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Kupffer Cell p38 MAPK Signaling Drives Post Burn Hepatic Damage and Pulmonary Inflammation when Alcohol Intoxication Precedes Burn Injury

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Abstract

Objective—Clinical and animal studies demonstrate that alcohol intoxication at the time of injury worsens post-burn outcome. The purpose of this study was to determine the role and mechanism of Kupffer cell derangement in exacerbating post-burn end organ damage in alcohol exposed mice.

Design—Interventional study.

Setting—Research Institute.

Subjects—Male C57BL/6 mice.

Interventions—Alcohol administered 30 minutes before a 15% scald burn injury. Antecedent Kupffer cell depletion with clodronate liposomes (0.5 mg/kg). p38 mitogen-activated protein kinase (MAPK) inhibition via SB203580 (10 mg/kg).

Measurements and Main Results—Kupffer cells were isolated 24 hours after injury and analyzed for p38 activity and IL-6 production. Intoxicated burned mice demonstrated a 2-fold ($p<0.05$) elevation of Kupffer cell p38 activation relative to either insult alone and this corresponded to a 43% ($p<0.05$) increase in IL-6 production. Depletion of Kupffer cells attenuated hepatic damage as seen by decreases of 53% ($p<0.05$) in serum ALT and 74% ($p<0.05$) in hepatic

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triglycerides, as well as a 77% reduction ($p<0.05$) in serum IL-6 levels compared to matched controls. This mitigation of hepatic damage was associated with a 54% decrease ($p<0.05$) in pulmonary neutrophil infiltration and reduced alveolar wall thickening by 45% ($p<0.05$). *In vivo* p38 inhibition conferred nearly identical hepatic and pulmonary protection after the combined injury as mice depleted of Kupffer cells.

Conclusions—Intoxication exacerbates post-burn hepatic damage through p38-dependent IL-6 production in Kupffer cells.

Keywords

Trauma; Burns; p38 MAPK; Intoxication; Alcohol; Inflammation

Introduction

Severe burns are a devastating injury affecting every major organ system, with the degree of systemic inflammation correlating to the size of the burn (1). Nearly 50% of patients admitted for burns have a positive blood alcohol content (BAC) and have worse clinical outcomes than individuals who sustain similar injuries not under the influence of alcohol (2–5). Specifically, they are twice as likely to acquire an infection, require more surgical procedures and have a longer duration of stay in the intensive care unit than their nonintoxicated counterparts (2, 6). With 1 million burn injuries in the US each year, resulting in 40,000 hospitalizations (7), this represents a substantial burden of morbidity, mortality and socioeconomic cost for which there is currently no specific treatments. Recent studies suggest the majority of intoxicated trauma patients are binge drinkers without evidence of chronic alcoholism (8), consistent with the majority of alcohol consumption in the US (9) and the design of the enclosed animal study.

Previous work in this animal model has demonstrated that the presence of alcohol at the time of a burn alters the gut-liver axis (10) leading to increased interleukin-6 (IL-6) which drives pulmonary inflammation (11). We therefore sought to examine the role of Kupffer cells in the response to the combined insult of intoxication and burn with the aim of identifying a mechanistic target for this aberrant response. We describe within novel findings that demonstrate intoxication augments Kupffer cell IL-6 production through increased p38 activation after burn, suggesting that specifically targeting Kupffer cell p38 activity may have therapeutic value in this at-risk patient population.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in sterile micro-isolator cages under pathogen-free conditions at Loyola University Chicago. All experiments were conducted with approval of the Loyola Institutional Animal Care and Use Committee, performed with mice weighing between 23–25 g, and during the hours of 8–10 AM to avoid confounding factors related to circadian rhythms.

Murine model of intoxication and burn

A murine model of ethanol exposure and burn injury was employed as described previously (12) with minor modifications. Mice were given a single dose of ethanol (1.12 g/kg) by oral gavage that resulted in a BAC of 180 mg/dl at 30 minutes (13). The mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum shaved and they were placed in a plastic template exposing 15% of the total body surface area and subjected to a scald injury in 92ºC water or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn (14). All mice were resuscitated with 1.0 ml of 0.9% normal saline and placed on warming pads until recovered from anesthesia. Mice were euthanized by CO_2 narcosis followed by exsanguination 24 hours after injury. For experiments involving *in vivo* p38 inhibition, the intoxicated, burn injured mice were split into two groups and at 30 minutes after injury, given an intraperitoneal injection of the selective p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580 (InvivoGen, 10 mg/kg) or vehicle control (saline). For experiments involving antecedent Kupffer cell depletion, clodronate liposomes (Encapsula NanoSciences, 0.5 mg/kg) or vehicle control (empty liposomes) were administered via tail vein injection. The control arms of sham vehicle, sham ethanol, and burn vehicle are presented herein for the clodronate and p38i experiments to demonstrate baseline results and provide injury context for the assessed parameters. The data from these control groups can be found in the manuscript figures and are consistent with previously reported results in the liver (10, 13, 15), and lungs (11, 13, 16). As this study examines the role of Kupffer cells in the established consequences of intoxication and burn injury in this animal model, the discussion of the *in vivo* studies will focus on the animals given the combined injury of ethanol and burn, with and without treatments. Furthermore, as the treatment vehicle controls given to intoxicated and burn injured mice (saline and empty liposomes for p38 inhibition and clodronate, respectively) did not differ from intoxication and burn without vehicle controls, only one set of results are shown for clarity.

Serum measurements

At 24 hours post injury, blood was collected via cardiac puncture, an aliquot was placed into a microcapillary tube, and read for a complete blood count with differential by Hemavet (Drew Scientific, Dallas, TX). The remaining blood was harvested for serum by centrifugation after clotting. Serum aliquots were used to measure IL-6 by ELISA (BD Biosciences, Franklin Lakes, NJ) or liver transaminase levels using a DRI-CHEM 7000 (HESKA, Loveland, CO) as described previously (13).

Liver histology

The whole liver was removed at the time of sacrifice, weighed and a portion embedded in OCT or snap frozen in liquid nitrogen. Frozen sections were cut at 7 μm, stained with Oil Red O, and examined for the presence of fat droplets as described previously (17). Sectioned tissue was rinsed in isopropyl alcohol and placed in a filtered working solution of Oil Red O for 20 minutes. The slides were rinsed in isopropyl alcohol, followed by tap water and counterstained with hematoxylin for 1 minute. The slides were serially rinsed in tap water, ammonia water (lithium carbonate), and finally tap water. Representative images were taken at 400x magnification.

Liver tissue measurements

Liver tissue was homogenized in 1 ml of BioPlex cell lysis buffer according to manufacturer's instructions (BioRad, Hercules, CA) and analyzed for cytokine production using an ELISA for IL-6 (BD Biosciences, Franklin Lakes, NJ). The results were normalized to total protein using the BioRad protein assay (BioRad, Hercules, CA). A portion of the frozen liver was used in a Triglyceride Quantification Kit according to manufacturer instruction (Abcam, Cambridge, MA).

Kupffer cell isolation

Kupffer cells were isolated 24 hour after injury as described by Kuriakose et al. (18) with minor modifications. Rinsed liver tissue was incubated at 37°C for 30 minutes in collagenase IV (5000 CDU/mL), run through a gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA), and passed through a sterile 100 um strainer into phosphate-buffered saline (Miltenyi Biotec, Auburn, CA) containing 0.5% bovine serum albumin. The hepatocytes were removed by centrifugation at 20xg for 4 minutes, and the residual cell suspension was centrifuged twice at 300xg for 10 minutes at 4°C with the cell pellet washed with Red Blood Cell Lysis Solution between centrifugations. $CD11b^+$ cells were enriched by positive selection using AUTOMACS (Miltenyi Biotec Auburn, CA). Enriched liver $CD11b^+$ cells isolated by this method have been shown to be greater than 96% positive for F4/80 expression as assessed by flow cytometry (18).

Cell culture

Isolated Kupffer cells were cultured at a concentration of 3×10^5 per well in RPMI medium supplemented with 10% fetal bovine serum and 50μg/ml gentamicin for 2 hours. Cells were pretreated with SB203580 (10 μM) for 1 hour before stimulation with lipopolysaccharide (LPS) (100 ng/mL) followed by incubation at 37°C with 5% CO_2 and 95% humidity for 18 hours. At the concentration used, SB203580 is highly selective for p38 (19). Supernatants were removed and analyzed for cytokine production using an enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6; BD Biosciences, Franklin Lakes, NJ). Activation of p38 was determined by cell-based ELISA (RayBiotech Inc, Norcross, GA) using primary antibodies against p38 and phospho-p38 (Thr180/Tyr182) as specified per manufacturer instructions.

Lung histology

The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously (20). The lung was embedded in paraffin, sectioned at 5μm, and stained with hematoxylin and eosin (H&E). The sections were analyzed microscopically in a blinded fashion for the number of neutrophils in 10 high power fields (HPF). Representative images were taken at 400x magnification. Ten HPF (400X) per animal were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). The images were converted to binary to differentiate lung tissue from air space and analyzed for the percent area covered by lung tissue in each field of view as described previously (11).

Statistical analysis

Statistical comparisons (GraphPad Instat, La Jolla, CA) were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol animals, resulting in four total groups analyzed treated as an independent variable with one factor. One-way analysis of variance (ANOVA) was used to determine differences between groups, and Tukey's post hoc test once significance was achieved ($p<0.05$). Statistical comparisons made between intoxicated burned mice given saline and intoxicated burned mice given a treatment (clodronate or SB203580) were performed using a Student's t-test ($p<0.05$). Data are reported as mean values ± the standard error of the mean (SEM).

Results

Kupffer cell p38 activation and IL-6 production

Total p38 was detectable in all samples and did not change between treatment groups. The combination of ethanol and burn injury led to a 4-fold ($p<0.05$) increase in p38 activation relative to sham, which was 2-fold ($p<0.05$) greater than either insult alone (Figure 1A). IL-6 levels paralleled p38 activation with the combined injury demonstrating a 3-fold ($p<0.05$) and 1.5-fold ($p<0.05$) increase over sham injury or isolated intoxication or burn, respectively (Figure 1B). *Ex vivo* p38 inhibition abrogated the increases in LPS-induced p38 activation (Figure 1C) or IL-6 production (Figure 1D) in Kupffer cells from any injury group.

Hepatic damage

The clodronate dose and administration route used is a well-established method to achieve relatively selective depletion of Kupffer cells ((21), Figure 2A&B), while leaving other macrophage populations such as the circulating monocytes (Figure 2C) and alveolar macrophages (Figure 2D&E.) intact. The dosage of p38i used in the present study has been shown to inhibit p38 activation in Kupffer cells 24 hours after burn (22) which we confirm through decreased p38 phosphorylation (Figure 2F) and IL-6 production (Figure 2G) after LPS stimulation.

Inhibition of p38 after the combined insult alleviated hepatic damage as measured by a 35% reduction ($p<0.05$) in serum AST, a 58% decrease ($p<0.05$) in serum ALT and a 22% lower ($p<0.05$) liver weight to body weight ratio compared to intoxicated and burned animals given saline (Figure 3A–C). Analogous to SB203580 treatment, antecedent Kupffer cell depletion reduced serum AST by 44% ($p<0.05$), ALT by 53% ($p<0.05$) and liver weight to body weight ratio by 28% ($p<0.05$), relative to animals given saline after ethanol and burn (Figure 3A–C). The decreased hepatic damage after p38 inhibition or the absence of Kupffer cells paralleled decreases in hepatic triglycerides of 59% ($p<0.05$) and 74% ($p<0.05$), respectively (Figure 3D) which can also be appreciated upon histologic examination (Figure 3E).

Hepatic and systemic levels of IL-6

After the combined insult of intoxication and burn, p38 inhibition led to a 57% reduction ($p<0.05$) in hepatic IL-6 levels (Figure 4A) which corresponded to a 68% decrease ($p<0.05$)

in serum IL-6 (Figure 4B). Similarly, IL-6 levels in mice that lacked Kupffer cells at the time of injury had reduced hepatic IL-6 by 65% ($p<0.05$) and serum IL-6 by 77% ($p<0.05$) compared to mice undergoing intoxication and burn with intact Kupffer cells.

Pulmonary inflammation

Clodronate or p38 inhibition lessened pulmonary congestion and cellularity upon visual examination (Figure 5A). Consistent with visual findings, p38 inhibition decreased alveolar wall thickness by 43% ($p<0.05$) compared to matched controls and similarly, Kupffer cell depletion lessened alveolar wall thickness by 45% ($p<0.05$) (Figure 5C). Neutrophil accumulation was reduced by 58% ($p<0.05$) with SB203580 treatment and 54% ($p<0.05$) with clodronate compared to matched controls (Figure 5B).

Discussion

Animal studies suggest hepatic derangement is dependent on the size of the burn with a threshold beyond which a qualitatively different response occurs (23). Work from our lab and others indicates that the presence of alcohol intoxication at the time of injury lowers the size of burn required to cause liver damage (10, 15, 24), though the mechanisms of how this ensues is currently unknown. One possibility is that alcohol and burn act synergistically on Kupffer cells to cause excessive cytokine production. Existing literature suggests alcohol consumption (25) or an isolated burn (26) can independently increase Kupffer cell sensitization to LPS, a potent stimulator of Kupffer cell activation. We now report that the combined insult of intoxication and burn leads to greater Kupffer cell activity after LPS stimulation than either insult alone. Taken together, this novel finding combined with previous work showing increased bacterial translocation after the combined injury (10), indicates that alcohol augments post-burn IL-6 through increasing both the stimulus and response to LPS. This is of clinical importance as elevated IL-6 correlates with mortality risk in burn patients (27), possibly explaining the worsened outcomes of patients who are intoxicated at the time of a burn (2). While it remains controversial if IL-6 in human burn patients plays a contributory role or is simply a biomarker of mortality risk, animal studies have conclusively demonstrated a causative role for IL-6 in end-organ damage after intoxication and burn (11, 28). We therefore sought to elucidate the signaling mechanisms responsible for Kupffer cell hyperactivity in this setting.

Kupffer cells produce IL-6 when LPS binds to Toll-like receptor 4 (TLR4), initiating a variety of potential down-stream signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway. A member of the MAPK family, p38 has a well-established role in post-burn remote organ damage (29–34) and enhances TLR4 reactivity following injury (35). Our observations from isolated Kupffer cells establish that ethanol augments p38 activation in Kupffer cells after burn, which corresponds to heightened IL-6 production and end organ damage. Furthermore, *ex vivo* inhibition of p38 abrogated LPS-induced IL-6 production suggesting that the increased IL-6 after intoxication and burn is produced in a p38-dependent manner.

Global p38 inhibition attenuated hepatic damage to an extent equal to that of antecedent Kupffer cell depletion, suggesting Kupffer cell p38 may be responsible for the increased

hepatic damage of the combined injury. Furthermore, both treatments reduced hepatic damage and steatosis to the extent seen after a burn alone, implying that alcohol may potentiate post-burn hepatic damage through Kupffer cell p38 activity. These findings do not rule out the possible influence of other etiologies of hepatic damage, such as oxidative stress (36) or changes in fat metabolism (37). However our *ex vivo* results of increased LPS-induced Kupffer cell activity and our *in vivo* findings of elevated p38-dependent IL-6 production allude to a more prominent role for Kupffer cell IL-6 in causing the hepatotoxicity of this setting. Indeed, excess IL-6 can be detrimental to hepatocytes (38) and promote steatosis (39).

Increased serum IL-6 is linked to poor survival in patients with acute respiratory distress syndrome (ARDS) (40), a common complication after a burn (41) which develops independently of inhalation injury (42). ARDS is characterized by inflammation and edema in the lung parenchyma leading to impaired gas exchange which is exacerbated when alcohol precedes burn (11, 13, 43, 44). The degree of reduction in pulmonary inflammation observed with p38i treatment matches the level of reduction found in studies of IL-6 deficient mice undergoing the same combined injury (11), suggesting the pulmonary benefit in p38i-treated animals may be related to decreased IL-6 levels. The parallel results in clodronate-treated mice imply the pulmonary benefits of p38i treatment stem from its effect on Kupffer cells and not a direct action of p38 inhibition in the lungs.

Conclusions

The studies above delineate a clear and reversible role for Kupffer cell p38 MAPK in the hepatic response to intoxication and burn. The ability to manipulate hepatic IL-6 production through specifically targeting Kupffer cell p38 may be a more viable clinical approach than global p38 inhibition or impairment of Kupffer cell function which work at the expense of other critical functions of this pathway and cell, respectively. Inhibition of p38 in animal models has been shown to benefit local wound inflammation and remote organ damage after a burn when applied topically (45) or given systemically (29–33). However, clinical trials of p38 inhibitors for chronic inflammatory conditions have been disappointing in efficacy and fraught with adverse events, likely due to the ubiquitous and multi-functional nature of p38. Therefore, specifically targeting Kupffer cell p38, perhaps through packaging into liposomes, may be a superior treatment for patients intoxicated at the time of a burn.

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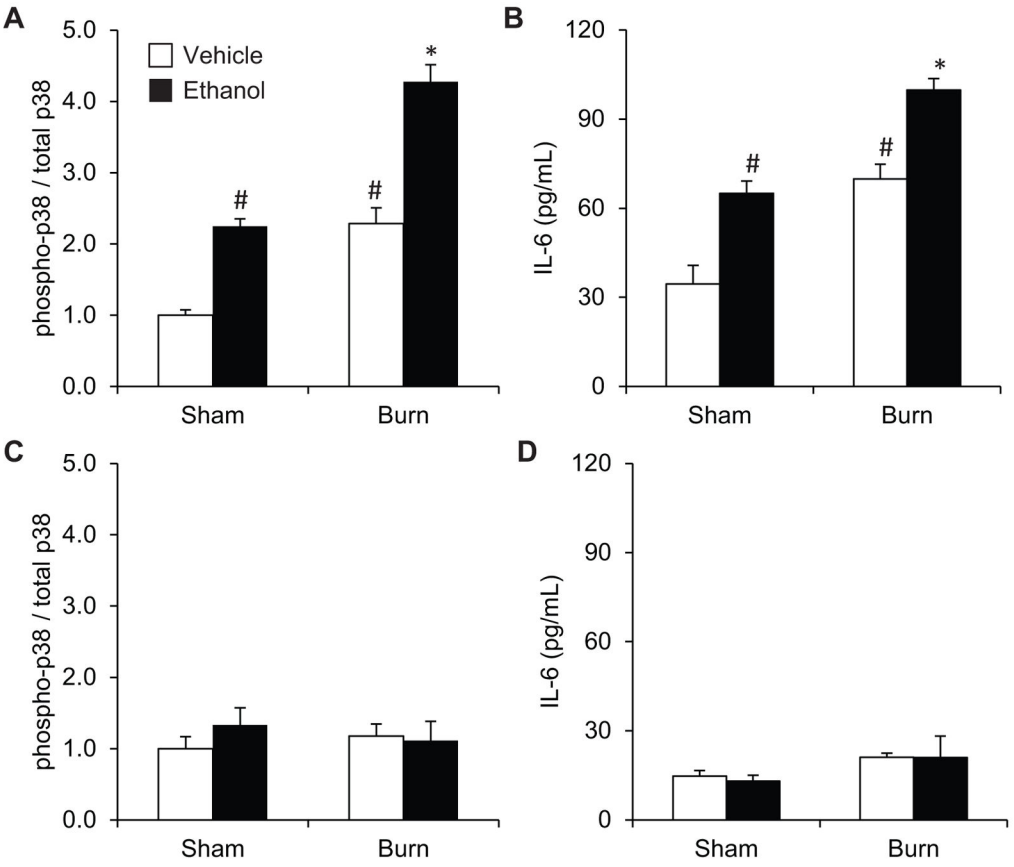

Figure 1. Kupffer cell p38 activation is increased when intoxication precedes burn

Kupffer cells were isolated 24 hours after injury and assessed for (A) p38 activation and (B) IL-6 production after LPS-stimulation, which were also assessed (C and D) in the presence of the p38 inhibitor, SB203580. *p<0.05 by compared to all groups by one-way ANOVA with Tukey's post hoc. #p<0.05 compared to Sham Vehicle by one-way ANOVA with Tukey's post hoc. N=4–6 animals per group.

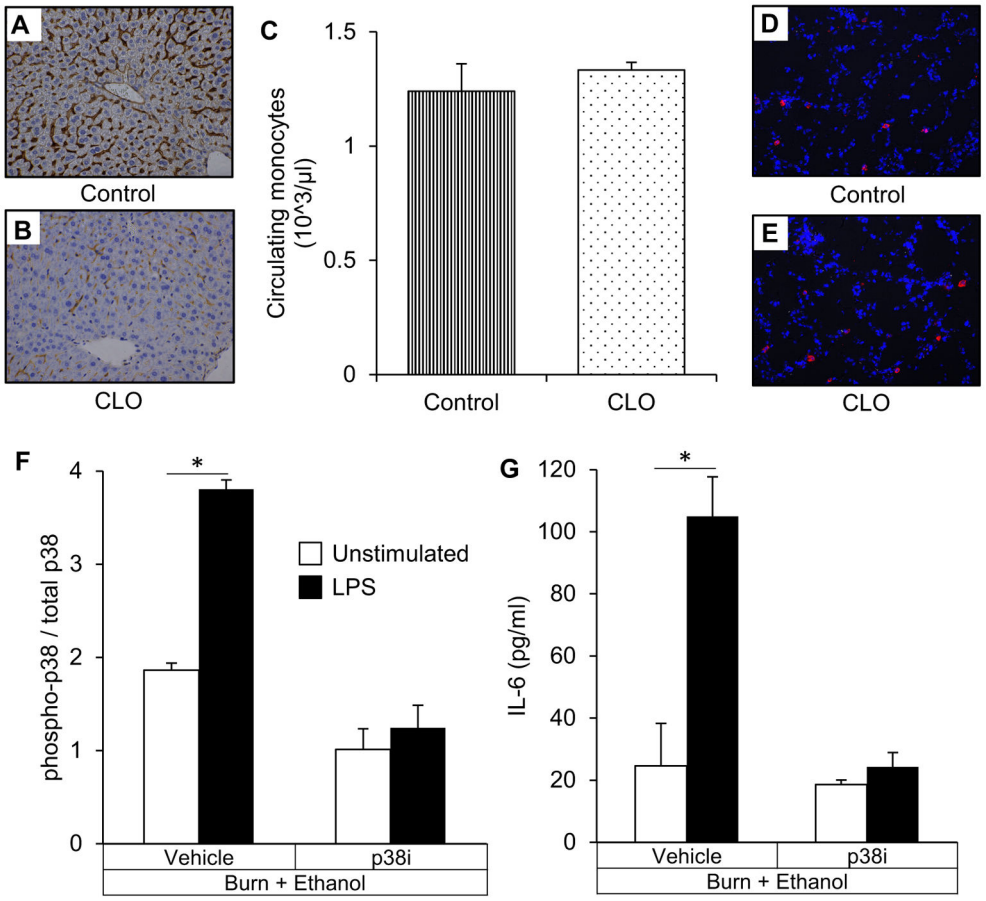

Figure 2. Selective depletion of Kupffer cells and *in vivo* Kupffer cell p38 inhibition

(A) Liver sections immunostained for CD68 from mice receiving empty liposomes. (B) Liver sections immunostained for CD68 from mice receiving clodronate liposomes. (C) Peripheral blood monocytes. (D) Lung sections stained for alveolar macrophage marker SiglecF from mice receiving empty liposomes. (E) Lung sections stained for alveolar macrophage marker SiglecF from mice receiving clodronate liposomes. (F) p38 activation in isolated Kupffer cells from animals of the Burn + Ethanol Subgroup with and without LPS stimulation. Within the subgroup, animals given *in vivo* SB203580 (p38i) or vehicle control are shown. (G) IL-6 production in isolated Kupffer cells from animals of the Burn + Ethanol Subgroup with and without LPS stimulation. *p<0.05 by Student's T test. N=3–6 animals per group.

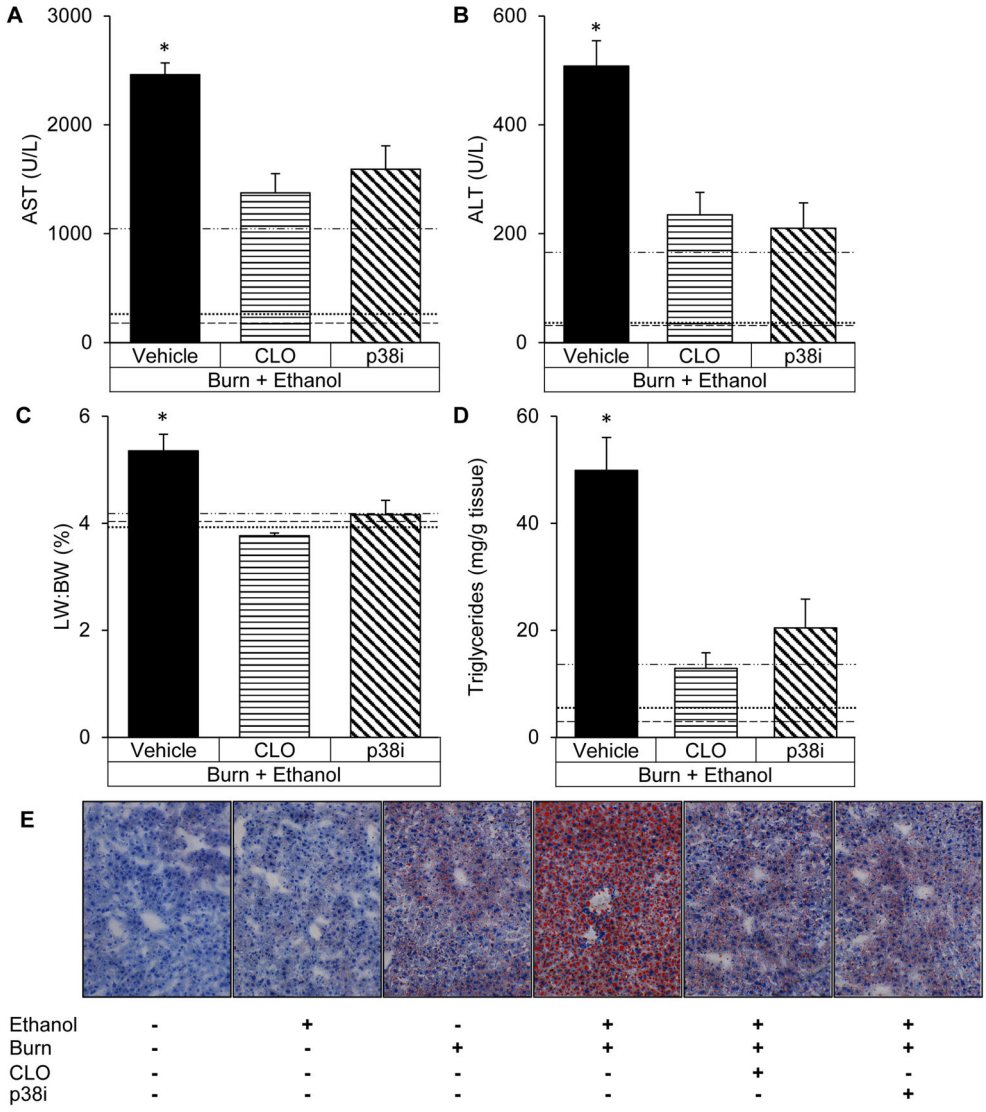

Figure 3. The absence of Kupffer cells or p38 inhibition equally attenuate hepatic damage

(A) Serum levels of aspartate aminotransferase (AST) after treatment with vehicle, clodronate liposomes (CLO) or SB203580 (p38i). (B) Serum levels of alanine aminotransferase (ALT). (C) Liver weight to total body weight ratio (LW:BW). (D) Triglycerides in liver homogenates after intoxication and burn. (E) Liver sections stained with Oil Red O to visualize triglyceride accumulation at 400x magnification. *p<0.05 compared to CLO and p38i by Student's T test. N=4–6 animals per group. ------ represents Sham Vehicle, -------- represents Sham Ethanol, and –·–·– represents Burn Vehicle.

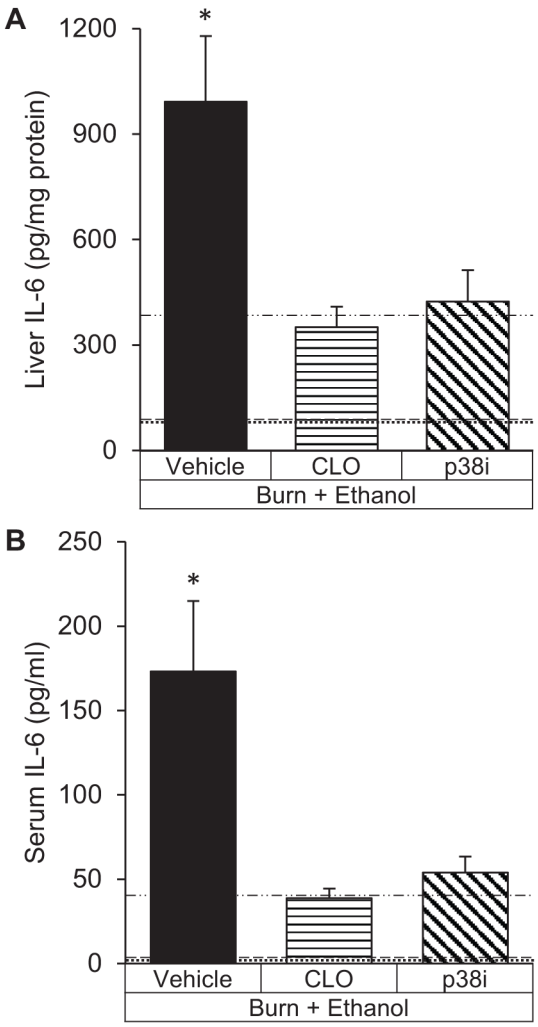

Figure 4. Interleukin-6 production is decreased with clodronate liposomes (CLO) or SB203580 (p38i) after intoxication and burn

(A) Interleukin-6 (IL-6) protein levels in liver homogenates. (B) Serum IL-6 levels. *$p<0.05$ compared to CLO and p38i by Student's T test. N=4–6 animals per group. ------ represents Sham Vehicle, ------- represents Sham Ethanol, and –·–·–·– represents Burn Vehicle.

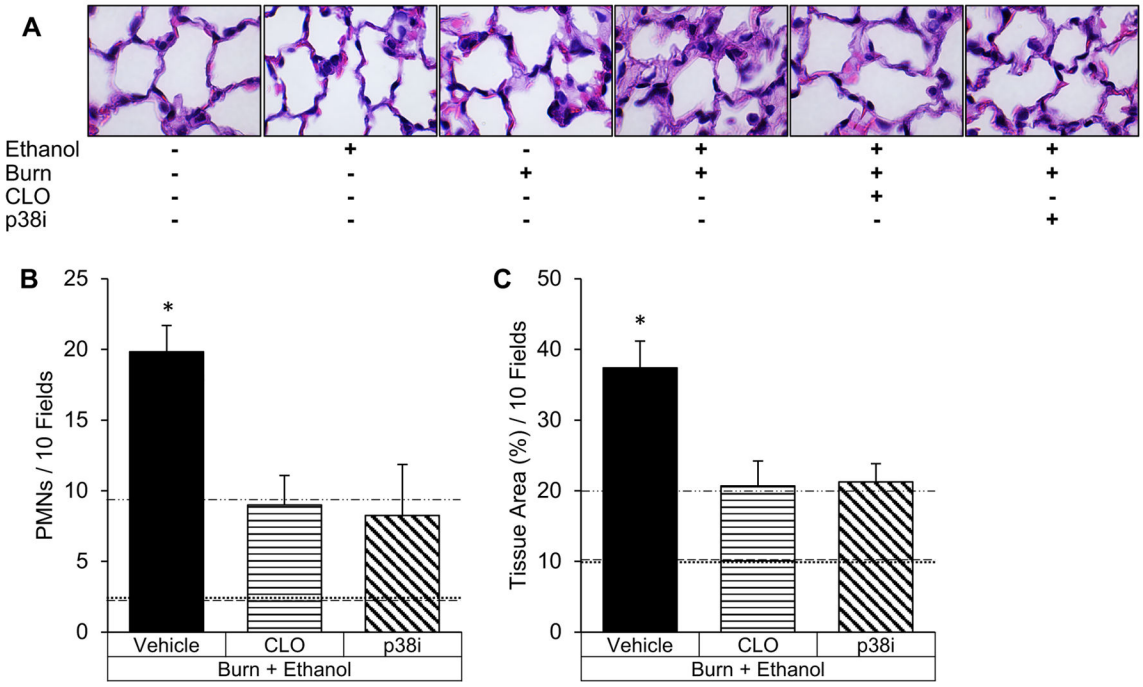

Figure 5. Pulmonary inflammation after intoxication and burn is attenuated by Kupffer cell depletion or p38 inhibition

(A) Lung histology (hematoxylin and eosin at 400x) of intoxicated and burn injured mice receiving clodronate liposomes (CLO) or SB203580 (p38i). (B) Neutrophil (PMN) quantification in 10 high power fields of view of intoxicated and burn-injured mice. (C) Pulmonary congestion quantified using imaging software to calculate the lung tissue area in 10 fields of view. *p<0.05 compared to CLO and p38i by Student's T test. N=4–6 animals per group. ------ represents Sham Vehicle, -------- represents Sham Ethanol, and –·–·– represents Burn Vehicle.