box) of CD45⁺ cells (data not shown) from all four treatment groups. B) Absolute number of neutrophils in lung isolates. * $p < 0.05$ versus

sham groups. Data are presented as mean of total neutrophils from 100,000 lung cells per group $\pm$ SEM. N = 3-6 animals per group.

Figure 5. Pulmonary neutrophil chemokine levels in lung tissue. Lung homogenates from all four treatment groups were analyzed for levels of A) KC and B) MIP-2. * $p < 0.05$ versus sham groups; # $p < 0.05$ versus all treatment groups. Data are presented as the mean in picograms per milligram of protein $\pm$ SEM. N = 3-6 animals per group.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5