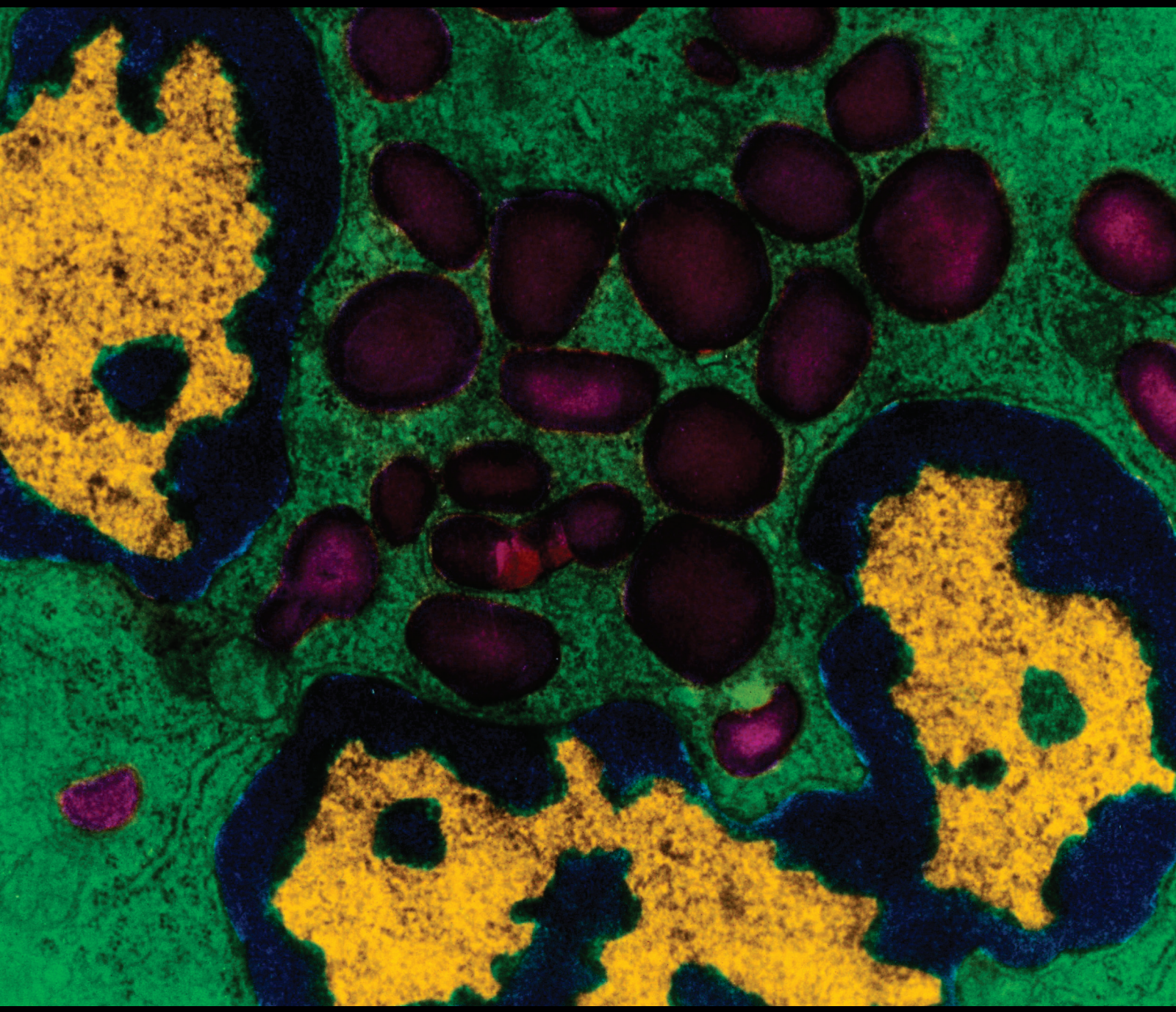
Mediators of Inflammation

Modulation of Inflammation by Alcohol Exposure

Guest Editors: Mark Lehnert, Elizabeth J. Kovacs, Patricia Molina, and Borna Relja



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Editorial Board

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Editorial

Modulation of Inflammation by Alcohol Exposure

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The morbidity and mortality resulting from alcohol-related diseases globally impose a substantive cost to society [1, 2]. Alcohol consumption is a major risk factor for all types of injuries [3] and excessive alcohol consumption is the third leading cause of preventable death worldwide. Besides its multiorgan system effects, ethanol exposure is known to cause changes in the physiological response following inflammatory stimuli leading to increased morbidity and mortality. Multiple studies have demonstrated that alcohol significantly affects the immune system and that modulation of inflammatory reactions seems not only to depend upon the pattern of exposure (acute ethanol intoxication—binge like ethanol consumption—chronic ethanol abuse) but also differs depending on the underlying insult that causes the inflammatory reactions (i.e., hemorrhagic shock-burns) [4]. In the present special issue, 5 original research reports as well as a clinical study and a review report elucidate the influence of alcohol on the pathophysiology of inflammation. This featured topic issue contains reports that highlight the impact of alcohol on responses to traumatic injury. In addition, it features reports describing the inflammatory responses associated with alcoholic liver disease and the impact of alcohol on lung epithelial cell function. Together these reports add to our body of knowledge of the significant detrimental impact of alcohol on systemic responses to injury and on its contribution to disease progression. The study-called "*Heavy ethanol intoxication increases proinflammatory cytokines and aggravates hemorrhagic shock-induced organ damage in rats*" by T. M. Hu and colleagues demonstrates that the high levels of proinflammatory mediators generated after the combined insult of acute ethanol administration and hemorrhagic shock cause both immunosuppression and resulted in excessive tissue damage relative to either insult alone. These studies examined liver, kidney, and pulmonary, responses.

In a study by M. M. Chen, entitled "*Intoxication by intraperitoneal injection or oral gavage equally potentiate post-burn organ damage and inflammation*" reveals that the route of administration of alcohol (oral gavage versus intraperitoneal injection) similarly effects postburn responses. M. M. Chen et al. found that intoxication potentiates post-burn damage in the ileum, liver, and lungs of mice to an equivalent extent when either ethanol administration route is used. Moreover, they showed comparable systemic changes including the hematologic response and serum levels of proinflammatory mediators, including interleukin-6 (IL-6).

In a review entitled "*The effect of inflammatory cytokines in alcoholic liver disease*," H. Kawaratani and colleagues compared the roles of pro- and anti-inflammatory mediators in the liver in a rat model of alcoholic liver disease. Their model suggests that cytokines, chemokines, oxidative stress, and microbial flora play a role in the development and progression of alcoholic liver disease. The review describes the link between the derived endotoxins and the activation of Kupffer cells through the LPS/Toll-like receptor (TLR) 4 pathways.

"*The study called Association of serum adiponectin, leptin and resistin concentrations with the severity of liver dysfunction and the disease complications in alcoholic liver disease*" by B. Kasztelan-Szczerbinska and coworkers investigate serum

levels of adiponectin, leptin, and resistin in patients with chronic alcohol abuse and different grades of liver dysfunction, as well as alcoholic liver disease (ALD) complications in inpatients. Their data revealed both changes in some of these mediators and in difference in sex, which shows that the leptin concentrations may play a role in the observed sex difference in the development of ALD.

"*The study called Alcohol-induced liver injury is modulated by Nlrp3 and Nlrc4 inflammasomes in mice*" by D. A. DeSantis and colleagues reported on the role of members of the Nod-like receptor (NLR) family in the development of alcoholic liver disease. The authors utilized gene targeted deletions for Nlrp3 ($Nlrp3^{-/-}$) and Nlrc4 ($Nlrc4^{-/-}$) in chronic alcohol consumption model and their data suggest that the Nlrp3 inflammasome is protective during alcohol induced liver injury.

M. Lehnert and his group describe how chronic ethanol modulates inflammatory mediators, activation of nuclear factor-κB in murine Kupffer cells, and circulating leukocytes. They investigated the effect of chronic ethanol feeding on *in vivo* activation of NF-κB in circulating leukocytes and whole liver of NF-κB-EGFP reporter gene mice. The work focuses on the production of proinflammatory mediators and activation of NF-κB in Kupffer cells after chronic ethanol feeding followed by *in vitro* stimulation with lipopolysaccharide (LPS).

Shifting to the lung, Todd Wyatt and his group report on the effects of "exhalation of volatized ethanol from the bronchial circulation on bronchial epithelial cells." Their data reveal that alcohol exposure leads to an early elevation in the cilia beat frequency of bronchial epithelial cells followed by a chronic desensitization of cilia stimulatory responses. This effect is controlled, in part, by nitric oxide mediated protein kinase A activation.

Taken together, this special issue highlights up to date research on the pathophysiology of alcohol associated inflammation. Every paper opens the door to further hypothesis-driven investigations emphasizing the need for ongoing preclinical and clinical studies.

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Research Article

Chronic Ethanol Feeding Modulates Inflammatory Mediators, Activation of Nuclear Factor-κB, and Responsiveness to Endotoxin in Murine Kupffer Cells and Circulating Leukocytes

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Chronic ethanol abuse is known to increase susceptibility to infections after injury, in part, by modification of macrophage function. Several intracellular signalling mechanisms are involved in the initiation of inflammatory responses, including the nuclear factor-κB (NF-κB) pathway. In this study, we investigated the systemic and hepatic effect of chronic ethanol feeding on *in vivo* activation of NF-κB in NF-κB^{EGFP} reporter gene mice. Specifically, the study focused on Kupffer cell proinflammatory cytokines IL-6 and TNF-α and activation of NF-κB after chronic ethanol feeding followed by *in vitro* stimulation with lipopolysaccharide (LPS). We found that chronic ethanol upregulated NF-κB activation and increased hepatic and systemic proinflammatory cytokine levels. Similarly, LPS-stimulated IL-1β release from whole blood was significantly enhanced in ethanol-fed mice. However, LPS significantly increased IL-6 and TNF-α levels. These results demonstrate that chronic ethanol feeding can improve the responsiveness of macrophage LPS-stimulated IL-6 and TNF-α production and indicate that this effect may result from ethanol-induced alterations in intracellular signalling through NF-κB. Furthermore, LPS and TNF-α stimulated the gene expression of different inflammatory mediators, in part, in a NF-κB-dependent manner.

1. Introduction

Every fifth patient treated in hospital has a history of alcohol abuse [1], about 11 million people in the UK are estimated to regularly have an alcohol intoxication [2], and alcoholic liver disease (ALD) is an important outcome factor after trauma and elective surgery [3, 4]. Interestingly, chronic but not acute alcohol abuse adversely affects outcome at least in trauma patients [1, 5], and, besides, about 20% of alcoholics develop fibrosis and subsequent cirrhosis [6]. In contrast to the beneficial effect of moderate alcohol consumption, above all red wine, namely, the reported decrease of cardiovascular diseases [7, 8], these patients cope with complications such as high blood pressure, stroke, and an increased susceptibility to infections. In these patients it is widely accepted that bacteremia in blood is one of the key causes of liver injury.

Chronic ethanol abuse is known to cause disruption of the intestinal mucosal layer, leading to an increased permeability to gut-derived bacteria [9–13]. Once in the liver, endotoxin (LPS), a component of the wall of Gram-negative bacteria, binds to Toll-like receptor 4 (TLR4) and affects an intracellular signalling cascade resulting in NF-κB activation, which in turn leads to release of hepatotoxic TNF-α [14, 15].

Another mechanism, by which liver damage is caused, is the activation of liver sessile Kupffer cells. A variety of subsequent reactions leading to cell injury exist, most

notably for this study the generation and release of reactive oxygen species (ROS) and of pro-inflammatory mediators [13, 16–20]. The former occurs by catalytic activity of the transmembrane NADPH oxidase superoxideanion, which is an intermediate of ethanol metabolism, and the cytosolic NADPH oxidase [9, 21]. Kupffer cells do not only express various receptors for phagocytosis but, on activation, also produce multiple inflammatory mediators [e.g., interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α)], mainly induced by TLR4 signaling. The TLR4 pathway downstream results in activation of transcription factors, such as nuclear factor-κB (NF-κB).

NF-κB can act as an early transcription factor by modulation gene expression as no de novosynthesis is required. In most cells it is located in the cytoplasm as latent inactive IκB-bound complex and as p50/p65 heterodimer [22]. NF-κB-activating agents can induce the phosphorylation of IκB inhibitory proteins, targeting them for rapid degradation through the ubiquitin-proteasome pathway and releasing NF-κB to enter the nucleus where it modulates gene expression [23, 24].

In the present study we wanted to determine which role NF-κB plays in ethanol-induced liver injury and furthermore in activated Kupffer cells after chronic ethanol feeding. Therefore we used a NF-κB enhanced EGFP (enhanced green fluorescent protein) reporter gene mouse. As a second goal we experimentally tried to investigate the influence of ethanol preexposure (*in vivo*) on the reactivity of Kupffer cells to an *in vitro* LPS challenge.

2. Material and Methods

Male *cis*-NF-κB^{EGFP} reporter gene mice and C57BL/6 mice were exposed to chronic EtOH intake. After the 4-weeks lasting pair-feeding regime, liver tissue samples were taken to measure steatosis, histopathological changes, NF-κB activity, release of pro-inflammatory cytokines, and expression of inflammatory NF-κB target genes. Blood samples were taken to measure systemic cytokines (IL-6, MCP-1, and TNF-α), AST (aspartate aminotransferase), and the expression of leukocyte surface markers (CD11b) and NF-κB. In another experimental approach, liver was perfused for isolation of Kupffer cells from ethanol-fed (EtOH) and pair-fed mice, which were subsequently stimulated with LPS or TNF-α, respectively. Cytokines (IL-6, TNF-α), inflammatory NF-κB target genes, and the receptor density of CD11b and CD68 (Scavenger) and NF-κB in the Kupffer cell populations were measured.

2.1. Animals. Male *cis*-NF-κB^{EGFP} mice (C57BL/6 background) were kindly provided by Christian Jobin, Chapel Hill, NC, USA, and bred in pathogen free conditions at Mfd Diagnostics (Wendelstein, Germany). In this gene targeted mouse strain, EGFP expression is under the transcriptional control of NF-κB *cis*-elements; therefore NF-κB binding results in transcription of EGFP [25]. At 6–8 weeks of age, weighing 20–25 g, they were delivered to our animal facility. Specific pathogen-free wild-type (WT) C57BL/6J mice (Janvier, Le Genest-Saint-Isle, France) served as controls. All animals were housed in separate individual, filter-top cages in an air flow, light (12 h light/12 h dark cycle), and temperature controlled room with free access to food and water. Animal protocols were approved by the Veterinary Department of the Regional Council in Darmstadt, Germany.

2.2. Experimental Model. Chronic ethanol feeding protocol: mice were acclimatized to our facility for 7 days after arrival, were randomly divided into pairs, and then assigned to a 4-week pair-feeding regime of standard Lieber-DeCarli diet (Ssniff Spezialdiäten; Soest, Germany) supplemented with either maltodextrin (control group) or ethanol 6.3% (vol/vol) (EtOH group) [26, 27]. Ethanol-fed mice were allowed free access to EtOH-supplemented diet. The amount of ingested diet was determined and an equal volume of maltodextrin-supplemented diet was supplied to the pair-fed animal. Accordingly, isocaloric feeding of each individual mouse was warranted. In selected experiments mice were fed standard laboratory chow, to control for the effects of the Lieber-DeCarli diet. As rodents naturally have an aversion against EtOH, the mice in this experiment were fed a liquid diet, with a gradual increase in the dose of EtOH starting with 1.75% (v/v) for 5 days, then increasing the dose to 2.63%, 3.5%, 4.38%, and finally 6.3% (v/v). This regimen reflects chronic ethanol abuse in humans, beginning with low volumes and increasing over time. Animal preparation: sacrifice and collection of tissue and blood samples: after 28 days of feeding Lieber DeCarli diet mice were weighed and anesthetized with isoflurane (Forane isoflurane, Abbott; Wiesbaden, Germany) under a continuous flow of 1.5 L/min by a mask. Laparotomy was carried out after sterilizing the abdomen and thorax with 70% EtOH by making a median incision 1 to 2 cm above the hind legs and continuing up to sternum, followed by a horizontal incision on each side ending at the rib cage. A 24-gauge needle was inserted in the IVC (inferior vena cava) and whole blood was withdrawn and collected. After disrupting portal vein, liver was perfused with Ringer's solution, excised, and weighed to determine the liver/body ratio. After removal of the gall bladder, a section of the liver's median lobe was embedded in Tissue-Tek O.C.T Compound (Sakura Finetek; Helsinki, Finland) for cryosections. Then the left lobe was infused and fixed with 4% buffered Zn-Formalin and subsequently embedded in paraffin, sectioned (7 μm), and stained with hematoxylin-eosin (HE). The remaining liver lobes were cut into small pieces, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent examination. In another experimental approach, liver was perfused with ice-cold Hank's Buffered Salt Solution (HBSS; Gibco; w/o Ca^{2+} and Mg^{2+}) for 5 min for isolation of Kupffer cells from ethanol-, pair-, and chow-fed mice. After removing the gall bladder, liver was transferred to a sterile Petri dish containing HBSS (w/o Ca^{2+} and Mg^{2+}) placed on ice until further preparation.

Groups: see Table 1.

2.3. Kupffer Cell Preparation and Culture. Isolation of NPC (nonparenchymal cells) from liver tissue: For Kupffer cell

Table 1: Experimental groups.

	Ethanol-fed (EtOH)	Pair-fed (Ctrl)	Chow-fed (chow)
Mice strain	*cis*-NF-κB^{EGFP}	*cis*-NF-κB^{EGFP}	C57BL/6
Examination of liver tissue, blood	$n = 15$	$n = 15$	$n = 15$
Soxhlet extraction	$n = 5$	$n = 5$	$n = 5$
Isolation of Kupffer cells	$n = 15$	$n = 15$	$n = 20$

preparation the removed liver tissue was gently cut into small pieces, approximately 2 mm × 2 mm in the Petri dish on ice, and washed in a 50 mL conical tube in cold HBSS (w/o Ca^{2+} and Mg^{2+}) until the supernatant was clear. After replacement in the Petri dish on ice, liver specimens were minced further finely using a scalpel and mashed with a plunger of a 10 mL syringe. Tissue was subdivided into 2 portions; each was transferred in a C tube (Miltenyi Biotec; Bergisch Gladbach, Germany) and subsequently resuspended in 15 mL Enzyme mix [50 mg/mL collagenase IV, 100U/mL DNaseI, 5 mM $CaCl_2$, and 96 mL HBSS (w/o Ca^{2+} and Mg^{2+})]. Then, tissuse was dissociated gently by using Gentle MACS, program mouse liver (modified: "m_liver_01.02", 73 s), followed by incubation at 37°C in a water bath with agitation mode set at the highest speed for 15 min. After repeating mechanical dissociation as described above followed by another incubation step for 15 min in water bath with agitation mode set at the highest speed, tissue was completely dissociated. Homogenizate was passed through a sterile nylon 70 μm cell strainer (BD Biosciences; Heidelberg, Germany), washed twice with ice-cold DMEM [supplemented with 20% FCS, 50 μg/mL gentamycin sulphate, 20 mM HEPES], pooled, and finally resuspended in 10 mL DMEM suppl. Cell suspensions were centrifuged at 17–21 ×g for 5 min to separate hepatocytes and the resulting supernatants from two mice per treatment group were pooled. Then they were layered carefully on a 50%/25% two-step Percoll gradient (GE Healthcare; Freiburg, Germany) in a 50 mL conical tube and centrifuged 15 min at 1800 ×g, 4°C (brake off) to separate nonparenchymal cells (NPCs) from parenchymal cells. Both interface fractions containing mainly Kupffer cells were transferred into DMEM suppl., washed twice, resuspended in 1 mL DMEM suppl. to determine cell number and viability by trypan blue exclusion, and found to be ~95%. Enrichment of F4/80$^{\pm}$ Kupffer cells from NPC fraction: for selection of the F4/80 positive mononuclear cells fraction, cells suspensions were incubated with mouse FcR Blocking Reagent (Miltenyi Biotec) together with PE-conjugated anti-F4/80 Ab (Biolegend; San Diego, CA, USA) for 25 min at 4°C. After washing with MACS buffer [containing 2 mM EDTA, 0.5% BSA in PBS (w/o Ca^{2+} and Mg^{2+})], cells were magnetically labeled with anti-PE Microbeads (Miltenyi Biotec) for 15 min at 4°C and washed with MACS buffer. F4/80^{+} cells were separated over to sequential columns by positive selection using the MACS system (Miltenyi Biotec) according to manufacturer's recommendations. The eluate (F4/80^{+} cells), and the flow through (F4/80^{-} cells) were collected and purity of magnetic separation was determined by flow cytometric analysis on FACSCalibur (BD Biosciences; Heidelberg, Germany). Additionally vitality of isolated cells was evaluated by 7-AAD staining (BD Biosciences). Isolated Kupffer cells were suspended in DMEM suppl. and plated onto 24-well culture plates. After 2 h the media were replaced to remove nonadherent cells. After 16–18 h, cells were stimulated or not with either LPS (10 μg/mL; Sigma; Deisenhofen, Germany) or TNF-α (500 ng/mL; R&D Systems), for 2, 4, or 24 h, respectively.

2.4. Measurement of Steatosis and Serum Enzyme Levels after Ethanol Feeding. Serum aspartate aminotransferase (AST) was detected using a dry chemistry analyzer (Spotchem EZ; Arkray, Philippines). Fat content was determined quantitatively by means of Soxhlet extraction technique as described elsewhere [28]. In brief, samples of dried and pulverized liver tissue were weighed and afterwards placed in an extraction thimble. Petroleum ether was used as solvent. By heating the water bath around the flask, the solvent is boiled and the vapour passes the condenser. Whereas ten reflux cycles were finished, whole fat has accumulated in the bottom flask and was weighed.

2.5. Analysis of Proinflammatory Changes due to Chronic Ethanol Intake. Whole blood stimulation assay: monocyte activity was evaluated by whole blood stimulation assay with 10 μg/mL endotoxin (LPS) from *Escherichia coli* 0127:B8 (Sigma) in RPMI 1640 medium (Sigma) and incubated for 24 h at 37°C and 5% CO_2. A negative control lacking LPS for every assay was performed. Afterwards, blood cells were sedimented by centrifugation (2000 ×g, 10 min) and supernatants were collected and stored at −80°C. The IL-1β concentration was monitored using a Quantikine Mouse IL-1β ELISA kit following the manufacturer's instructions (R&D Systems). Quantification of cytokine levels: the release of IL-6, MCP-1, and TNF-α in plasma or culture supernatants was measured using flow cytometry with FACSCalibur (BD Biosciences; Heidelberg, Germany) and Mouse IL-6, MCP-1, and TNF-α Flex Set with a cytometric bead array according to the manufacturer's instructions (BD Biosciences). Concentrations of hepatic IL-6 in protein lysates extracted from snap-frozen liver tissue samples were determined using a Quantikine Mouse-IL-6 ELISA kit according to the manufacturer's instructions (R&D Systems). The ELISA 96-well microtiter plates were analyzed using a microplate reader Bio-Tek Ceres UV900C (Bio-Tek; Winooski, VT, USA). Determination of EGFP and CD11b cell surface expression in circulating neutrophils: Flow cytometry was performed to detect NF-κB enhanced GFP and CD11b expression on the surface of leukocytes, as described in detail elsewhere [29]. Briefly, RBC-depleted peripheral blood cells were stained with anti-CD11b-PerCP-Cy5.5 (BD Biosciences). After washing with PBS containing 0.5% bovine serum albumin, cells were analyzed by a FACSCalibur (BD Biosciences). Polymorphonuclear neutrophils (PMNLs) were identified by their forward/side scatter characteristics (R2, Figure 1(a)). EGFP (FL-1) versus CD11b (FL-3) of the isotype control is presented (Figure 1(b):

pair-fed; Figure 1(c): EtOH-fed). Data analysis was carried out using CellQuest Pro (BD Biosciences).

2.6. Visualization of cis-NF-κB^{EGFP} Transcriptional Induction in Liver Tissue. EGFP in tissue specimens from *cis*-NF-κB^{EGFP} mice was detected by epifluorescence microscopy. Tissue samples were fixed with 10% Zinc-Formalin for 24 h and paraffin-embedded. Sections were cut 5 μm and EGFP expression was visualized by using the FITC reflector of Axio Observer Z1 (Carl Zeiss MicroImaging; Jena, Germany) with identical exposure times for each data point. Localization and cellular expression pattern of activated NF-κB/GFP were further assessed by immunocytochemistry. Liver sections were fixed and cut as described and then incubated with anti-GFP antibody (1 : 400, 60 min, RT; Abcam; Cambridge, UK). An anti-rabbit horseradish peroxidase linked secondary antibody (30 min, RT; Histofine; Nichirei, Tokyo, Japan) and diaminobenzidine (Peroxidase EnVision Kit, DakoCytomation; Hamburg, Germany) were used to detect specific binding, followed by counterstaining with hematoxylin.

2.7. Detection of NF-κB Activated Kupffer Cells. Paraffin-embedded liver sections (5 μm) were deparaffinized and rehydrated. Macrophages were visualized using anti-F4/80-PE monoclonal antibody (Biolegend; San Diego, CA, USA) diluted 1 : 100 in phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumin for 1 h. After washing with PBS, nuclei were counterstained with mounting medium containing 1.5 μg/mL DAPI (Vector Laboratories; Burlingame, CA, USA). Fluorescence was visualized using multichannel fluorescence capturing with the reflectors DAPI-DNA (nuclei), FITC (EGFP), and Rhodamine (F4/80) of the Axio Observer Z1 microscope (Carl Zeiss MicroImaging; Jena, Germany). Representative images were captured from ten random fields with identical exposure times for each data point (×400).

2.8. Analysis of Kupffer Cell Subtypes by Staining of Characteristic Coreceptors. After isopycnic centrifugation with Percoll, a portion of the collected cell suspensions (~ 5×10^5 cells) was stained with combinations of fluorochrome-conjugated antibodies against CD11b, CD68, and F4/80: F4/80-PE (Biolegend), CD11b-PerCP-Cy5.5 (BD Biosciences), and CD68-Alexa Fluor 684 (AbD Serotec). Fluorochrome-labeled isotype identical antibodies served as control. After 25 min of incubation at 4°C, cells were washed and percentage of Kupffer cell subtypes and amount of NF-κB activation (EGFP, FITC channel) were determined using FACSCalibur flow cytometer (BD Biosciences).

2.9. Quantification of NF-κB Activation in LPS Stimulated Kupffer Cells. To determine the proportion of NF-κB activated Kupffer cells, 2 h, 4 h, or 24 h, respectively, after LPS stimulation, EGFP$^+$ (green), F4/80$^+$ (red), and colabeled (orange) cells were counted. Fluorescence was visualized using multichannel fluorescence. Images were taken with the reflectors FITC (EGFP) and Rhodamine (F4/80) of Axio Observer Z1 (Carl Zeiss MicroImaging). Representative images were captured from ten random fields with identical exposure times for each data point (×400).

Table 2: Primers used for qRT-PCR of Kupffer cells.

Gene name	RefSeq accession no.	UniGene no.
Mouse CXCL-1	NM_008176.2	Mm.21013
Mouse IL-6	NM_031168.1	Mm.1019
Mouse MMP9	NM_013599.2	Mm.4406
Mouse NOS2	NM_010927.3	Mm.2893
Mouse TNF	NM_013693.2	Mm.1293

CXCL-1: chemokine (C-X-C motif) ligand 1; IL-6: interleukin 6; MMP9: matrix metalloproteinase 9; NOS2: nitric oxide synthase 2 (inducible); TNF: tumor necrosis factor.

2.10. Investigation on Gene Expression of Inflammatory NF-κB Target Genes in Kupffer Cells of Ethanol-Fed Mice after LPS and TNF-α Challenge. To examine the expression of TNF-α, IL-6, matrix metalloproteinase-9 (MMP-9), CXCL-1, and NOS2, total RNA was extracted using the RNeasy-system (Qiagen; Hilden, Germany) according to the manufacturer's instructions, after collecting the supernatants from the LPS stimulated Kupffer cells. The residual amounts of DNA remaining were removed using the RNase-Free DNase Set according to the manufacturer's instructions (Qiagen). Quality and amount of the RNA were determined photometrically using the NanoVue Plus device (GE Healthcare; Munich, Germany). Reverse transcription was carried out subsequently with Omniscript (Qiagen; Hilden, Germany) using the AffinityScript PCR cDNA Synthesis Kit (Stratagene; La Jolla, CA, USA). qRT PCR reactions were performed using Stratagene MX3005p QPCR system (Stratagene) with specific primers for target genes (Table 2) and 18S ribosomal RNA as a reference gene, all purchased from SA Bioscience (SuperArray; Frederick, MD, USA). PCR reaction mixtures (25 μL) were performed using 1X RT^2 SYBR Green/Rox qPCR Master mix (SA Bioscience) according to manufacturer's instructions. Amplification of cDNA was initiated with 10 min of denaturation at 95°C followed by 40 cycles with 15 s denaturation at 95°C and 60 s annealing/extension at 60°C. A melting-curve analysis was applied to control the specificity of amplification products. Relative expression of each target gene's mRNA level was then calculated using the comparative threshold-cycle (CT) method ($2^{-\Delta\Delta CT}$ method). In brief, the amount of target mRNA in each sample was first normalized to the amount of *18S ribosomal* mRNA to give ΔCT and then to a calibrator consisting of samples obtained from the stimulation-Ctrl group. The relative mRNA expression of target genes is presented as fold increase calculated in relation to stimulation control (medium) after normalization to *18S ribosomal RNA*.

2.11. Statistical Analysis. Data are presented as mean ± SEM (standard error of the mean). A P value of less than 0.05 was considered significant. Differences between means were

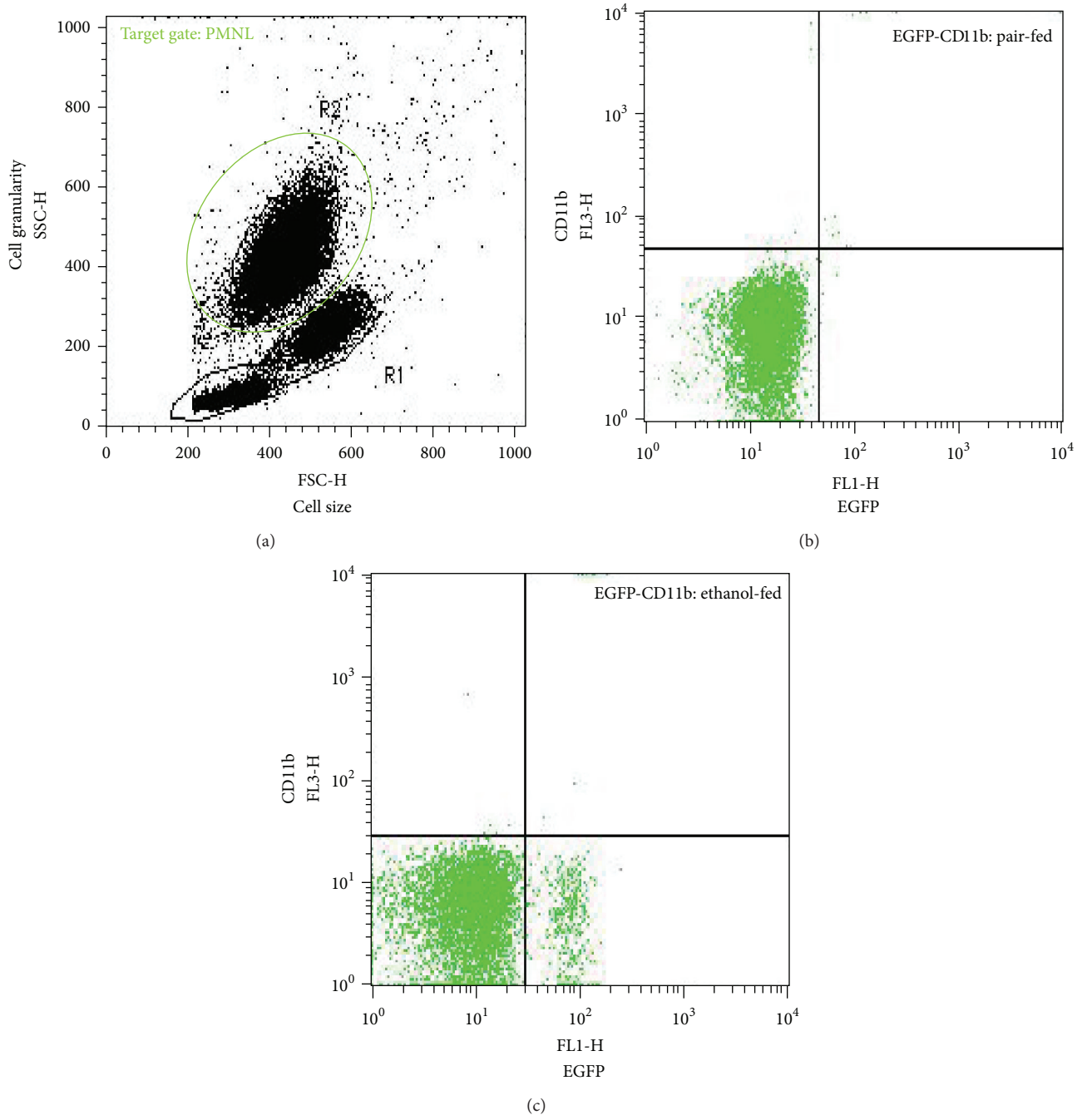

Figure 1: Gating strategy for the determination of PMNL. Representative FACS diagrams are shown. In (a), blood samples, obtained from *cis*-NF-κB^{EGFP} after feeding Lieber-DeCarli diet for 4 weeks, were analyzed and the region (R2) was set according to the forward/side scatter characteristics (FSC/SSC) of PMNL. Dot plots depict EGFP expression versus isotype control CD11b staining from (b) pair- and (c) ethanol-fed mice.

determined by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons.

3. Results

3.1. Liver Injury due to Ethanol Feeding. Feeding of ethanol containing Lieber DeCarli diet for 28 days increased the relative liver weight (liver/body weight ratio 4.3 ± 0.1 versus 5.85 ± 0.2, $P < 0.05$; Figure 2(a)). Quantification of dry fat content of whole liver tissue revealed an increase when compared to the control group ($P < 0.05$, Figure 2(b)). EtOH feeding caused an elevation in serum AST to 132.2 ± 8.1 U/L when compared to pair-fed mice ($P < 0.05$, Figure 2(c)). Feeding the maltodextrin containing Lieber-DeCarli diet revealed the same effect on the aforementioned

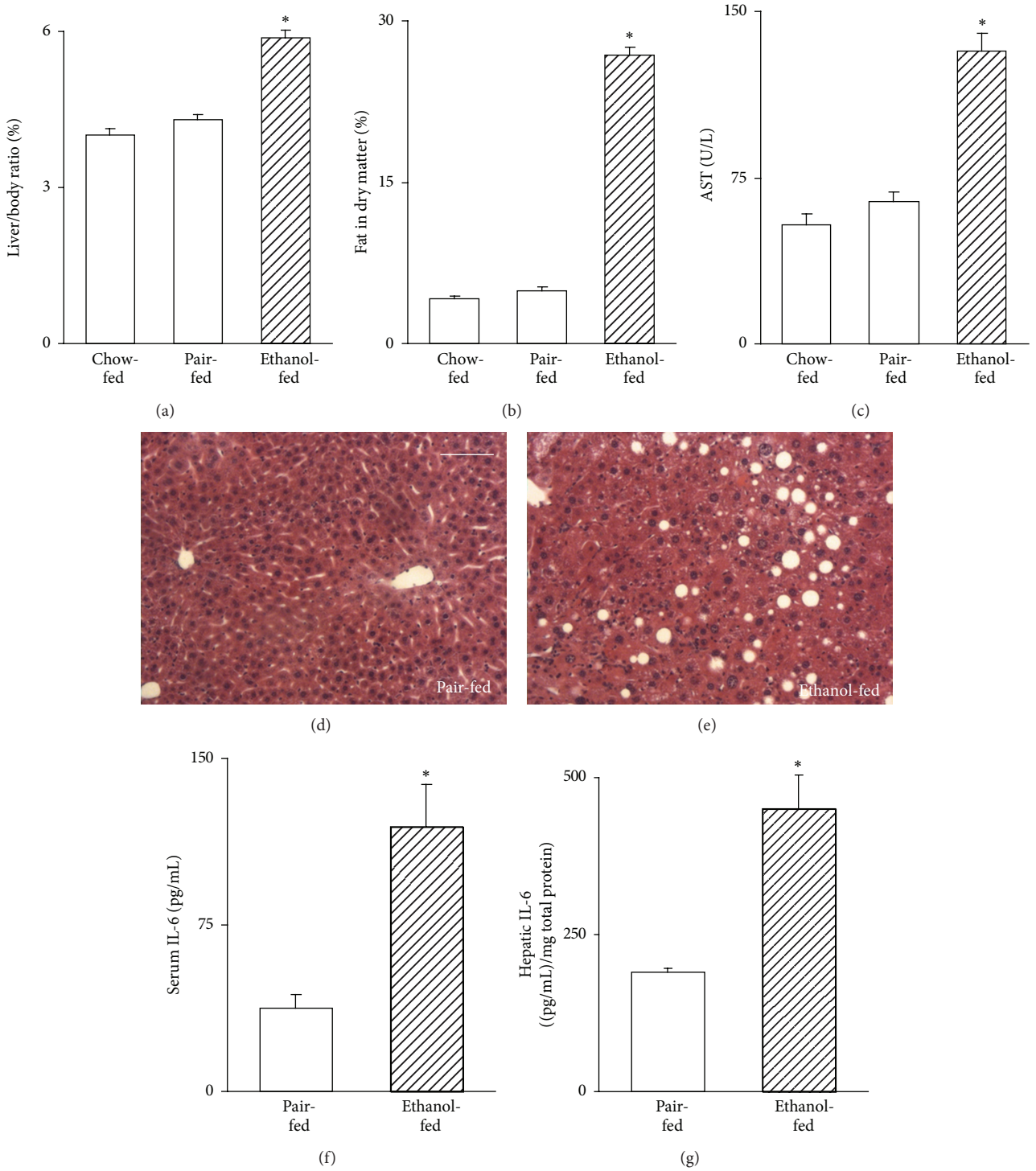

Figure 2: EtOH-containing liquid diet affects fatty liver and increased proinflammatory IL-6 release 4 weeks after feeding mice an ethanol (EtOH-fed) or control (pair-fed) Lieber DeCarli diet; blood samples and livers were harvested as described in Section 2. Chow-fed animals served as internal controls for the pair feeding approach. Data are given as mean ± SEM. $P < 0.05$ versus all. In (a), liver body ratio from pair-fed mice is presented. Hepatic dry fat content was quantified by means of Soxhlet technique as described in Section 2. section (b). Serum aspartate aminotransferase levels were measured (c). Representative photomicrographs of HE stained liver sections from (d) pair- and (e) ethanol-fed mice are presented. Bar equals 100 μm. In (f), systemic levels of IL-6, and in (g), hepatic IL-6 proteins are shown. $^{*}P < 0.05$ versus pair-fed Ctrl.

physiological liver markers as feeding a regular chow food diet. These results demonstrate the effectiveness of the pair feeding approach to study the effects of ethanol feeding while an equicaloric condition is maintained and no hepatic changes are induced by the maltodextrin containing diet.

3.2. Effects of Ethanol Diet on the Local and Systemic Inflammatory Response. Chronic EtOH feeding caused a systemic inflammatory response, as determined by circulating levels of IL-6, MCP-1, and TNF-α. The concentration of IL-6 rose markedly in the EtOH-fed group when compared to the control group (119.14 ± 19.4 versus 37.73 ± 6.1 pg/mL, respectively, $P < 0.05$; Figure 2(f)). The same effect was observed for levels of MCP-1 (287.2 ± 45.4 versus 85.75 ± 28.7 pg/mL, $P < 0.05$; data not shown) as well as TNF-α (8.26 ± 2.2 versus 2.72 ± 1 pg/mL, $P < 0.05$; data not shown). Interestingly, chronic EtOH intake caused a local hepatic IL-6 release when compared to pair-fed mice with EtOH-free diet (450.87 ± 52.8 versus 189.39 ± 7.5 (pg/mL)/mg protein, $P < 0.05$; Figure 2(g)) but without histopathological evidence for steatohepatitis (Figure 2(e)).

LPS-stimulated monocyte cytokine production: the *in vitro* production of IL-1β in whole blood was higher in ethanol-fed *cis*-NF-κB^{EGFP} mice when compared to pair-fed controls after LPS stimulation which was comparable to mice fed a regular chow diet (84.43 ± 31.4 versus 18.5 ± 3.3 pg/mL; $P < 0.05$; data not shown).

Ethanol feeding primed peripheral blood neutrophils: chronic EtOH intake activates circulating polymorphonuclear leukocytes (PMNLs) as indicated by FACS analysis. The expression of a prerequisite surface marker to migrate through the endothelium, the integrin Mac-1 (CD11b/CD18), and the expression of EGFP, representing NF-κB activation, were investigated in peripheral blood samples collected after chronic ethanol feeding. After pair feeding, only 0.3% of PMNLs showed coexpression of CD11b/CD18 and EGFP and this part rose markedly to 7.7% after ethanol pretreatment. Total EGFP expression was increased in ethanol-primed CD11b$^+$ PMNL (Figure 3(c)).

3.3. Intensified Expression of cis-NF-κB^{EGFP} in Liver Tissue. To assess the time and site specific expression of EGFP representing sites of NF-κB activation after chronic ethanol abuse, paraffin-embedded liver sections were analyzed by epifluorescence microscopy. An increased NF-κB transcriptional activity was present in EtOH treated mice (Figure 4(b)) compared to pair-fed mice (Figure 4(a)). Bias from hepatic autofluorescence was eliminated by immunostaining liver sections with an anti-GFP antibody and again more GFP was present after ethanol feeding (Figures 4(c)–4(f)). Furthermore, tissue was immunostained with F4/80 to identify mouse macrophages, mostly liver sessile Kupffer cells. After pair feeding, only F4/80 positive Kupffer cells were detected whereas, after ethanol feeding, the proportion of cells coexpressing EGFP and F4/80$^-$ resulting in a yellow type fluorescence was largely elevated (Figures 4(g) and 4(h)). Interestingly, in ethanol-fed mice, EGFP-positive Kupffer cells were mainly located in periportal and midzonal areas, whereas EGFP-positive hepatocytes could be found mostly in pericentral and midzonal areas. These observations indicate that the ethanol-containing diet influences both quantity and topography of NF-κB activation in hepatocytes and macrophages.

Table 3: CD11b and CD68 expression of F4/80$^+$ cells in the liver.

	Ethanol-fed (EtOH)	Pair-fed (Ctrl)
%CD11b$^+$ of F4/80$^+$ cells	24.4 ± 2.3	12.3 ± 1.2
%CD68$^+$ of F4/80$^+$ cells	41.5 ± 5.1	12.9 ± 1.8

CD11b and CD68 expression of liver F4/80$^+$ cells, isolated from EGFP reporter gene mice after 4 weeks lasting Lieber-DeCarli pair-feeding regime. Data are percentages (mean ± SEM) of five mice in each group with similar results.

3.4. Expression of CD11b and CD68 on F4/80$^+$ Kupffer Cells. Freshly isolated Kupffer cells demonstrated a significant surface expression of CD11b and CD68 with expression of all receptors more pronounced in macrophages from ethanol-fed mice (Table 3).

3.5. NF-κB in Kupffer Cells Is Activated by Both Ethanol Feeding and In Vitro LPS Stimulation. NF-κB activation in isolated KC was enhanced after chronic ethanol feeding when compared to pair-fed mice (unstimulated Ctrl: Figure 5(a), unstimulated EtOH: Figure 5(b)). Both after pair feeding and ethanol diet, the proportion of activated KC was largely enhanced at 4 h after LPS stimulation (Ctrl: Figure 5(c), EtOH: Figure 5(d)). Interestingly, the percentage of KC with activated NF-κB does not further increase at 24 h after LPS stimulation (Figure 5(e)).

3.6. NF-κB Activation in Kupffer Cells Is Associated with Release of Proinflammatory Cytokines. To analyze the inflammatory potential of Kupffer cells obtained from ethanol-fed mice and pair- and chow-fed controls, we measured the concentrations of several cytokines in the supernatants 2 h, 4 h, and 24 h after endotoxin stimulation. IL-6 release was the highest after 24 h LPS and EtOH diet (Figure 6(a)); TNF-α rose to the highest levels 2 h after LPS treatment and EtOH feeding (Figure 6(b)). Again, cytokine production of Kupffer cells from mice fed with the maltodextrin augmented Lieber DeCarli diet did not differ from mice fed with a regular chow diet. These results indicate that the increased NF-κB activation in KC is associated with an increased production of inflammatory cytokines and, that the pair feeding of Lieber-DeCarli diet is a valuable tool to analyze even subtle changes in individual cell subsets with results that can be transferred to animals fed with the standard chow food. No significant differences were observed in both control groups.

3.7. LPS- and Ethanol-Induced Alterations in Expression of NF-κB Controlled and Proinflammatory Genes. LPS stimulation of macrophages is known to induce among others TNF-α mRNA expression through activation of the canonical NF-κB pathway [22]. To investigate whether expression of TNF-α and of other NF-κB related genes is increased in

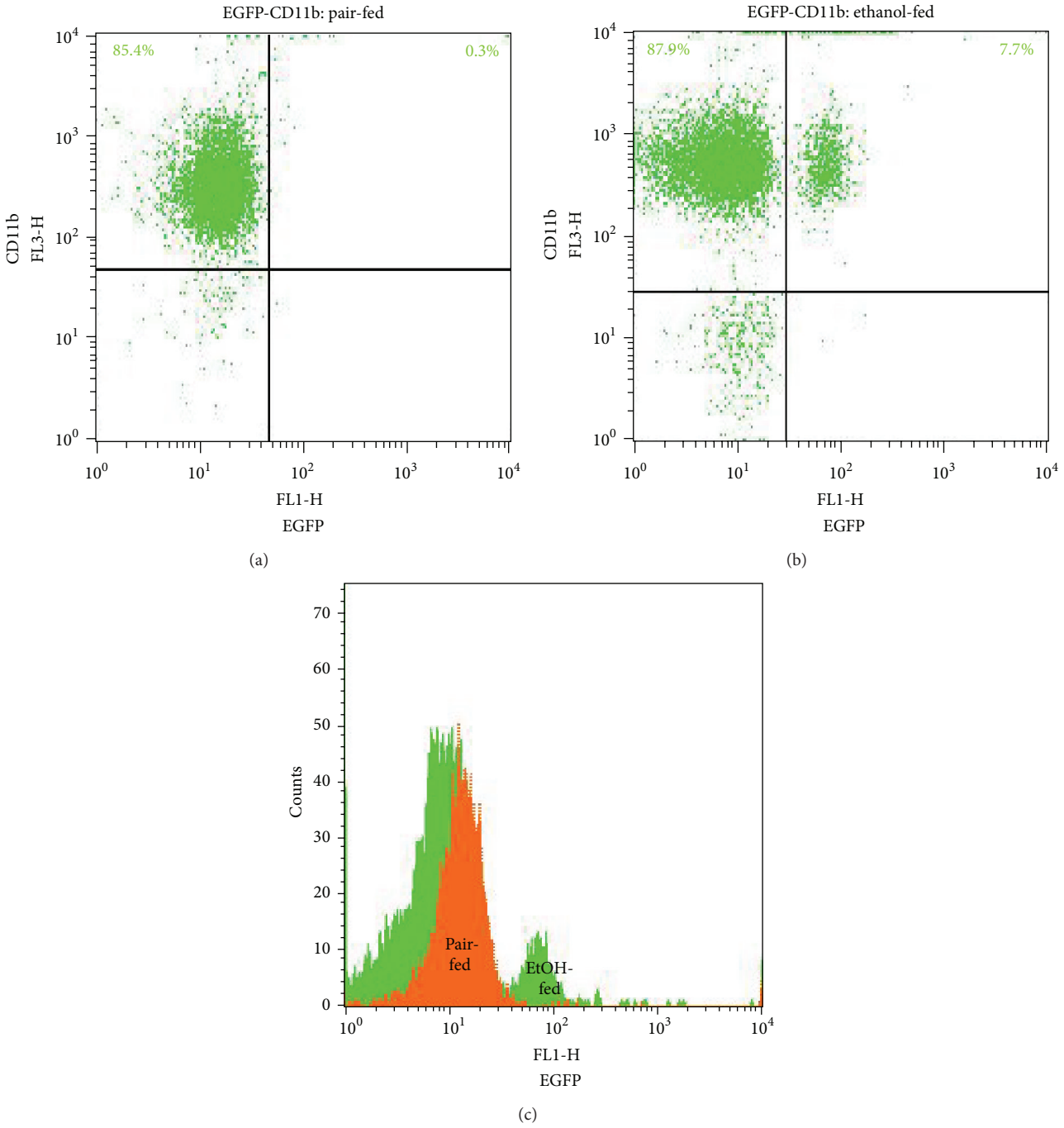

FIGURE 3: Surface expression of CD11b and transcriptional induction of the *cis*-NF-kB^{EGFP} transgene after chronic ethanol exposure. Representative FACS diagrams are shown. EGFP expressing versus CD11b^{+} PMNL, gated by their FSC/SSC properties as shown in Figure 1, were identified in a FL-1-FL-3 scattergram. The percentage of the indicated populations was determined through quadrant analysis of (a) pair- and (b) ethanol-fed mice. In (c), an overlay of FACS histograms, representative of five independent experiments, demonstrates elevated EGFP expression in PMNLs from ethanol pretreated mice. The orange-filled histogram depicts pair-fed controls and the green-filled graph ethanol-fed mice.

ethanol-primed Kupffer cells, gene expression was assessed by RT PCR. Nearly all analyzed genes, either after LPS stimulation (Figure 7) or after TNF-α stimulation (Figure 8), showed higher expressions after ethanol feeding alone and in combination with stimuli. Interestingly, stimulation with LPS and TNF-α resulted in a different activation pattern. 2 h after LPS stimulation the production of IL6 and TNF-α was largely elevated when compared to pair-fed controls (IL-6 mRNA: 164.1 ± 45.1 versus 46.0 ± 27.6, TNF mRNA: 113.0 ± 43.2 versus 18.9 ± 13.7, $P > 0.05$; Figures 7(a) and 7(b)).

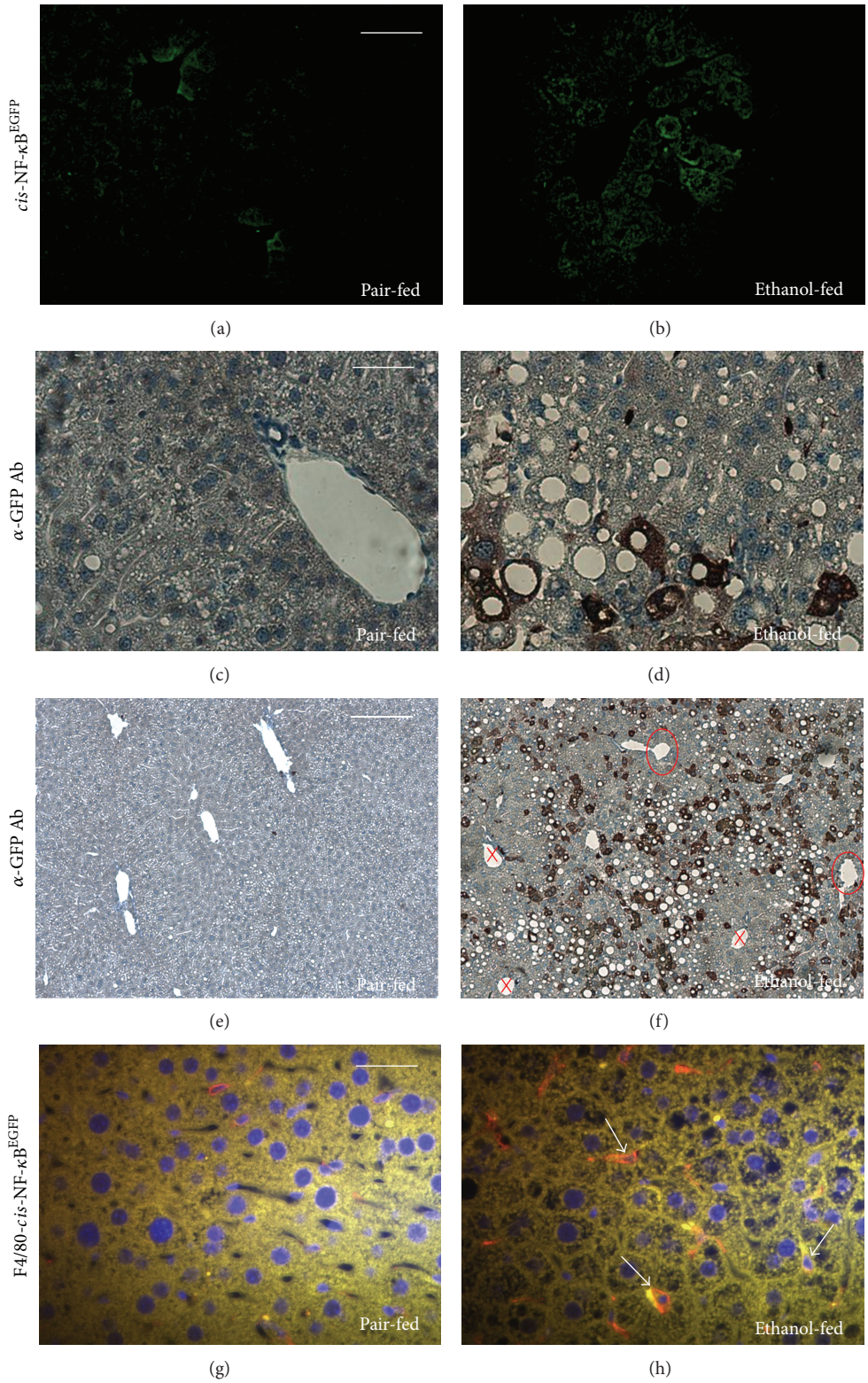

FIGURE 4: Effect of chronic ethanol on hepatic topography of *cis*-NF-κB^{EGFP} transcriptional induction and hepatic macrophage activation of NF-κB dependent EGFP expression was analyzed using fluorescence microscopy in livers harvested from *cis*-NF-κB^{EGFP} mice and prepared as described in Section 2. Representative liver lobes from pair-fed ((a), (c), (e), and (g)) and ethanol-fed ((b), (d), (e), and (h)) mice are shown. Green fluorescence by EGFP represents NF-κB activity ((a), (b): bar equals 100 μm). Additional GFP antibody staining identifies the topography of NF-κB activation ((c), (d): bar equals 50 μm; (e), (f): bar equals 200 μm). The encircled areas mark central veins; the crosses mark portal fields. Immunostaining for F4/80 (red fluorescence) identifies Kupffer cells in *cis*-NF-κB^{EGFP} mice livers; overlay images show colabeled cells, marked by arrows ((g), (h): bar equals 20 μm).

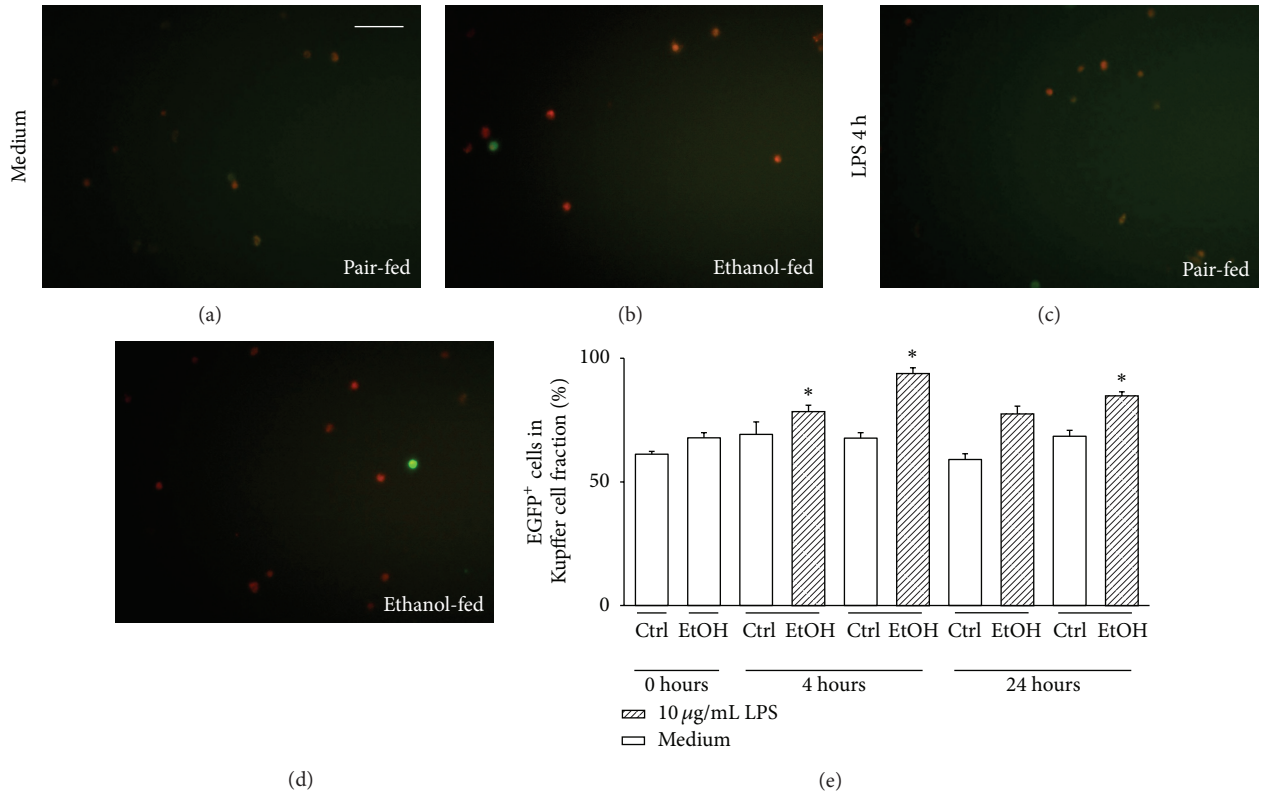

FIGURE 5: *cis*-NF-κB^{EGFP} transcriptional induction in F4/80$^+$ hepatic macrophages at 4, 24 h after *in vitro* LPS challenge prior to chronic ethanol feeding. Livers of *cis*-NF-κB^{EGFP} mice were harvested 4 weeks after Lieber-DeCarli diet treatment and Kupffer cells were purified and cultured as described in Section 2. Cells were then stimulated with 10 μg/mL LPS over a period of 24 hours. Red fluorescence (F4/80) labels Kupffer cells and green fluorescence of EGFP identifies cells expressing NF-κB transcriptional activity at 4, 24 h after LPS stimulation. (a) and (b) show representative unstimulated controls whereas in (c) and (d) representative overlay images at 4 h after LPS stimulation are given (magnification, ×200). Colabeled (yellow) cells and F4/80 positive (red) cells were counted. The percentage of F4/80$^+$EGFP$^+$ cells in total F4/80$^+$ cells is depicted in (e) at 4, and 24 h after LPS stimulation. Data shown are representative of five to eight separate experiments and are presented as mean ± SEM. $^*P < 0.05$ versus Ctrl.

4. Discussion

NF-κB plays an integral role in liver injury and inflammation as the main consequence of acute and chronic ethanol consumption [12, 16, 18, 19, 30–33]. Our study demonstrates that the inflammatory response following chronic ethanol abuse is characterized by the activation of hepatic macrophages (Kupffer cells, Figures 4–8), monocytes (data not shown), and PMNL (Figure 3) and the upregulation of pro-inflammatory mediator synthesis (Figures 2 and 6). Using a transgenic NF-κB^{EGFP} mouse model we present a strong association of these observations to the activation of NF-κB *in vivo* (Figures 3–5). Furthermore, the percentage of Kupffer cells (KC) with activated NF-κB does increase after LPS stimulation *in vitro* in ethanol-fed mice, indicating that chronic ethanol feeding does at least partially improve the ability of KC to react to a secondary stimulus such as incubation with LPS (Figure 5).

To investigate the hepatotoxic effect of chronic ethanol abuse, we used a voluntary, EtOH diet-feeding model, first described by Lieber and DeCarli, in 1967. This ad libitum model causes signs of steatosis and mild steatohepatitis [10, 34–38] that closely simulates that seen in humans following chronic ethanol consumption [39]. The final dose of 6.3% (v/v) in our model corresponds to 35% of the calorie intake as carbohydrates [35, 36]. Advantages compared to the intragastric gavage (IG) model are defined by better simulation of chronic EtOH consumption after voluntary feeding, avoidance of repeated surgery or stomach intubation, and preservation of a continuous metabolic rate in rodents [40]. Accordingly in our study, chronic EtOH intake led to a fatty liver, with increased liver to body weight ratios in ethanol-fed mice (Figure 2). Fatty liver also is the hallmark of ethanol induced liver injury in humans with EtOH metabolism in hepatocytes causing hyperplasia by the accumulation of free fatty acids in cytosol and in interstitial space (Figures 2(a) and 2(e)). Liver fat content (Figure 2(b)) was comparable to studies by others in C57BL/6 mice [10, 35]. HE staining of liver parenchyma (Figure 2(e)) as well as serum transaminase release (Figure 2(c)) revealed marked signs of steatosis and hepatocellular damage.

An increased systemic release of proinflammatory mediators such as IL-6, TNF- and macrophage chemoattractant protein- (MCP-) 1, activation of adhesion molecules such as CD11b in circulating neutrophils an increased hepatic IL-6

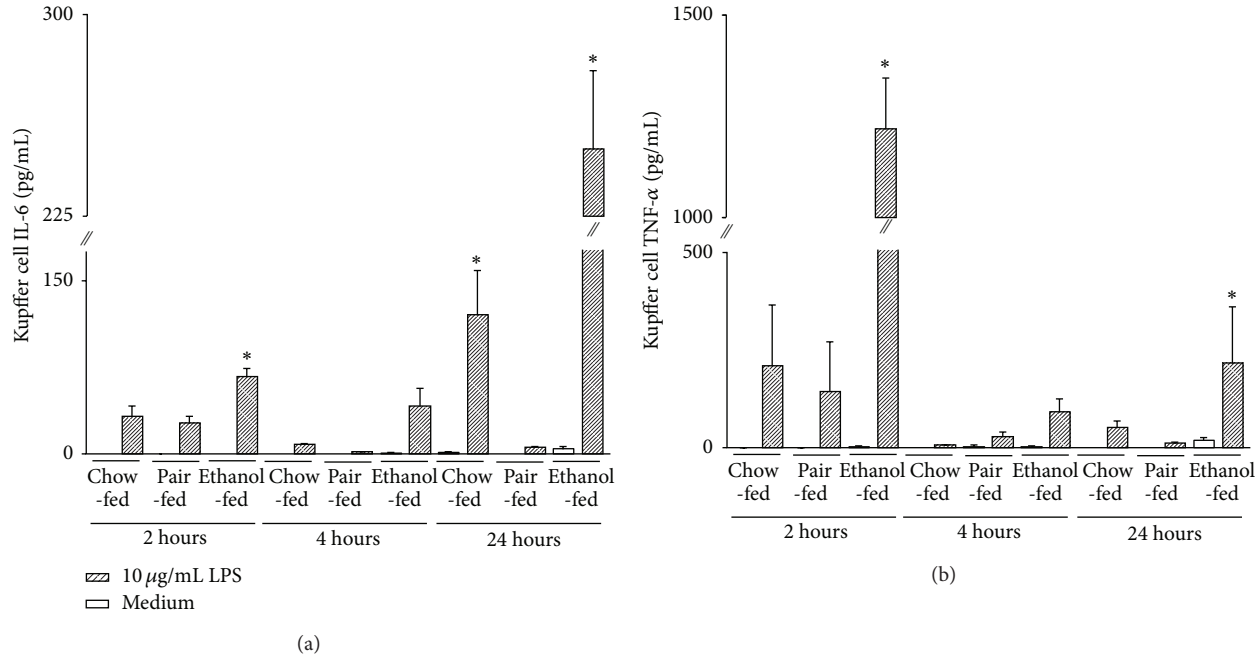

Figure 6: Proinflammatory cytokine production of F4/80^{-} hepatic macrophages following ethanol feeding in response to LPS or TNF-α. Kupffer cells were isolated from *cis*-NF-κB^{EGFP} mice after 4 weeks of pair feeding regime, as described in Section 2, and stimulated with 10 μg/mL LPS or 500 ng/mL TNF-α. Culture supernatants were collected at 2 h, 4 h, and 24 h after stimulation. IL-6 (a) and TNF-α (b) were measured by cytometric bead array (CBA). Data (mean ± SEM) are representative of five to eight independent experiments. $^{*}P < 0.05$ versus Ctrl.

level was present in ethanol-fed animals, clearly reflecting steatohepatitis; however histomorphological sequelae were not present consistent with previous reports using the Lieber DeCarli-ethanol diet (Figure 2(e) versus Figure 2(g)) [34, 37]. Further, the amount of activated circulating leukocytes after ethanol feeding that coexpressed EGFP and the binding receptor CD11b/CD18 (Mac1) was elevated (Figure 3). Interestingly, these pathophysiological changes were also associated with an enhanced EGFP expression reflecting NF-κB activation in ethanol-fed mice livers (Figures 4(b), 4(d), 4(f), and 4(h)). Hence, NF-κB activation after ethanol pretreatment is also present in circulating neutrophils and seems to correlate with the local and systemic synthesis of NF-κB-dependent mediators.

Our results also demonstrate a NF-κB-dependent priming effect of an ethanol diet on F4/80 positive macrophages in the liver (Kupffer cells); incubation with both LPS and TNF-α resulted in a largely exaggerated release of pro-inflammatory mediators and expression of NF-κB-dependent target genes (Figures 6–8). However, differences in the amount of NF-κB positive KC are only seen at 4 h after LPS stimulation (Figure 5(e)). Accordingly, this study presents evidence that Kupffer cells are an important player in initiating an overwhelming pro-inflammatory immune reaction after chronic ethanol feeding.

Although NF-κB is broadly accepted as a crucial factor in the regulation of the intracellular mechanisms after chronic ethanol abuse, there is, to our knowledge, no study that directly visualizes NF-κB spatial and temporal activation pattern in the liver and in circulating and hepatic immune cells. However, the function and involvement of NF-κB in different liver cell populations might be quite different. Using the NF-κB^{EGFP} transgenic mice, we visualized the spatial NF-κB activation in hepatic nonparenchymal cells and this differed profoundly compared to the topographical activation of NF-κB in hepatocytes. EGFP expressing hepatocytes were typically found in the midzonal and pericentral regions in livers of ethanol-fed mice (Figures 4(b), 4(d), and 4(f)). Hepatocytes in the pericentral zones of the liver lobe are involved in ethanol metabolism. In contrast, hepatic Kupffer cells showed a strong NF-κB activity in periportal and midzonal regions of the liver after chronic ethanol intake (Figure 4(h)) where they form the first line of defense against bacterial pathogen-associated molecular patterns (PAMPs), entering the portal circuit via the gut-liver axis, and endogenous damage-associated molecular patterns (DAMPs) generated at sites of sterile inflammation [41–44].

LPS stimulation of Kupffer cells increases TNF-α release and results in hepatocyte cell death and increased local synthesis of pro-inflammatory mediators, such as IL-6, IL-1, and TNF-α [13, 20, 45–47]. In our study, IL-6 release from ethanol pretreated Kupffer cells peaks at 24 h after KC stimulation, whereas peak of TNF-α release occurs already at 2 h after stimulation of ethanol pretreated KC (Figure 6). In parallel, detection of early inflammatory changes in isolated KC (mRNA expression: Figures 7(a)–7(e)) is also elevated after

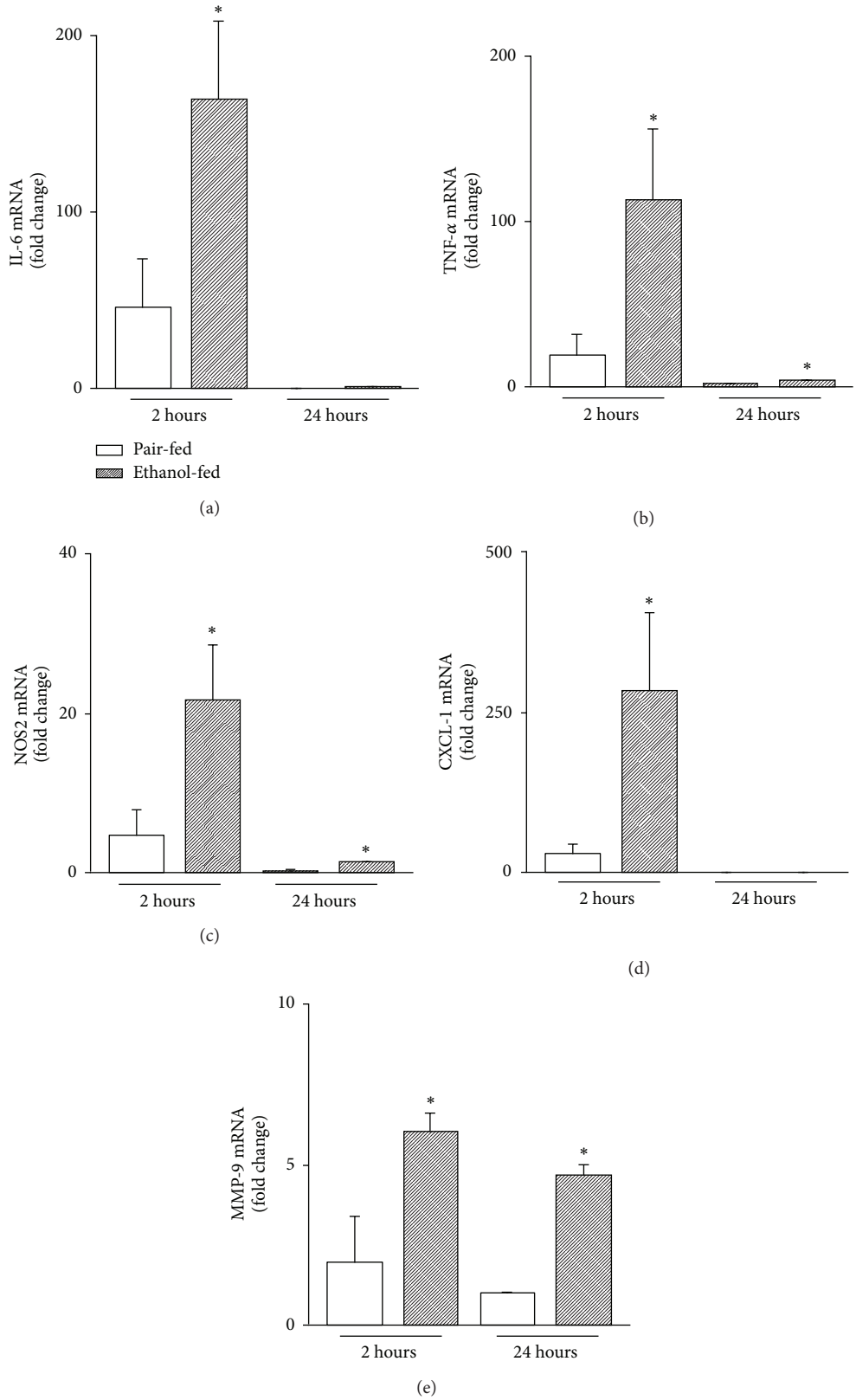

Figure 7: NF-κB target gene expression in isolated Kupffer cells in response to LPS after ethanol feeding F4/80$^+$ macrophages were isolated from livers of ethanol-, pair-fed mice, respectively, and stimulated with LPS (10 μg/mL, Figure 7) for 2 h and 24 h. Supernatants were measured for mRNA levels of IL-6 (a), TNF-α (b), NOS2 (c), CXCL-1 (d), and MMP-9 (e) by RT-PCR. Data (mean ± SEM) are expressed as fold change compared with corresponding medium controls (unstimulated) and are representative of five to eight separate experiments. $^*P < 0.05$ versus pair-fed control.

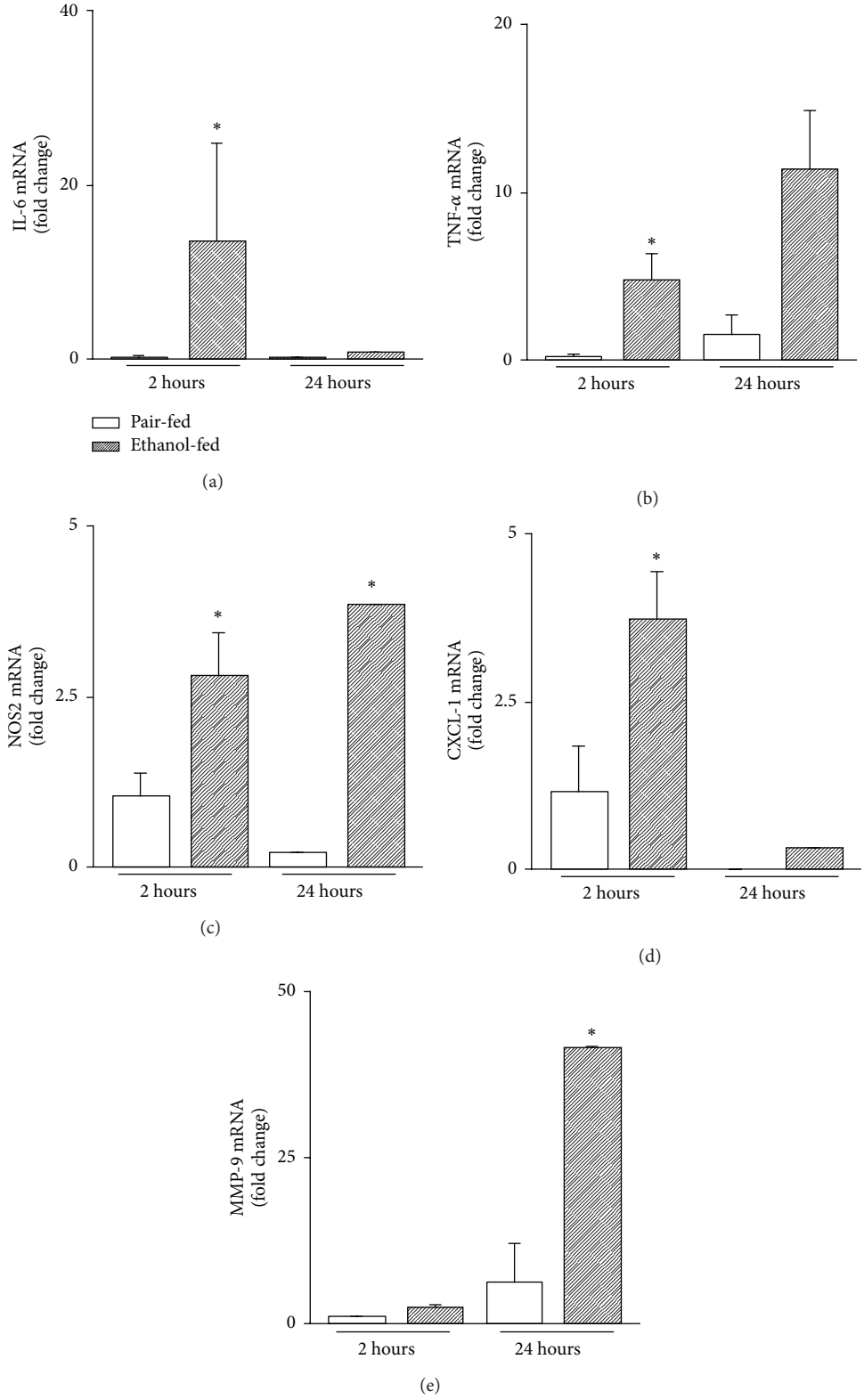

Figure 8: NF-κB target gene expression in isolated Kupffer cells in response to TNF-α after ethanol feeding F4/80$^+$ macrophages were isolated from livers of ethanol-, pair-fed mice, respectively, and stimulated with TNF-α (500 ng/mL) for 2 h and 24 h. Supernatants were measured for mRNA levels of IL-6 (a), TNF-α (b), NOS2 (c), CXCL-1 (d), and MMP-9 (e) by RT-PCR. Data (mean ± SEM) are expressed as fold change compared with corresponding medium controls (unstimulated) and are representative of five to eight separate experiments. $^*P < 0.05$ versus pair-fed control.

ethanol pretreatment. These differences in cytokine expression and production may be due, in part, to activation of NF-κB in Kupffer cells since after 4 h of LPS stimulation a larger percentage of EGFP positive Kupffer cells in ethanol-fed mice are present, an effect that is attenuated at 24 h after LPS stimulation (Figure 5(e)). Therefore, ethanol pretreatment of KC affects cytokine expression and production profiles when compared to control fed animals and this effect may be partly due to activation of NF-κB in Kupffer cells. Interestingly, acute ethanol intoxication exerts anti-inflammatory effects in the setting of resuscitated blood loss [19, 31, 48, 49]. Further studies are certainly needed to more specifically dissect the contribution of various cell types to the modulation of inflammatory responses after ethanol exposure.

Alcohol abuse plays a particular role in patients admitted to emergency services, for example, suffering from traumatic injury or massive bleeding, or after surgical interventions. The outcome and the incidence for multiple organ failure (MOF) or sepsis in acute ethanol intoxicated individuals differ from those of patients with a history of chronic ethanol abuse, mostly as a result of impaired host response [1, 16, 50–53]. Thus, the 24 h survival after trauma and the in-hospital mortality were worse in chronic ethanol abusers, and the percentage of individuals, suffering from a multiple organ failure (MOF), was 2-fold higher in victims with a cirrhotic liver when compared to acutely intoxicated patients [1]. A better outcome after binge-like ethanol consumption might be due to the activation status of innate immune cells. However, chronic ethanol affects a stimulation of the responsiveness of PMNL after a second challenge and therefore leads to an overwhelming multifactorial immune response [19].

5. Conclusions

Taken together NF-κB activation in Kupffer cells seems to be of critical importance in the response of the innate immune system after chronic ethanol feeding. There is accumulating evidence that ethanol-primed macrophages show an altered cytokine and chemokine expression after additional stimuli, such as trauma or endotoxemia. Our data indicate that chronic ethanol feeding increased Kupffer cell TNF-α release by sensitization to LPS. This may explain the increased susceptibility to infections of trauma victims with a history of chronic ethanol abuse.

Conflict of Interests

None of the authors have any conflict of interests regarding the material used here.

Acknowledgments

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Research Article

Alcohol-Induced Liver Injury Is Modulated by Nlrp3 and Nlrc4 Inflammasomes in Mice

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Alcoholic liver disease (ALD) is characterized by increased hepatic lipid accumulation (steatosis) and inflammation with increased expression of proinflammatory cytokines. Two of these cytokines, interleukin-1β (IL-1β) and IL-18, require activation of caspase-1 via members of the NOD-like receptor (NLR) family. These NLRs form an inflammasome that is activated by pathogens and signals released through local tissue injury or death. NLR family pyrin domain containing 3 (Nlrp3) and NLR family CARD domain containing protein 4 (Nlrc4) have been studied minimally for their role in the development of ALD. Using mice with gene targeted deletions for Nlrp3 ($Nlrp3^{-/-}$) and Nlrc4 ($Nlrc4^{-/-}$), we analyzed the response to chronic alcohol consumption. We found that $Nlrp3^{-/-}$ mice have more severe liver injury with higher plasma alanine aminotransferase (ALT) levels, increased activation of IL-18, and reduced activation of IL-1B. In contrast, the $Nlrc4^{-/-}$ mice had similar alcohol-induced liver injury compared to C57BL/6J (B6) mice but had greatly reduced activation of IL-1β. This suggests that Nlrp3 and Nlrc4 inflammasomes activate IL-1β and IL-18 via caspase-1 in a differential manner. We conclude that the Nlrp3 inflammasome is protective during alcohol-induced liver injury.

1. Introduction

Alcoholic liver disease (ALD) represents a variety of clinical and morphological changes that range from steatosis to inflammation and necrosis (alcoholic hepatitis) to progressive fibrosis (alcoholic cirrhosis) [1]. Most chronic heavy drinkers exhibit steatosis characterized by a greater amount of macrovesicular fat content than microvesicular fat. In addition, hepatocyte ballooning degeneration with mixed lobular inflammation is evident [2, 3]. Patients with ALD also have elevated serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which is evidence of liver injury. The severity of disease is not always correlated with the amount of alcohol consumed. In fact, most long-term heavy drinkers develop steatosis, but only 20–30% of these patients develop hepatitis, and less than 10% will progress to cirrhosis [4–6].

Activation of the immune system plays a critical role in the pathogenesis of ALD. Presently the current hypothesis for ethanol-induced liver injury proposes that ethanol results in leakage of bacterial products from the gut. Furthermore, chronic ethanol exposure alters the jejunal microflora leading to an increase in Gram-negative bacteria. Together these alterations cause an increase in circulating lipopolysaccharide (LPS) from Gram-negative bacteria in alcoholics [7].

The integrated human immune response has traditionally been divided into 2 branches: innate and adaptive (or acquired) immunity. The innate immune system is responsible for the initial task of recognizing and eradicating potentially dangerous microorganisms. A critical property of the

innate immune system is its ability to discriminate microbes from itself through recognition of conserved microbial structures called "pathogen"-associated molecular patterns (PAMPs) such as LPS, peptidoglycan, flagellin, and microbial nucleic acids [8].

Recognition of PAMPs is accomplished by membrane bound Toll-like receptors (TLRs) and cytoplasmic nucleotide oligomerization domain-like receptors (NLRs) [9]. The mammalian NLR family is composed of >20 members that contain a C-terminal leucine-rich repeat domain, a central nucleotide-binding NACHT domain, and a N-terminal protein-protein interaction domain composed of a caspase activation and recruitment domain or pyrin domain [10]. These proteins promote the assembly of multiprotein complexes, termed inflammasomes, which are required for the activation of inflammatory caspases. Upon sensing of PAMPs, NLR forms a complex with the effector molecule, procaspase-1 with or without the contribution of an adapter molecule apoptosis-associated speck-like Card-domain containing protein (ASC) [11–13]. Assembly of the inflammasome complex leads to cleavage of procaspase-1 to its active form of caspase-1. Once activated, caspase-1 promotes proteolytic maturation and activation of IL-1β, IL-18, and caspase-7 as well as deactivation of IL-33 [9] to mediate pyroptosis or cell death [14].

Nlrp3 and Nlrc4 are the best characterized NLR molecules. Nlrp3 controls caspase-1 activation in response to a range of stimuli such as ATP, pore-forming toxins, or uric acid crystals [15–17]. Nlrc4 is important for the activation of caspase-1 in macrophages infected with several pathogenic bacteria including *Salmonella enteric* server Typhimurium (*Salmonella*), *Legionella pneumophila* (*Legionella*), and *Pseudomonas aeruginosa* (*Pseudomonas*) [18–23]. However the role of these NLR molecules in the development of ALD has been investigated minimally. Because activation of proinflammatory cytokines is increased in ALD [24], we hypothesized that deletion of the inflammasome would prevent development of ALD. In this paper we analyzed the role of Nlrp3 and Nlrc4 in the development of ALD using the Lieber-DeCarli ethanol-containing diet model in B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice.

2. Experimental Procedures

2.1. Husbandry. Nlrp3$^{-/-}$ (generous gift from Dr. Amy G. Hise) and Nlrc4$^{-/-}$ (generous gift from Dr. Gabriel Nunez) mice were maintained at Case Western Reserve University. All mice were in the C57BL/6J (B6) background. The control B6 mice were originally from Jackson labs but have been bred and maintained at Case Western Reserve University for over 8 generations. Mice were raised in microisolator cages with a 12 hour light: 12 hour dark cycle. All mice were weaned at 3-4 weeks of age and raised on LabDiet number 5010 autoclavable rodent chow (LabDiet, Richmond, IN) *ad libitum* until studies were initiated.

2.2. Ethanol Feeding Diet Study and Ethanol Gavage. Eight to ten week-old female B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice were fed either Lieber-DeCarli ethanol-containing diet (EtOH-Fed) or pair-fed control diet (Pair-Fed) (Dyets Inc., Bethlehem, PA). Mice were randomized into ethanol-fed and pair-fed groups and then adapted to control liquid diet for 2 days. The ethanol-fed group was allowed free access to ethanol-containing diet with increasing concentrations of ethanol: 1% (vol/vol) and 2% for 2 days, then 4% ethanol for 7 d, and finally 5% ethanol for a further 2 weeks. For chronic alcohol study, we measured the volume of ethanol-containing diet consumed daily and fed the control mice pair-fed diets which isocalorically substituted maltose dextrin for ethanol over the entire feeding period. For measurements of serum ethanol concentrations, blood was taken from the tail vein 2 hours into the feeding cycle. At the end of the feeding trial, mice were sacrificed and blood was collected by cardiac puncture. Plasma was isolated using Microtainer plasma separator tubes (Becton Dickinson, Franklin Lakes, NJ). For acute administration of ethanol, rates of ethanol clearance were determined using a spectrophotometric enzyme assay [25]. Female mice were administered an oral gavage of ethanol (5 g ethanol/kg body weight of ethanol) as described in [25, 26]. Blood samples (50 uL) were taken from tail vein (at 30 min post injection) and serum was isolated. The serum was added to 2 mL 3% perchloric acid and centrifuged for 10 min at 1000 $\times g$. Resulting supernatants were used to determine serum ethanol concentration using an alcohol dehydrogenase enzyme assay as described in [25]. Female mice were used for this study because they are more susceptible to alcohol-induced liver injury and have a significantly higher risk of developing cirrhosis for any given level of alcohol intake [27].

2.3. Hepatic Triglycerides, Plasma Alanine Aminotransferase (ALT) Activity. For measurement of liver triglycerides, we saponified 100–200 mg of liver with an equal volume by weight of 3 M KOH/65% ethanol as described by Salomon and Flatt [28]. We measured glycerol concentration against glycerol standards using a commercially available triglyceride glycerol phosphate oxidase (GPO) reagent set (Pointe Scientific, Lincoln Park, MI) as previously described in [29]. We measured alanine aminotransferase (ALT) using commercially available enzymatic assay kit (Sigma-Aldrich, St. Louis, MO) as per manufacturer's directions.

2.4. Liver Histology and Inflammatory Score. Formalin-fixed tissues were paraffin-embedded, sectioned, coded, and stained with hematoxylin and eosin. Histological examinations were performed in a blinded fashion by our experienced pathologist (Xiuli Liu, M.D., Anatomic Pathology, Cleveland Clinic, Cleveland, OH) with histological scoring system for NAFLD [30]. Steatosis and inflammation scores ranged from 0 to 3 with 0 being within normal limits and 3 being the most severe.

2.5. Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR). Total RNA from 30 mg of liver was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA) and synthesized to single-strand cDNA from 500 ng of total RNA using random hexamer primers and MMTV reverse

transcriptase (Applied Biosystems, Foster City, CA). Real-time qRT-PCR analysis was performed using Bullseye Eva-Green SYBR qPCR reagent (MidSci, St. Louis, MO) on a Chromo4 Cycler (MJ Research/Bio-Rad, Hercules, CA) using specific primer sequences (see Supplementary Table 1 in supplementary in supplementary material available online at http://dx.doi.org/10.1155/2013/751374). Data was normalized using the comparative Ct method with load variations normalized to 18S rRNA. A ΔΔCT value is obtained by subtracting control ΔCT values from experimental ΔCT. The ΔΔCT values are converted to fold difference compared to control by raising two to the ΔΔCT power [31–33].

2.6. TNF-α, IL-1β, and IL-18 ELISA. The concentration of TNF-α in the liver was assessed using an enzyme-linked immunosorbent assay (ELISA) binding assay from liver protein homogenate derived from pair-fed and ethanol treated mice at the end of the feeding study as previously described [34]. The TNF-α ELISA was performed according to manufacturer's directions (BioLegend, San Diego, CA). The concentration of TNF-α was normalized by liver weight used for protein homogenization. Plasma isolated from pair-fed and ethanol-fed animals was used to measure IL-1β (R&D Systems, Minneapolis, MN) and IL-18 (Affymetrix eBioscience, San Diego, CA) by ELISA according to manufacturer's directions.

2.7. Protein Isolation and Western Blotting. Proteins were isolated and western blot analysis was performed from liver samples as previously described [34]. The membranes were incubated with antibodies to CYP2E1 (1 : 10,000; Fitzgerald Industries International Concord, MA), P-STAT (1 : 5,000; Abcam, Cambridge, MA), and total STAT3 (1 : 5,000; Cell Signaling Technology, Danvers, MA). The immunoreactive proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Rockford, IL) and the density of the immunoreactive bands was measured by scanning densitometry (UN-SCAN-IT gel software, Orem, Utah). The membranes were stripped using ReView Buffer Solution (Amresco, Solon, OH) and normalized for loading differences using heat shock cognate-70 (HSC70) (1 : 16,000; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described [34].

2.8. Caspase-3/7 Activity. Caspase-3/7 activity in the liver was assessed using an ELISA binding assay from protein homogenate derived from ethanol-fed mice livers at the completion of the feeding study. The assay was performed according to manufacturer's directions (Promega, Madison, WI) and was normalized by homogenate protein concentration.

2.9. Hydroxyproline Assay. Hydroxyproline content in liver was measured as previously described [35]. Briefly, liver tissues were homogenized in phosphate buffered saline and then hydrolyzed for 4 hours in 0.5 mL of 12 N Hydrochloric acid at 120°C. A portion of the hydrolysate (5 ul) was mixed with citrate/acetate buffer (238 mM citric acid, 1.2% glacial acetic acid, 532 mM sodium acetate, and 85 mM sodium hydroxide). Chloramine-T reagent (100 uL) was added (0.282 g chloramine-T into 16 mL sodium/acetate buffer, 2 mL n-Propanol, and 2 mL ddH_2O) and incubated for 30 min at room temperature. Ehrlich's Reagent (100 uL) (2.5 g p-dimethylaminobenzaldehyde added to 9.3 mL n-Propanol and 3.9 mL of 70% Perchloric acid) was added and incubated at 65°C for 30 min. The absorbance was then measured at 560 nm and a standard curve generated using commercially available hydroxyproline stock (Sigma-Aldrich, St. Louis, MO).

2.10. Statistical Analysis. The values reported are means ± standard error of the mean (SEM). Data were analyzed with Student's *t*-test using GraphPad Prism (GraphPad Software, San Diego CA).

3. Results

3.1. Nlrp3$^{-/-}$ Mice Are More Susceptible to Alcohol-Induced Liver Injury. We tested the susceptibility of female B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice to alcohol-induced liver injury after chronic administration of Lieber-DeCarli ethanol-containing diet. No differences in daily food intake were found in any of the strains (Table 1). To ensure that the various strains metabolized ethanol in a similar manner, serum ethanol levels were measured 2 hours into the feeding cycle. Nlrc4$^{-/-}$ mice had reduced plasma ethanol concentrations (0.5-fold compared to B6 mice fed ethanol-containing diet), while Nlrp3$^{-/-}$ mice showed similar increased plasma ethanol concentrations compared to B6 mice (Table 1). To confirm that Nlrc4$^{-/-}$ mice metabolized ethanol in a similar manner to the other strains, B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice were given an acute ethanol gavage and blood was taken from the tail vein thirty minutes later (Supplementary Data, Figure 1). Blood alcohol levels were measured and found to be similar between the strains and thus the strains metabolized ethanol in a comparable manner.

Liver injury was characterized as an increase in hepatic triglyceride and an increase in plasma ALT after ethanol consumption compared to pair-fed controls as well as histological analysis for steatosis and inflammation [36]. At the end of the ethanol-diet study, liver sections were analyzed by H&E staining (Figure 1). All strains had accumulation of lipid droplets with ethanol feeding. The concentration of hepatic triglycerides were measured biochemically and found to be increased in ethanol-fed mice for each strain of mice (Figure 2(a)). Histological analysis for steatosis in liver sections from each strain indicated that Nlrp3$^{-/-}$ mice had greater NAFLD activity scoring for steatosis in the pair-fed animals yet had a similar score as B6 when fed Lieber DeCarli ethanol-containing diet (Figure 2(c)). In contrast, the Nlrc4$^{-/-}$ mice had the lowest NAFLD activity scoring for steatosis after ethanol feeding compared to B6 and Nlrp3$^{-/-}$ mice. To examine inflammation, the pathologist analyzed the stained liver sections and scored them using NAFLD activity scoring [30]. There was mild appearance of inflammation in B6 and Nlrp3$^{-/-}$ mice and Nlrp3$^{-/-}$ mice had greater NAFLD

Table 1

	C57BI/6J		NIrp3−/−		NIrc4−/−	
	PF	+E	PF	+E	PF	+E
Food intake (mL/mouse/day)	12.51 ± 0.3[a]	12.27 ± 0.2[a]	12.6 ± 0.2[a]	12.8 ± 0.5[a]	12.56 ± 0.27[a]	12.27 ± 02[a]
Blood alcohol (mM)	0.42 ± 0.08[a]	62.49 ± 15.74[b]	0.08 ± 0.03[c]	54.51 ± 2.72[b]	0.29 ± 0.05[d]	30.58 ± 4.83[e]

Values are the mean ± SEM for $n = 4$–6 female mice per group. The means in a row with superscripts without a common letter differ from each other, $P < 0.05$ as determined with ANOVA and Bonferroni's correction for multiple testing. (PF: pair-fed, +E: ethanol-fed diet).

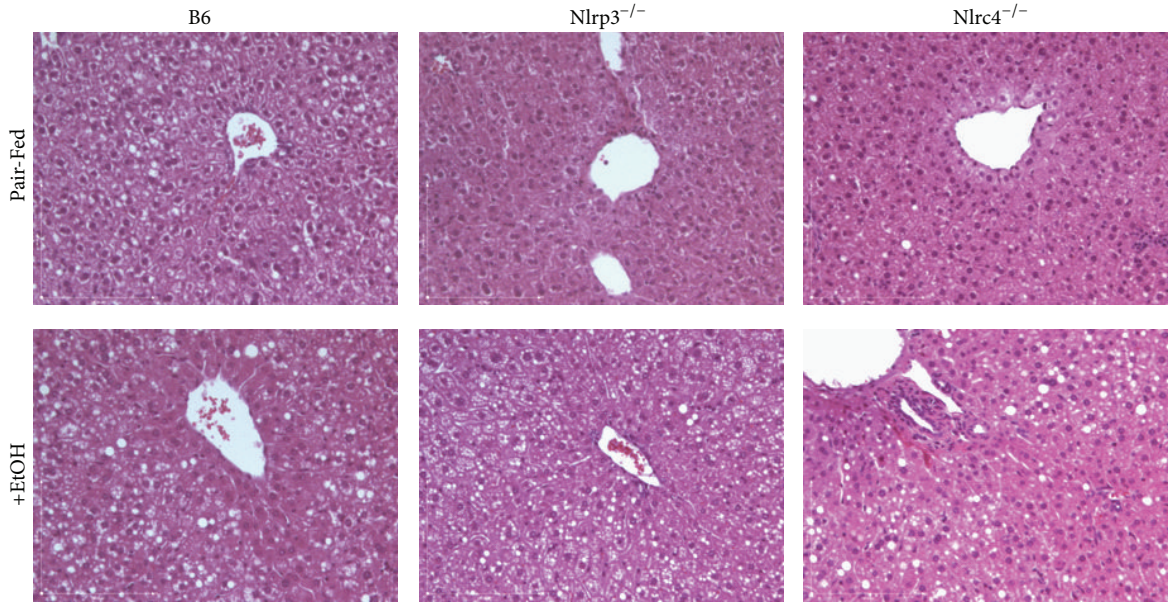

Figure 1: Analysis of steatosis with alcohol feeding. Hematoxylin and Eosin (H&E) staining of livers from C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed pair-fed control diets (Pair-Fed) or ethanol-containing diets (+EtOH). Figures are representative of 6 mice per group. Original magnification, ×200.

activity scoring for inflammation (Figure 2(c)). The Nlrc4$^{-/-}$ mice, on the other hand, had the highest NAFLD activity scoring for inflammation (Figure 2(c)). To further analyze liver injury plasma ALTs were measured. Liver injury was evident in all mice, as they exhibited increased plasma ALT concentrations. However, Nlrp3$^{-/-}$ mice showed the greatest increase in ALTs (3.0 fold) over its pair-fed control, while Nlrc4$^{-/-}$ and B6 mice had similar increases over their pair-fed controls (2.2-fold and 2.3-fold, resp.) (Figure 2(b)). This data suggests that Nlrp3$^{-/-}$ mice have increased alcohol-induced liver injury with greater plasma ALT, while Nlrc4$^{-/-}$ mice have greater inflammation after alcohol consumption compared to B6 mice.

Since ethanol consumption induces CYP2E1 expression and activity [37], we measured the induction of CYP2E1 by western blot analysis. All strains had increased expression of CYP2E1 with ethanol feeding (6.2-fold, 7.5-fold, and 3.7-fold over pair-fed controls for B6 and Nlrp3$^{-/-}$ mice and Nlrc4$^{-/-}$ mice, resp.) (Figure 3). The oxidative stress generated by increased CYP2E1 promotes alcohol liver disease and liver fibrosis [38]. In mice, it is difficult to induce frank liver fibrosis with 4 only weeks of alcohol feeding. To determine if deletion of Nlrp3 or Nlrc4 influences known components in development of liver fibrosis, we measured α-SMA mRNA and hydroxyproline expression. The α-SMA mRNA was induced with alcohol feeding in B6 and Nlrp3$^{-/-}$ mice. However, in Nlrc4$^{-/-}$ mice the basal expression of α-SMA mRNA was greater and ethanol feeding resulted in repression of α-SMA mRNA (Figure 4). Liver hydroxyproline content was measured using a hydroxyproline assay. In B6 and Nlrc4$^{-/-}$ mice there was an increase in hydroxyproline with ethanol feeding, while the Nlrp3$^{-/-}$ mice had a blunted induction of hydroxyproline (Figure 4(b)). The hydroxyproline values measured in Figure 4 were very low compared to previous studies of mice that had frank fibrosis (~18 mg/g protein for hydroxyproline after treatment with ethanol and carbon tetrachloride) [39].

It has been proposed that activation of the resident liver macrophages, Kupffer cells, has a pivotal role in the inflammation associated with alcohol liver disease (ALD) by secreting TNF-α as well as other cytokines [7, 40]. In addition the chemokine, monocyte chemoattractant protein-1

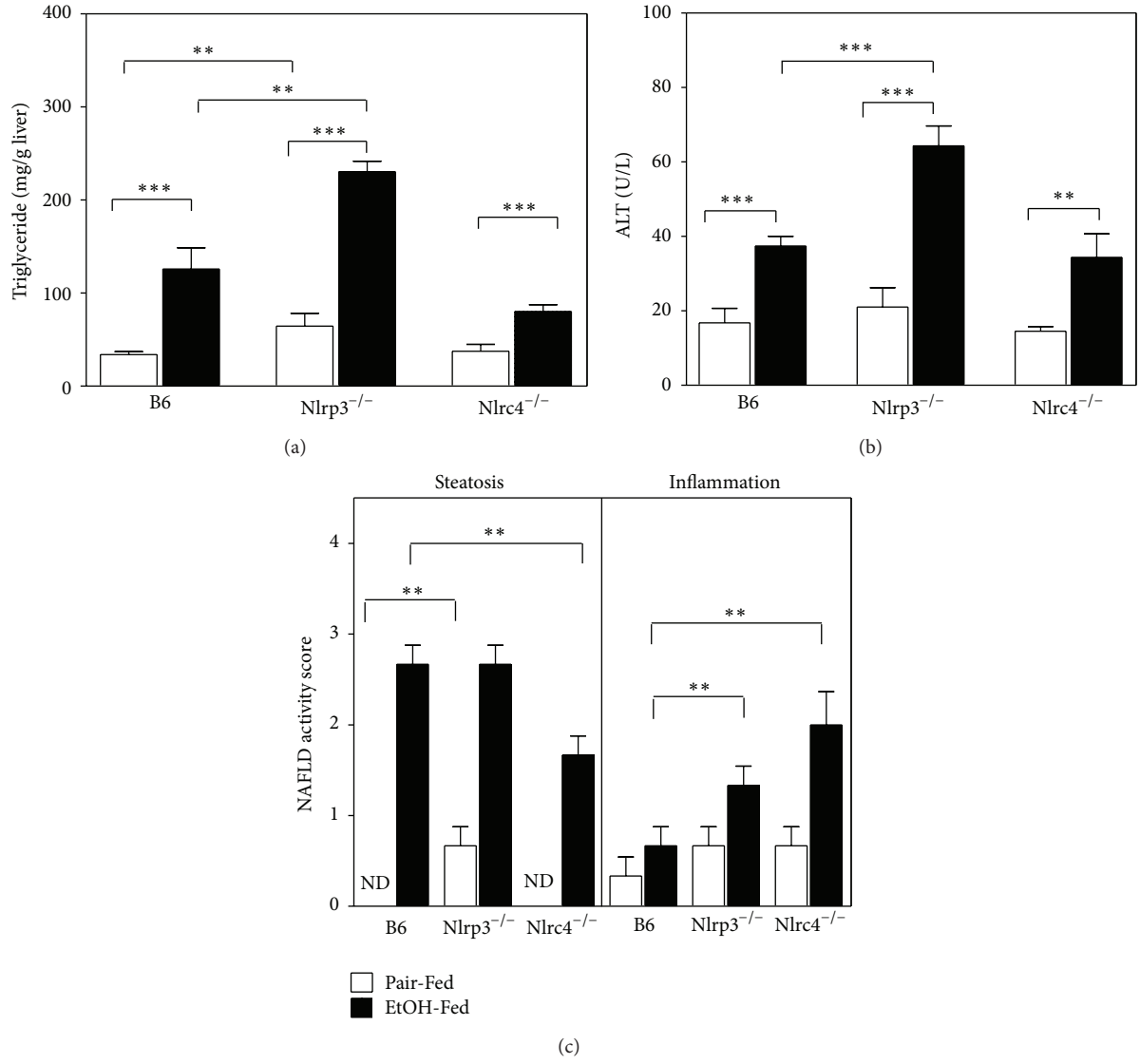

FIGURE 2: Measurements of liver injury. (a) Hepatic triglycerides were measured biochemically from C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed the ethanol-containing diet (EtOH-Fed) or pair-fed diet (Pair-Fed). (b) Plasma ALTs were measured with enzymatic assays from mice at the completion of the ethanol feeding trial. (c) NAFLD histological scores for steatosis and inflammation in B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice. Values represent the mean ± SEM with $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's t-test for $n = 4$–6 mice per group. ND is abbreviation for NAFLD activity score of 0.

(MCP-1) also contributes to alcohol-induced fatty liver likely via downregulation of PPAR-α and its target fatty acid metabolism genes [41]. Because TNF-α and MCP-1 influence the development of ALD, we measured hepatic TNF-α and MCP-1 in B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice (Figure 5). B6 mice had increased TNF-α with ethanol consumption, but both Nlrp3$^{-/-}$ and Nlrc4$^{-/-}$ did not have an increase of TNF-α in response to alcohol. The Nlrp3$^{-/-}$ mice had blunted levels similar to B6 pair-fed mice, while Nlrc4$^{-/-}$ mice had higher levels similar to B6 ethanol-fed mice. For MCP-1 expression, B6 mice had induced levels of MCP-1 with ethanol feeding and Nlrp3$^{-/-}$ mice had a dramatic increase of the chemokine with ethanol feeding (Figure 5(b)). However, Nlrc4$^{-/-}$ had levels similar to B6 ethanol-fed mice of MCP-1 for both pair-fed and ethanol-fed mice.

IL-1β is a potent proinflammatory cytokine that is elevated in patients with ALD [24, 42]. To determine if there was compensation for the deleted NLR molecules, we measured Nlrp3, Nlrc4, and Naip5 mRNA in B6 and knockout mice. In the Nlrp3$^{-/-}$ and Nlrc4$^{-/-}$ mice, the other NLR member of the inflammasome mRNA was reduced (Figure 6). Naip5 is the sensor component of the Nlrc4 inflammasome that specifically recognizes and binds flagellin from pathogenic bacteria such as *Legionella* or *Salmonella* [43]. Naip5 mRNA was found to be reduced in both Nlrp3$^{-/-}$ and Nlrc4$^{-/-}$ mice. This suggests that Nlrp3 expression is not compensating

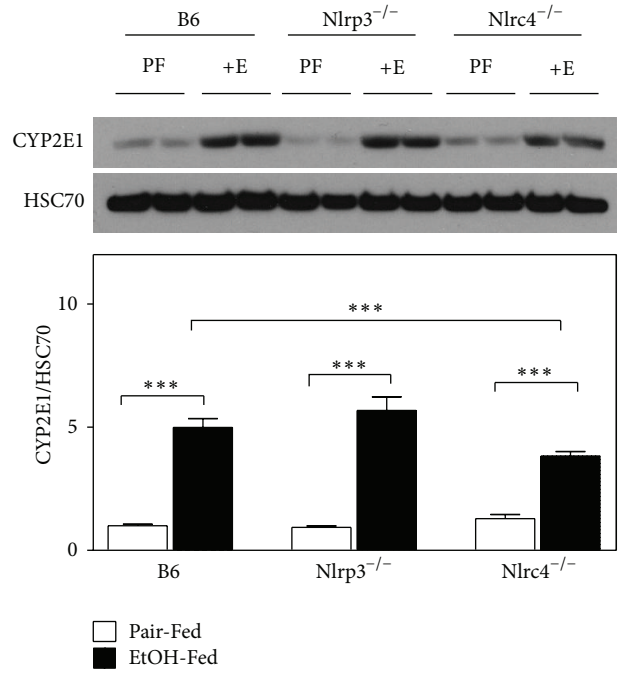

Figure 3: CYP2E1 induction with alcohol consumption. (a) western blot analysis of proteins from livers isolated from C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed ethanol-containing diet (EtOH-Fed) or pair-fed diet (Pair-fed). Westerns were normalized with heat shock cognate-70 (HSC70) as a loading control. In the graph, densitometric scans of western blots were performed and analyzed. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test for $n = 4$–6 mice per group.

for the loss of Nlrc4 gene expression in Nlrc4$^{-/-}$ mice and Nlrc4 expression is not compensating for loss of Nlrp3 gene expression in Nlrp3$^{-/-}$ mice.

To determine the consequence of deleting Nlrp3 or Nlrc4 genes in the production of proinflammatory cytokines IL-1β and IL-18, we measured the active form of these cytokines by ELISA (Figure 7). IL-1β was induced in B6 mice fed ethanol, but it was greatly reduced in Nlrp3$^{-/-}$ mice. IL-18 was induced in B6 mice fed ethanol, and it was greatly increased in Nlrp3$^{-/-}$ mice, while IL-18 was reduced in Nlrc4$^{-/-}$ mice. This suggests that Nlrp3 may play a more important role in the activation IL-1β, while Nlrc4 may play a more important role in the activation of IL-18.

Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [44]. Since Nlrp3$^{-/-}$ mice have elevated serum ALT, we measured caspase-3/7 activity (Figure 8). Nlrp3$^{-/-}$ mice had a 2-fold increase of caspase-3/7 activity suggesting increased apoptosis in both pair-fed and after ethanol feeding. Cell death can also be a result of decreased liver regeneration. For liver regeneration to occur, Kupffer cells release TNF-α and IL-6 which then activate STAT3 phosphorylation and initiate hepatocyte regeneration [45]. We measured STAT3 phosphorylation in B6, Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ pair-fed and ethanol-fed mice (Figure 9). The Nlrp3$^{-/-}$ mice did not have increased phosphorylation of STAT3 with ethanol feeding and Nlrc4$^{-/-}$ had reduced phosphorylation of STAT3 compared to B6 mice, suggesting that the liver regeneration pathway may also be impaired in these knockout mice.

4. Discussion

Previous work has studied the role of Nlrp3 and another member of the NLR inflammasome family, Nlrp6, in the context of nonalcoholic fatty liver disease (NAFLD) [46]. Using a variety of mice deficient for Nlrp3 gene (Nlrp3$^{-/-}$), Nlrp6$^{-/-}$ gene (Nlrp6$^{-/-}$), ACC (ASC$^{-/-}$), IL-18 (IL-18$^{-/-}$), and caspase-1 (caspase-1$^{-/-}$) the role of the inflammasome was investigated. The mice were fed methionine choline deficient diet (MCDD) to induce NASH. The investigators found that Nlrp6 and Nlrp3 inflammasomes and its downstream target IL-18 modulate the development of MCDD-induced liver injury. Because inflammasomes can also act as sensors and regulators of colonic microbiota [47], Mejia et al. analyzed the effects of gut microbiota in the development of NASH using these genetically modified strains. Antibiotic treatment with ciprofloxacin and metronidazole abolished the gut microbiota associated activity with development of NASH in ASC$^{-/-}$ mice. In addition cohousing ASC$^{-/-}$ or IL-18$^{-/-}$ mice with wild-type mice for 4 weeks before feeding MCDD diet to transfer microbiota from one strain to the other resulted in more severe liver injury in the wild-type mice compared to singly housed wild-type mice [46]. However not all NLR deficient mice developed liver injury in the same manner. Henao-Mejia et al. [46] also cohoused Nlrc4$^{-/-}$ and Nlrp12$^{-/-}$ mice with wild-type mice, but these strains did not alter the severity of liver disease with MCDD. This suggests a potential role for the Nlrp3 and Nlrp6 inflammasomes altering gut microbiotica that may in turn alter the development of nonalcoholic induced liver injury.

We have previously reported that the genetic contribution for the development of alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH) is unique [48] and multifactorial. In ALD, LPS derived from gut microflora has been extensively studied as a key inducer of inflammation in alcohol-related conditions. Alcohol stimulates LPS translocation across the gut via a number of mechanisms, and alcoholics with liver diseases are known to have significantly elevated circulating LPS [49]. In mice fed the Lieber DeCarli ethanol-containing diet, alcohol-induced liver injury is associated with increased plasma endotoxin (LPS) and hepatic lipid peroxidation. Treatment with an endotoxin neutralizing protein significantly suppressed alcohol-induced elevation of plasma endotoxin, hepatic lipid peroxidation, and inhibited TNF-alpha production [50]. These studies suggest the importance of the gut microbiotica and gut permeability in producing LPS in the plasma during ethanol consumption.

In the current study we analyzed the role of two NLR inflammasomes, Nlrp3 and Nlrc4, in the development of ALD. Recent studies have investigated the critical importance of IL-1 signaling in ALD [51] using caspase-1$^{-/-}$, ASC$^{-/-}$, or IL-1 receptor knockout mice (IL-1R$^{-/-}$). Loss of downstream signaling resulted in attenuation of alcohol-induced liver

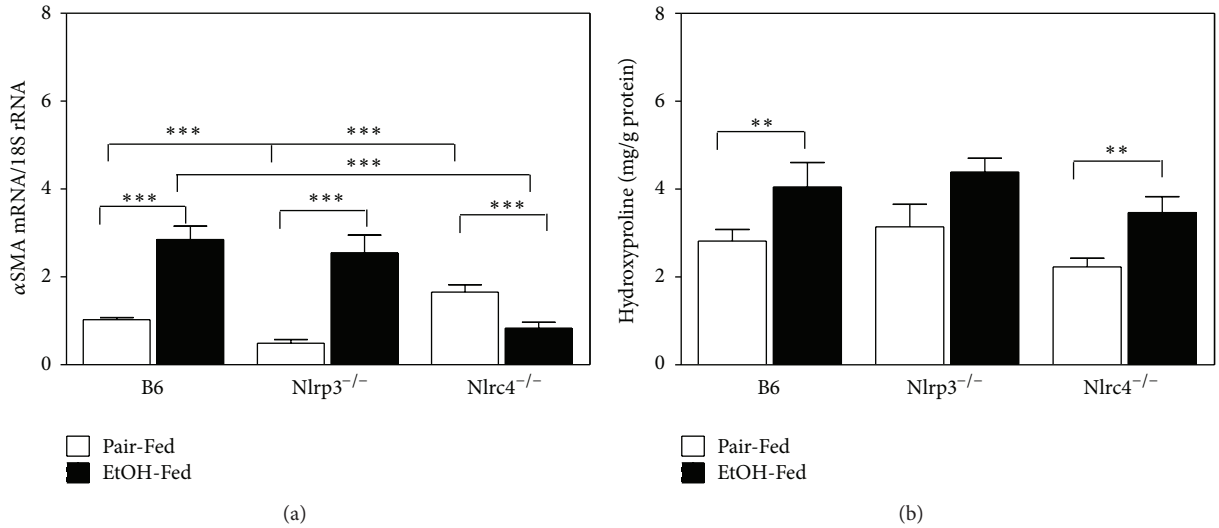

Figure 4: Measurement of αSMA and hydroxyproline. Total RNA was isolated from livers of C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed Lieber-DeCarli ethanol-containing diet (+EtOH) or pair-fed controls (Pair-Fed). Expression of (a) αSMA mRNA was measured and normalized by 18S rRNA. (b) Hydroxyproline was measured in protein homogenates by ELISA and normalized by protein concentration in the homogenates. The values are the means ± SEM for $n = 4$–6 mice per group. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test.

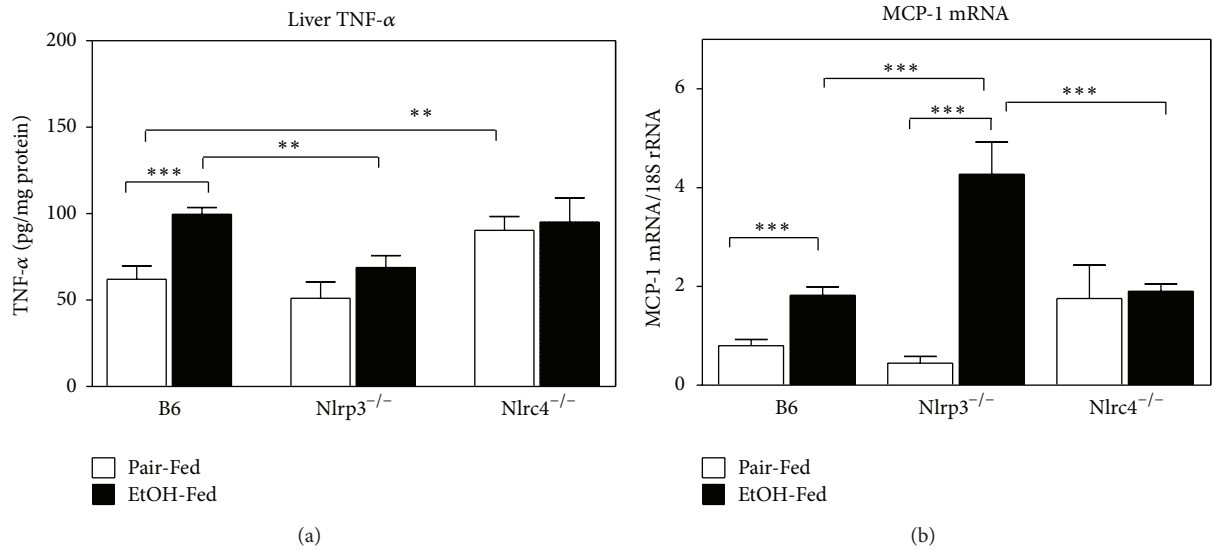

Figure 5: Expression of hepatic TNF-α. Protein was isolated from livers of C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed Lieber-DeCarli ethanol-containing diet (EtOH-Fed) or pair-fed controls (Pair-Fed). Expression of hepatic TNF-α was measured. The values are the means ± SEM and normalized with protein concentration in the homogenate for $n = 4$–6 mice per group. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test.

inflammation, steatosis, and damage [51]. The role of IL-18 in development of ALD has been studied in the context of a combined insult of ethanol and burn injury. In mice, the combined insult resulted in the suppression of immune responses with decreased host resistance and enhanced susceptibility to infection [52, 53]. However, the role of Nlrp3 and Nlrc4 inflammasomes in the development of ALD has not been fully elucidated.

Since previous studies showed that caspase-1 mediated activation of IL-1β was required for ALD, we hypothesized that deletion of either Nlrp3 or Nlrc4 genes would prevent ALD. Yet Nlrc4$^{-/-}$ mice had similar alcohol-induced injury compared to B6 mice, while Nlrp3$^{-/-}$ had more severe alcohol-induced liver injury compared to B6 mice (Figure 2(b)). In Nlrp3$^{-/-}$ mice, the loss of the Nlrp3 inflammasome reduced the amount of active IL-1β but dramatically

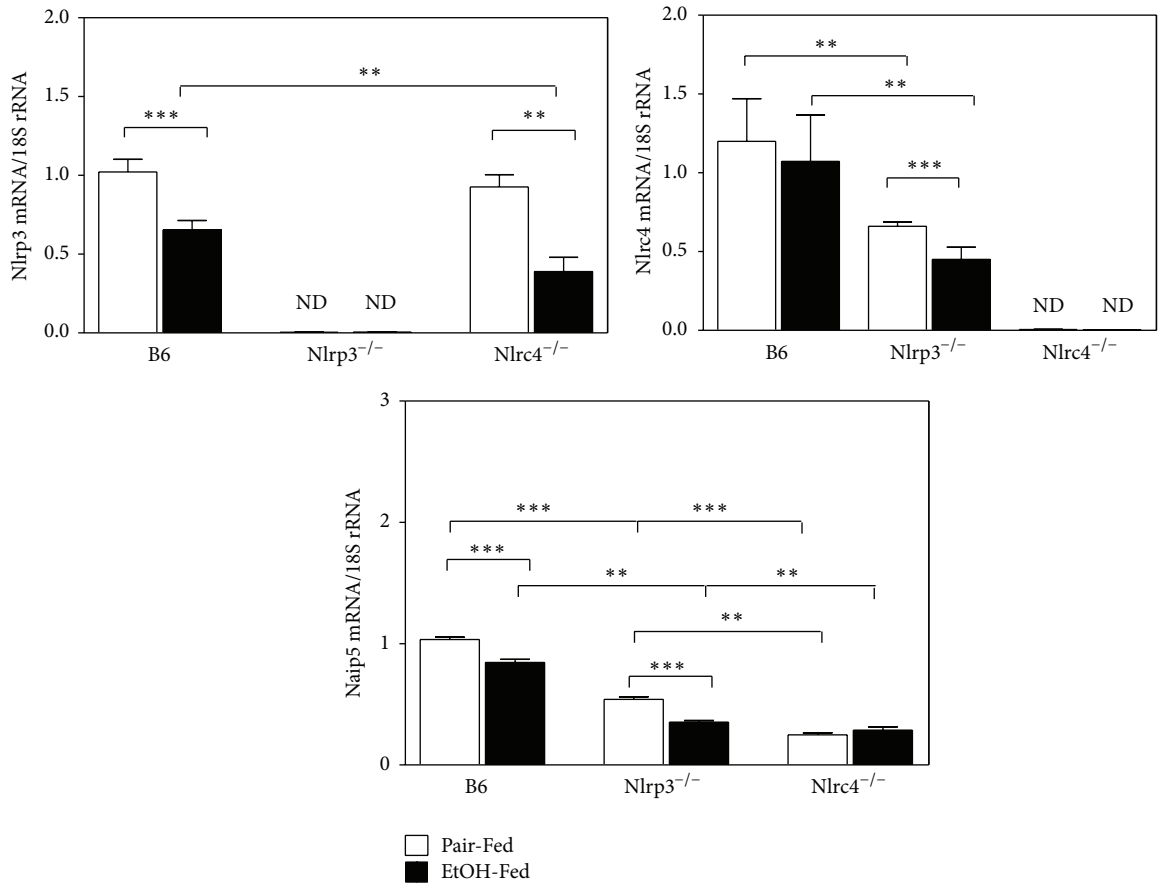

Figure 6: Hepatic expression of Nlrp3, Nlrc4, and Naip5 mRNA. RNA was isolated from C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed Lieber-DeCarli ethanol-containing diet (EtOH-Fed) or pair-fed controls (Pair-Fed). Expression of Nlrp3, Nlrc4, and Naip5 was measured. The values are the means ± SEM and normalized with 18S rRNA for $n = 4$–6 mice per group. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test.

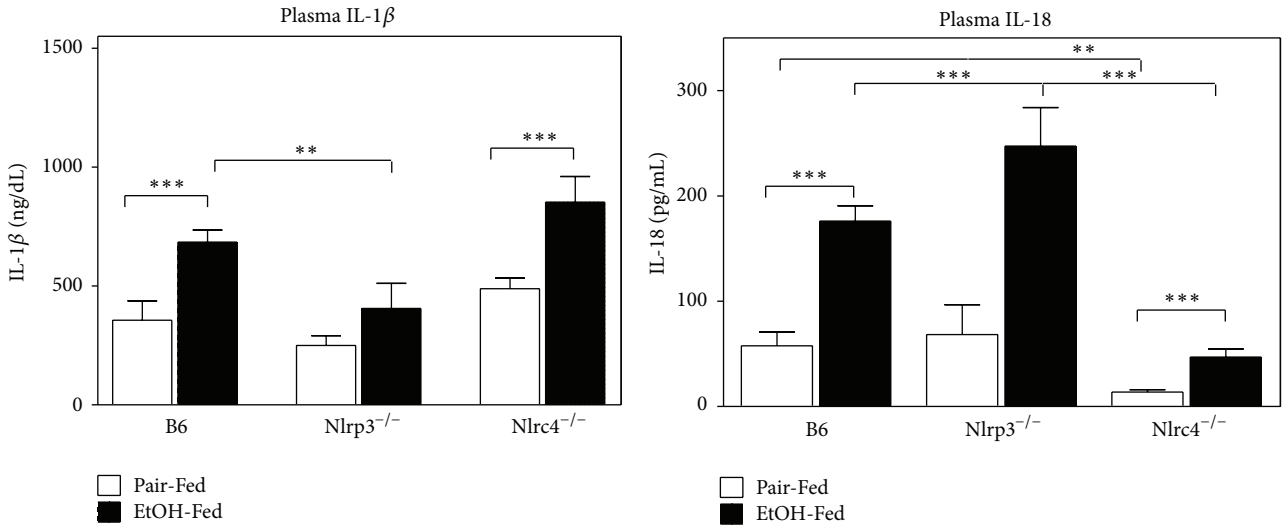

Figure 7: Altered IL-1β and IL-18 expression in the liver in Nlrp3$^{-/-}$ and Nlrc4$^{-/-}$ mice. Protein was isolated from livers of C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed Lieber-DeCarli ethanol-containing diet (EtOH-Fed) or pair-fed controls (Pair-Fed). Levels of IL-1β and IL-18 were measured using specific ELISA. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test for $n = 4$–6 mice per group.

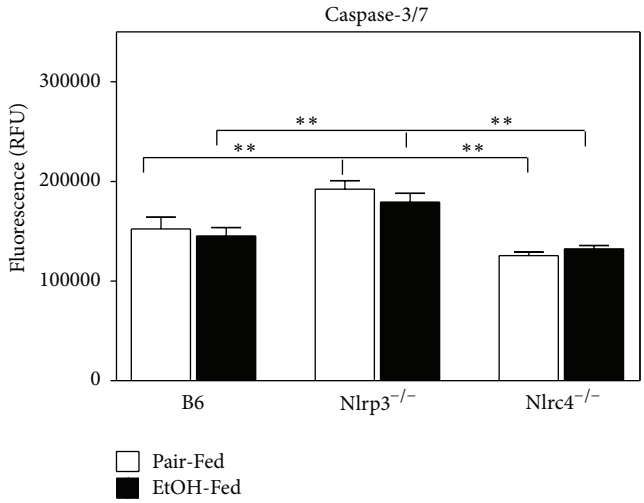

Figure 8: Increased caspase-3/7 in the liver of Nlrp3$^{-/-}$ mice. Protein was isolated from C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed Lieber-DeCarli ethanol-containing diet (EtOH-Fed) or pair-fed controls (Pair-Fed). Expression of hepatic caspase-3/7 was measured as relative fluorescence. The values are the means ± SEM for $n = 4$–6 mice per group. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test for $n = 4$–6 mice per group.

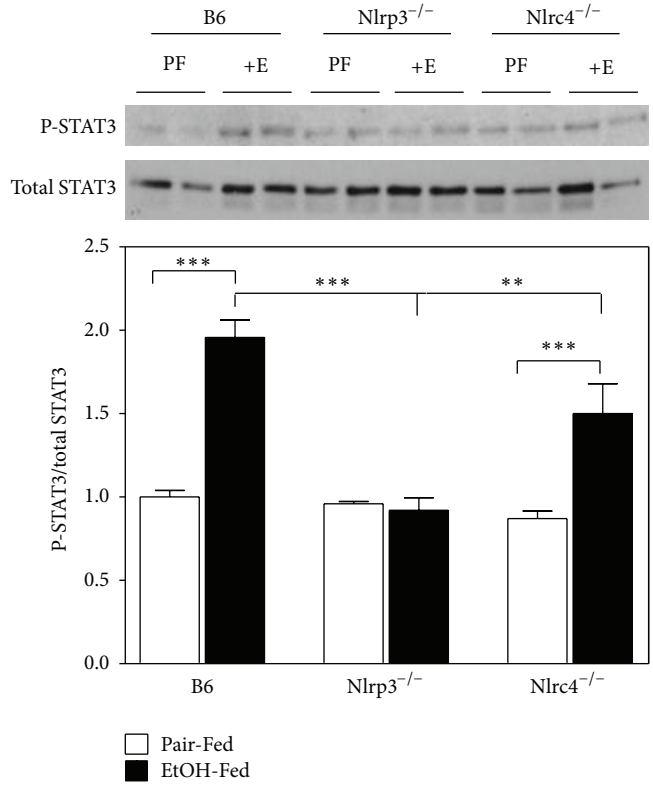

Figure 9: Decreased phosphorylation of STAT3 in the liver in Nlrp3$^{-/-}$ and Nlrc4$^{-/-}$ mice. Protein was isolated from livers of C57BL/6J (B6), Nlrp3$^{-/-}$, and Nlrc4$^{-/-}$ mice fed Lieber-DeCarli ethanol-containing diet (EtOH-Fed) or pair-fed controls (Pair-Fed). Expression of hepatic total STAT3 and phosphorylated STAT3 was measured by western blot analysis. In the graph, densitometric scans of western blots were performed and analyzed. The values are the means ± SEM. The phosphorylated STAT3 was normalized by total STAT3. Values represent the mean ± SEM with $^{**}P < 0.05$, $^{***}P < 0.001$ by Student's t-test for $n = 4$–6 mice per group.

increased the amount of active IL-18. This result is different from previously published studies that have shown that IL-18 was not induced in B6 mice fed ethanol [51]. Our results may be different because the B6 mice used in our study had been bred within an animal colony at Case Western Reserve University for over eight generations, resulting in slightly different inbred strains from Jackson labs. The other possibility is that Petrasek et al. initiated their ethanol feeding study in 6–8-week-old female mice, while we began our feeding study in 10–12-week-old adult female mice. In addition as Henao-Mejia et al. [46] have shown, Nlrp3 inflammasome but not the Nlrc4 inflammasome impacts microbiota in the gut which in turn modulated development of NASH with MCDD. This could also be a mechanism by which Nlrp3$^{-/-}$ mice have increased liver injury with alcohol consumption, while Nlrc4$^{-/-}$ mice have similar injury to B6 mice in our study. Nlrp3$^{-/-}$ mice may have increased leakage of LPS from the gut possibly due to altered microbiota that would impact the degree of liver injury. Further studies are needed to fully understand the role of Nlrp3 inflammasome and its impact on microbiota in the gut with alcohol feeding.

Could the increase of IL-18 contribute to the increased ALD in Nlrp3$^{-/-}$ mice? Finotto et al. analyzed IL-18 transgenic mice that expressed IL-18 under the control of CD2 promoter (express in T cells and B cells) [54]. The transgenic mice had increased hepatocyte apoptosis by spontaneous activation of the Fas associated death pathway [54]. Binding of IL-18 to the high affinity IL-18R leads to nuclear factor $\kappa\beta$ (NF$\kappa\beta$) activation through myeloid differentiation primary response 88 (MyD88) and TNF-α and subsequent phosphorylation of I$\kappa\beta$ via I$\kappa\beta$ kinases (IKK-1 and IKK-2). IL-18 also triggers NK cell activity and expression of FasL by natural killer cells (NK) [55, 56]. In our study, we suggest that Nlrp3 plays a key role in production of IL-1β and loss of the Nlrp3 inflammasome increases hepatocyte apoptosis possibly through FasL mediated mechanism.

The decrease of IL-1β may also impact cell survival. Previous studies have shown that active IL-1β but not IL-18 is induced after ethanol feeding in mice [51]. Could the lack of IL-1β contribute to increase ALD in Nlrp3$^{-/-}$ mice? IL-1β is thought to mediate its inflammatory actions by inducing the expression of proinflammatory genes (such as IL-6), recruiting immune cells to the site of injury (liver), and modulating infiltrating cellular immune-effector actions [57]. The proinflammatory cytokine, IL-1β, exerts a prominent effect on the expression of proinflammatory genes primarily by activation of intracellular signaling pathways involving NF-$\kappa\beta$ and p38 mitogen-activated protein kinase (MAPK) [58, 59]. The transcription factor NF-$\kappa\beta$ is inactive when associated with the inhibitory protein I$\kappa\beta$. Upon cytokine activation, I$\kappa\beta$ is degraded and NF-$\kappa\beta$ translocates to the nucleus [60]. Both NF-κB and p38 MAPK are involved in

the regulation of the expression of genes encoding E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), IL-6, IL- 8, and cyclooxygenase (COX)-2 [61–63]. When IL-6 binds to its receptor, gp130 is dimerized and associates with Janus kinases (JAKs) and phosphorylation of JAKs and gp130 occurs. This receptor-kinase complex then recruits and phosphorylates cytoplasmic STAT3. Once phosphorylated, STAT3 forms a dimer and translocates into the nucleus initiating transcription of many genes that play significant roles in inducing acute phase responses, promoting hepatocyte survival and liver regeneration [64]. In the present study we found blunted phosphorylation of STAT3 in response to ethanol in Nlrp3$^{-/-}$ mice (Figure 9). In addition the amount of caspase-3/7 was dramatically increased in Nlrp3$^{-/-}$ mice due to the loss of Nlrp3 (Figure 8). Therefore, we conclude that the Nlrp3 inflammasome contributes to the activation of JAK/STAT3 pathway and may promote liver regeneration. However further studies are needed to fully understand the mechanism for increased ethanol-induced liver injury in Nlrp3$^{-/-}$ mice.

Since both Nlrp3 and Nlrc4 inflammasomes activate caspase-1 and produce IL-1β and IL-18, can these pathways have nonredundant roles? As stated above, each has their own activators and Nlrc4 has a narrower spectrum of activators, primarily flagellin. In a study that infected bone marrow derived macrophages with *Burkholderia pseudomallei* (Gram-negative bacteria), differences in activation of inflammasomes and the amount of active IL-1β and IL-18 produced were found [65]. Using B6, Nlrp3$^{-/-}$, Casp1$^{-/-}$, Nlrc4$^{-/-}$, and ASC$^{-/-}$ mice, Ceballos-Olvera et al. found that Nlrc4 contributes to IL-1β production in the early phase of infection. This is important for early induction of pyroptosis, which would then restrict bacterial growth. Nlrp3 does not regulate pyroptosis and primarily controls IL-1β secretion. Most importantly they found that IL-1β and IL-18 were present at high levels in lungs of Nlrc4$^{-/-}$ mice that were infected with *B. pseudomallei* intranasally. In contrast, Nlrp3$^{-/-}$ and ASC$^{-/-}$ mice had little to no IL-1β produced after infection [65]. What determines this specificity? In this same study, the authors suggest the Nlrc4 can form two distinct Nlrc4 inflammasomes, one Nlrc4 inflammasome that contains ASC and regulates IL-1β production and the other lacking ASC which would activate caspase-1 and initiate pyroptosis [65, 66]. Finally members of NLR family, Naip family, have been shown to determine the specificity of Nlrc4 for its activators [67]. For example, activation of Nlrc4 inflammasome by bacterial PrgJ from *Salmonella* Typhimurium requires Naip2, while activation of Nlrc4 by flagellin from L. *pneumophila* requires Naip5 [67]. In the present study, we found that Nlrp3$^{-/-}$ mice had reduced formation of active IL-1β with increased formation of active IL-18. In Nlrc4$^{-/-}$ mice, the reverse was found. Nlrc4$^{-/-}$ mice had more active IL-18 but less active IL-1β. This data supports the hypothesis that each inflammasome may preferentially produce either active IL-1β or IL-18 adding to the complexity of regulation in the development of ALD.

5. Conclusions

In summary, we present evidence that Nlrp3 inflammasome is protective during alcohol-induced liver injury. The data presented in this study analyzed whole liver homogenates, but the liver is composed of several cell types (hepatocytes, Kupffer cells, NK cells, endothelial cells, and hepatic stellate cells). Because previous studies have shown the importance of Nlrp3 and ASC in hepatic stellate cells for the development of liver fibrosis [68], future studies will determine the role of the Nlrp3 inflammasome in the specific cell types for the development of ALD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

David A. DeSantis and Chih-wei Ko contributed equally to this work.

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Review Article

The Effect of Inflammatory Cytokines in Alcoholic Liver Disease

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Alcohol is the most common cause of liver disease in the world. Chronic alcohol consumption leads to hepatocellular injury and liver inflammation. Inflammatory cytokines, such as TNF-α and IFN-γ, induce liver injury in the rat model of alcoholic liver disease (ALD). Hepatoprotective cytokines, such as IL-6, and anti-inflammatory cytokines, such as IL-10, are also associated with ALD. IL-6 improves ALD via activation of the signal transducer and activator of transcription 3 (STAT3) and the subsequent induction of a variety of hepatoprotective genes in hepatocytes. IL-10 inhibits alcoholic liver inflammation via activation of STAT3 in Kupffer cells and the subsequent inhibition of liver inflammation. Alcohol consumption promotes liver inflammation by increasing translocation of gut-derived endotoxins to the portal circulation and activating Kupffer cells through the LPS/Toll-like receptor (TLR) 4 pathways. Oxidative stress and microflora products are also associated with ALD. Interactions between pro- and anti-inflammatory cytokines and other cytokines and chemokines are likely to play important roles in the development of ALD. The present study aims to conduct a systemic review of ALD from the aspect of inflammation.

1. Introduction

Alcohol-related liver disease is a major cause of morbidity and mortality worldwide. Chronic alcohol consumption leads to hepatocellular injury, fat accumulation, and liver inflammation and sometimes leads to liver cirrhosis or hepatocellular carcinoma (Figure 1). The pathogenesis of alcoholic liver disease (ALD) is a consequence of chronic alcohol consumption. The clinical syndrome of ALD carries a particularly poor prognosis, such as liver cirrhosis [1] or hepatocellular carcinoma [2]. The pathogenesis of ALD is uncertain, but the relevant factors include metabolism of alcohol to toxic products, oxidative stress, acetaldehyde adducts, abnormal methionine metabolism, malnutrition, the activation of endotoxin, and impaired hepatic regeneration (Figure 2) [3]. Kupffer cells, the resident macrophages in the liver, play the role of an innate immune system; they produce various cytokines and are known to be involved in the pathogenesis of liver diseases [4]. The inflammatory cytokine, tumor necrosis factor-alfa (TNF-α), is involved in acute alcoholic liver injury [5]. Moreover, it is also well known that chronic alcohol consumption increases TNF-α production and leads to liver injury [6]. The consumption of alcohol leads to an augmented permeability of the intestinal membrane, which increases the portal concentration of blood endotoxin (lipopolysaccharide; LPS) [7]. This causes the Kupffer cells to be activated and exhibit enhanced sensitivity to LPS-stimulated inflammatory cytokine production [8]. Chronic alcohol consumption leads to injury of the hepatocytes by TNF-α, with consequent apoptosis and phagocytosis by the Kupffer cells. The Kupffer cells are activated by phagocytosing the apoptotic cells and their inflammatory cytokine production is increased [9]. Alcohol consumption promotes liver inflammation by increasing the translocation of gut-derived endotoxins to the portal circulation and activating Kupffer cells through the LPS/Toll-like receptor (TLR) 4 pathways. ALD is associated with imbalanced immune responses and increased production of proinflammatory cytokines and chemokines [10, 11]. Various cytokines are associated with ALD, including hepatoprotective cytokines, such as lnterleukin-6 (IL-6), and anti-inflammatory cytokines, such as IL-10 [12]. These two cytokines are produced by ethanol-induced LPS-stimulated Kupffer cells and can attenuate alcohol-induced liver injury. Inflammasome and several chemokines also contribute to ALD.

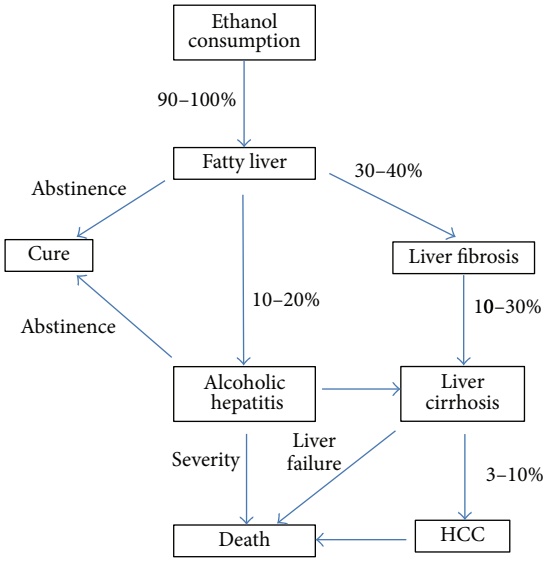

Figure 1: The natural history of alcoholic liver disease. Chronic ethanol consumption leads to fatty liver for more than 90%. But only up to 40% of this population develops more severe forms of alcoholic liver disease (ALD), including fibrosis and alcoholic hepatitis. Continuous ethanol consumption finally leads to liver cirrhosis or hepatocellular carcinoma and leads to death.

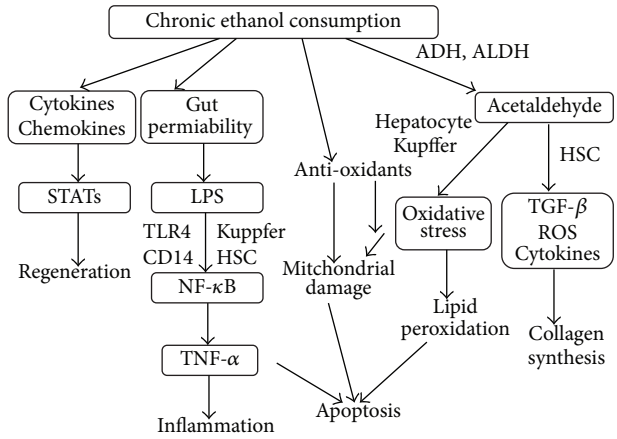

Figure 2: The pathogenic mechanisms of alcoholic liver disease. Chronic ethanol consumption promotes the translocation of LPS from the intestine to the portal vein, where it binds to the lipopolysaccharide-binding protein (LBP). STATs induces liver regeneration. Ethanol consumption alters the intracellular balance of antioxidants with subsequent decrease in the release of mitochondrial damage, leading to hepatic apoptosis. Hepatocytes and activated Kupffer cells are suggested to be the sources of oxidative stress, which are responsible for lipid peroxidation and further apoptotic damage. Activation of hepatic stellate cells also contributes to the production of TGF-β, ROS, and cytokines, leading to liver fibrosis.

Thus, the present paper aims to conduct a systemic review of ALD from the aspect of inflammation.

2. Metabolism of Alcohol

When alcohol is consumed, it passes from the stomach and intestines into the blood, a process referred to as absorption. Alcohol then enters the liver through the portal vein. We show the pathway of alcohol metabolism in Figure 3. In the liver, alcohol dehydrogenase (ADH), the key enzyme in alcohol metabolism, mediates the conversion of alcohol to acetaldehyde [13]. Acetaldehyde is rapidly converted to acetate by acetaldehyde dehydrogenase (ALDH) and is eventually metabolized in the muscle to carbon dioxide and water. There is another pathway independent of ADH, which is called the microsomal ethanol oxidizing system (MEOS) [14]. Alcohol is metabolized in the liver by the enzyme cytochrome P450 2E1 (CYP2E1). CYP2E1 is mainly expressed in the liver, with hepatocytes showing the highest expression, but it is also located in other organs, such as the brain and intestine. CYP2E1 is mainly located within the endoplasmic reticulum (ER) although it is also expressed in the mitochondria [15] and is increased after chronic alcohol consumption [16] and an increase in acetaldehyde. Acetaldehyde has a stronger toxicity than ethanol and leads to liver injury. Most of the alcohol consumed is metabolized in the liver, but the small quantity that remains is not metabolized and is excreted in the breath and urine.

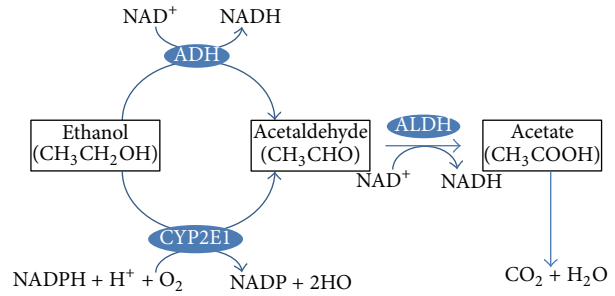

Figure 3: The pathway of ethanol metabolism. Ethanol is metabolized into acetaldehyde by alcohol dehydrogenase (ADH) and the microsomal enzyme cytochrome P450 2E1 (CYP2E1). The ADH enzyme reaction is the main ethanol metabolic pathway involving an intermediate carrier of electrons, namely, nicotinamide adenine dinucleotide (NAD^+). Acetaldehyde is rapidly metabolized by aldehyde dehydrogenase (ALDH) in the mitochondria to acetate and NADH. And acetate is eventually metabolized in the muscle to carbon dioxide and water.

2.1. TNF-α. TNF-α is a cytokine involved in systemic inflammation and is a member of a cytokine family that stimulates acute inflammation. TNF-α is produced by various types of cells in the body. In the liver, TNF-α is mainly produced by Kupffer cells, and TNF-α is also an important mediator in various physiological processes, such as inflammation, cell proliferation, and apoptosis [10]. The role of TNF-α as a critical inflammatory cytokine in the progression of ALD is now well known [5]. However, the mechanism of alcohol enhancement of TNF-α has not been clarified yet. Kupffer cells secrete inflammatory cytokines [4] and reactive oxygen species (ROS) [17], which activate cells such as hepatocytes, hepatic

stellate cells, and endothelial cells [18]. In alcoholic hepatitis (AH), inflammatory cytokines, such as TNF-α or IL-6, induce liver injury [19]. After chronic alcohol consumption, Kupffer cells exhibit enhanced sensitivity to LPS-stimulated TNF-α production [20]. Elevated serum levels of TNF-α inducible cytokines or chemokines, including IL-6, IL-8, and IL-18, have also been reported in patients with AH [21]. Serum TNF-α is increased in patients with ALD and correlates with mortality. Administration of excessive ethanol to TNF-α knockout mice does not cause liver injury. Thus TNF-α is thought to be the main cytokine of inflammation. Furthermore, increased serum levels of TNF-α have also been noticed in rat models of nonalcoholic steatohepatitis (NASH) [22] and in patients with NASH [23]. TNF-α is associated with the development of liver injury in both ALD and NASH.

Recently, it has become known that platelet aggregation activity is associated with ALD. The platelet adhesive protein, von Willebrand factor (VWF), and its cleavage protease, ADAMTS13, have been gaining attention. In previous studies, our group showed that plasma ADAMTS13 activity decreased in ALD or severe AH and was inversely proportional to TNF-α [24–26]. Treatment with pentoxifylline, an inhibitor of TNF-α synthesis, improved the survival of patients with severe AH [27]. Anti-TNF-α antibodies prevented inflammation and necrosis in the rat model of alcohol feeding [6]. Anti-TNF-α antibody, infliximab, is also effective in severe AH patients [28]. Multiple cytokine modulator, Y-40138, is known to inhibit the production of inflammatory cytokines, such as TNF-α or IL-6, and to enhance the production of anti-inflammatory cytokines, such as IL-10. Our results showed that Y-40138 reduced the inflammatory cytokines in ALD [29]. These results suggest that TNF-α plays an important role in the progression of ALD.

2.2. IL-6. The role of IL-6 in ALD is complex and not well understood. It appears to have some beneficial effects on the liver. IL-6 may protect against hepatocyte apoptosis and participate in mitochondrial DNA repair after alcoholic liver injury [30, 31]. IL-6 may promote human Th17 differentiation and IL-17 production, therefore contributing to ethanol-induced liver inflammation. IL-6 is also released along with IL-10, TNF-α, and other cytokines by Kupffer cells after alcohol consumption. IL-6 and IL-10 are two cytokines that play roles in reducing alcoholic liver injury and inflammation through activation of the signal transducer and activator of transcription (STAT3) [12]. Elevated IL-6 is found in chronic alcohol-fed animals and in alcoholics, with or without liver disease [32]. On the other hand, IL-6 knockout mice fed chronic alcohol showed increased liver fat accumulation, lipid peroxidation, mitochondrial DNA damage, and sensitization of hepatocytes to TNF-α induced apoptosis, which was prevented by the administration of recombinant IL-6 [31, 33, 34]. Furthermore, blocking of IL-6 signalling in mice reduced the infiltration of neutrophils and mononuclear cells and inflammation [35]. These findings suggest that IL-6 has a protective effect at the early phase of ALD.

2.3. IL-10. IL-10 is an anti-inflammatory cytokine that controls the endogenous production of TNF-α during endotoxemia and reduces LPS stimulation when added exogenously [36]. IL-10 is produced by macrophages, lymphocytes, and Kupffer cells, and the liver is considered to be the main source of IL-10 production [37]. IL-10 decreases the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, from activated macrophages or monocytes. IL-10 also possesses a hepatic protective effect on proliferation and fibrosis [38]. In the liver, Kupffer cells are the main producers of IL-10. Kupffer cells produce IL-10 in response to LPS stimulation and downregulate the release of TNF-α and IL-6. Endotoxin administration is an extensively studied model of IL-10 induction from monocytes and macrophages [39]. Human monocytes activated by LPS are able to produce a high level of IL-10 in a dose-dependent manner [40]. The activated monocytes inhibit production of proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β. Moreover, proinflammatory cytokine levels were also increased by LPS treatment in IL-10 knockout mice [41]. Alcohol-fed IL-10 knockout mice have increased hepatic and systemic inflammatory conditions, and LPS enhanced alcohol-induced liver injury compared with wild-type mice [42]. On the other hand, IL-10 knockout mice have a reduced fatty liver and lower serum AST and ALT levels after ethanol feeding compared with wild-type mice [42]. This may be because IL-10 knockout mice have elevated levels of IL-6, and STAT3 activation in the liver, which lead to steatosis and hepatocellular damage. At the early stage of ALD, inflammation involving IL-6/STAT3 has a protective effect against alcoholic steatosis and liver injury. These findings suggest that IL-10 as well as IL-6 plays a protective role in the progression of ALD.

2.4. Other Interleukins. Nuclear regulatory factor kappa B (NF-κB) is a protein complex that controls the transcription of DNA and a central regulator of cellular stress in all cell types in the liver. NF-κB plays a key role in regulating the immune response to infection and in both acute and chronic inflammation. Activation of NF-κB in rats can induce the expression of IL-1β, which increases the expression of proinflammatory molecules [43, 44]. IL-1β and IL-6 were found to be essential for the induction of Th17 lymphocyte differentiation from human naive CD4+ T cells [45]. Furthermore, LPS-stimulated human monocytes induced Th17 polarization of naive $CD4^+$ T cells in an IL-1β signalling-dependent manner. IL-8 is a critical proinflammatory cytokine involved in many steps of neutrophil mobilization, from bone marrow to tissue infiltration or activation. IL-8 is induced by TNF-α and by ligands for TLRs via the activation of NF-κB. Serum IL-8 is highly elevated in patients with AH and is linked to neutrophil infiltration. In contrast, IL-8 is only moderately elevated in alcoholic cirrhosis patients or alcoholics. IL-17 plays a key role in enhancing the host immune response against microorganisms as well as in autoimmune diseases [46]. IL-17 stimulates multiple types of nonparenchymal hepatic cells to produce proinflammatory cytokines and chemokines [47] with a relatively weak TNF-α-like function. Furthermore, IL-17 can act with other cytokines to activate NF-κB and induce

IL-8. Recently it was shown that patients with ALD had higher IL-17 plasma levels compared with healthy subjects [48]. The functions of Th17 cells are also mediated via the production of IL-22. IL-22 is a member of the IL-10 family of cytokines and plays an important role in promoting hepatocyte survival and proliferation [49]. IL-22 administration to alcohol-fed mice also prevented liver steatosis and liver injury through the activation of hepatic STAT3 [50].

IL-1β is also a potent proinflammatory cytokine [51]. In both animal model and patient with ALD, the levels of pro-IL-1β are significantly increased in the liver and in the serum [52, 53]. IL-1β is produced as inactive pro-IL-1β in response to inflammatory stimuli, including both microbial products and endogenous danger-associated molecules. IL-1β gene expression and synthesis of pro-IL-1β occur after activation of pattern recognition receptors (PRRs). IL-1β acts in an autocrine or paracrine manner via the type I IL-1 receptor (IL-1R1). Activation of IL-1R1 is inhibited by its binding to the IL-1 receptor antagonist (IL-1Ra). Treatment with IL-1Ra significantly improves symptoms in patients with rheumatoid arthritis [54], or autoinflammatory syndromes.

3. Toll-Like Receptors

Toll-like receptors (TLRs), a family of PRRs, are transmembrane proteins originally identified in mammals on the basis of their homology with Toll, a Drosophila receptor that contributes to the production of antimicrobial peptides that act against microorganism invasion in the fly. 11 TLRs have been identified in humans and 13 in the mouse [55]. TLRs recognize pathogen-derived molecules, such as structural components unique to bacteria, virus, and fungi, and activate inflammatory cytokines and type I interferon (IFN) production. TLRs are expressed on the surface of immune cells, such as macrophages, dendritic cells, and nonimmune cells, including epithelial cells. Expression of TLR1, 2, 6, 7, and 8 was elevated in chronic ethanol-feeding model. The treatment with ethanol resulted in sensitization to liver inflammation and damage by TLR1, 2, 4, 6, 7, 8, and 9 ligands due to increased expression of TNF-α [56]. However, deficiency in TLR2 had no protective effect in a chronic ethanol-feeding mouse model [57]. TLRs play important roles in the pathophysiology of a variety of liver diseases [58], which may attribute to wide expression of TLRs on hepatocytes [59]. TLR4 is a functional receptor expressed on the surface of macrophages and various other types of cells that transmit endotoxin signals. Cluster of differentiation 14 (CD14) is a protein that is a component of the innate immune system. CD14 binds to LPS, thereby subsequently presenting it to TLR4 and MD-2, which activate the intracellular signalling pathway via myeloid differentiation factor 88 (MyD 88), resulting in NF-κB activation [60]. Both MyD88 and TRIF (MyD88 independent) signalling are associated with TLR3 and TLR4 (Figure 4). Recruitment of the TRIF adapter activates phosphorylation of interferon regulatory factor 3 (IRF3) that results in type I IFN production [61]. It is known that mice deficient in IRF3 or TLR4 expression are protected from alcohol-induced liver inflammation and hepatocyte injury

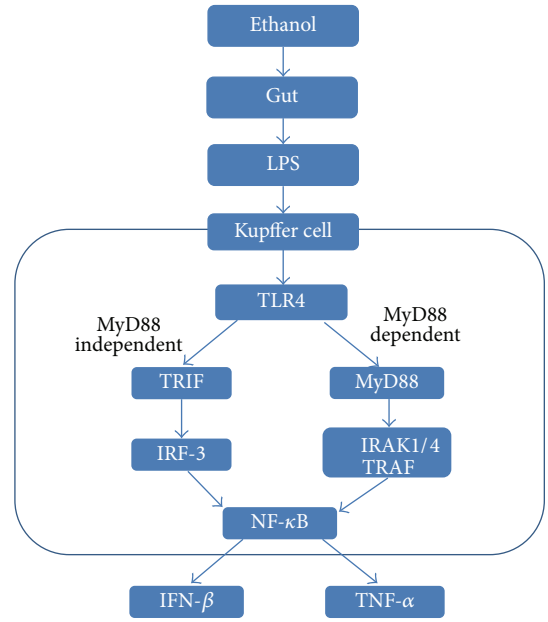

Figure 4: Toll-like receptor 4 signaling pathway in alcohol consumption. Ethanol promotes the translocation of lipopolysaccharide from the gastrointestinal lumen to the portal vein, where it binds to the lipopolysaccharide-binding protein. In Kupffer cells, lipopolysaccharide binds to CD14, which combines with TLR4 activating multiple cytokine genes. TLR4 are activated by MyD88 dependent or independent manner, leading to secretion of TNF-α or IFN-β.

[62]. The LPS/TLR4 signalling pathway consists of activation of transcription factors, such as NF-κB, which induces proinflammatory cytokine expression in the Kupffer cell. In the liver, TLR4 is expressed not only on innate immune cells such as Kupffer cells, but also on hepatocytes, hepatic stellate cells, sinusoidal endothelial cells, and biliary epithelial cells. Blockade of TLR4 or CD14 reduces liver pathology and inflammation in a mouse model of alcoholic liver injury [63, 64], which indicates the importance of the TLR4 signalling pathway. LPS recognition by TLR4 expressed on hepatic stellate cells and sinusoidal epithelial cells also contributes to the progression of ALD [65]. Alcohol stimulates Kupffer cells and monocytes to produce increased TNF-α in response to endotoxin [66]. In previous studies, our group showed that endotoxemia plays an important role in the initiation and aggravation of ALD through the enhancement of proinflammatory cytokines, including IL-6, IL-8, and TNF-α [67, 68]. Hepatic expression of TLR1, 2, 4, 6, 7, 8, and 9 mRNA was increased in the mouse model of chronic alcohol feeding [69]. Alcohol feeding also resulted in sensitization to liver damage and inflammation because administration of TLR1, 2, 4, 6, 7, 8, and 9 ligands resulted in increased expression of TNF-α mRNA [69]. Acute alcohol exposure inhibited TLR4 signalling in macrophages after alcohol treatment in mice leading to decreased LPS-induced TNF-α production [70]. In ALD, TLR3 activation in HSCs and Kupffer cells plays an antagonistic role against the TLR4-mediated signal pathway via the production of IL-10 [71].

A certain double-stranded RNA virus, a ligand of TLR3, triggered the expression of IL-10 through IRF3 signaling [72] and that activation of TLR3 signaling induced IRF3 activation [73]. TLR3 and IL-10 participate in the suppression or killing of activated HSCs and Kupffer cells in a variety of models of liver injury. Recent investigations suggest that TRIF-regulated IRF3 binds to the promoter region of the TNF-α gene and upregulates transcription in chronic ethanol-exposed macrophages contributing to alcohol-induced steatosis [74]. These findings suggest that TLRs play a "gate keeper" role in ALD.

4. Chemokines

Members of the CXC family of chemokines include IL-8 and growth-regulated α-protein (Gro-α). These mediators attract polymorphonuclear leukocytes which are the predominant inflammatory cells that infiltrate the livers of patients with ALD. In patients with AH, expression of these chemokines in the liver correlates with the severity of portal hypertension and patient survival [75, 76].

CCL2, referred to as monocyte chemotactic peptide-1 (MCP-1), is a member of the CC chemokine family. Its expression can be induced in many cell types, including inflammatory cells, hepatocytes, and hepatic stellate cells. CCR2 is the only known receptor for CCL2 and is expressed on monocytes, T lymphocytes, and basophils [77]. MCP-1 regulates adhesion molecules and proinflammatory cytokines TNF-α, IL-1β, and IL-6 [78]. The pivotal role of MCP-1 in ALD was recognized by showing higher amounts of MCP-1 as compared to other CC chemokines, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β, in the liver and mononuclear cells of patients with AH [79]. And MCP-1 is important in the modulation of proinflammatory cytokines [80]. Deficiency of MCP-1 protects mice against ALD, independent of CCR2, by inhibition of proinflamma-tory cytokines and induction of fatty acid oxidation, linking chemokines to hepatic lipid metabolism [81].

5. Inflammasomes

The inflammasome is a multiprotein oligomer consisting of caspase-1, PYCARD, and NALP that mediate the response to cellular danger signals activating and recruiting inflammatory cells. Procaspase-1 is activated by the inflammasome and cleaves pro-IL-1β into the bioactive IL-1β [82]. The inflammasome also promotes the cleavage of pro-IL-18 into IL-18 to induce IFN-γ secretion and natural killer cell activation, cleavage and inactivation of IL-33 [83, 84]. Inflammatory stimuli also drive activation of cytosolic caspase activation and recruitment domain (CARD) that recruit ASC and caspase-1 to assemble into the inflammasome. Inflammasome and IL-1β are activated in ALD patient or rodent animal model [85]. Recent studies demonstrated mRNA expression of several inflammasomes in the liver thus suggesting that inflammasome activation is a component of the liver pathophysiology in ALD [86].

6. Oxidative Stress

Oxidative stress is caused by excess ROS production, which leads to apoptosis and necrosis. ROS can also lead to a free radical chain reaction with unsaturated fatty acids generating toxic lipid intermediates. Oxidant stress is a pathogenic factor for the onset of ALD and nonalcoholic fatty liver disease (NAFLD). In vivo models of alcohol infusion induce lipid peroxidation because of increased free radical formation and decreased hepatic antioxidants, such as glutathione (GSH) [87]. In addition to GSH, other liver antioxidants, such as vitamin A, vitamin C, and bilirubin, and enzymes, such as superoxide dismutase and catalase, remove ROS. Moreover, treatment with an inhibitor of alcohol oxidation, such as 4-methylpyrazole, or an antioxidant, such as trolox, effectively prevented or reduced alcohol-induced toxicity, thereby demonstrating the importance of oxidant stress in the pathogenesis of ALD [88]. The catalytic activity of the cytochrome P450 enzymes requires oxygen activation, which results in the generation of ROS, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH). Activated Kupffer cells are responsible for the release of various mediators, such as proinflammatory cytokines including TNF-α, IL-1, and ROS. ROS participates in inflammation and modulation of hepatocyte metabolism [89]. There is increased production of ROS in ALD. ROS can be released from a variety of sources, such as activated Kupffer cells, CYP2E1, and NADPH oxidase. NADPH oxidase can enhance the activation of NF-κB and phosphorylate the ERK1/2 and p38 MAPK kinases that amplify Kupffer cell production of TNF-α [90]. Thus, ROS is highly involved in ALD.

7. Microflora Products

The importance of bacterial translocation in the pathogenesis of ALD has been demonstrated in many reports. Intestinal tight junction dysfunction [91–94] or bacterial proliferation [95, 96] caused by alcohol or its metabolites such as acetaldehyde enhance bacterial translocation into the liver, which induces the activation of Kupffer cells to release various proinflammatory cytokines and chemokines [97, 98] and increase acute LPS translocation. Chronic alcohol feeding causes structural changes in the gastrointestinal tract that might contribute to LPS translocation [99]. Continuous intragastric feeding of alcohol results in intestinal bacterial overgrowth and enteric dysbiosis, which is due to alcohol-induced downregulation of expression of several intestinal antimicrobial molecules [100]. In the intestine, disruption of tight junctions may lead to increased permeability to pathogens, which appears to be a common mechanism involved in the pathogenesis of ALD. Zonula occludens 1 (ZO-1), occludin, and claudin-5 are the transmembrane proteins that are expressed at the tight junction. Alcohol-treated mice showed loss of ZO-1, occludin, and claudin-5 expression in the colon. Gut-derived endotoxins play a crucial role in liver inflammation. Indeed, gastrointestinal permeability is higher in alcoholics than in normal [92, 101]. Alcohol-induced leaky gut results in the translocation of gram-negative bacteria from the intestinal lumen into the portal

blood, elevating lipopolysaccharide (LPS) levels and triggering significant inflammation and liver injury [102–104]. The gut-liver axis is associated with alcohol-induced liver injury, both in experimental animal models and in patients of ALD. Intestinal sterilization with antibiotics prevents alcohol-induced liver injury [105]. Probiotic therapy reduces circulating endotoxin derived from intestinal gram-negative bacteria in ALD. These findings indicate that there are close interactions between the liver and intestinal bacteria in ALD.

8. Treatment

Abstinence from alcohol is the essential treatment for ALD [106, 107], but many ALD patients have difficulty remaining abstinent. Thus, there is an urgent need to develop novel therapeutic interventions. In the experimental setting, there are many animal models of ALD [108–111], but these have some advantages and disadvantages. In the clinical setting, alcoholics with acute hepatitis or cirrhosis have identifiable symptoms and receive treatment. Severe AH patients have a high mortality rate of about 50%, and those who survive have a 70% probability of developing liver cirrhosis. Nutritional supplementation is necessary for AH patients because of the prevalence of malnutrition. Only two pharmacological agents, which are corticosteroids and pentoxifylline, are recommended for treating AH. Both are aimed at reducing inflammatory conditions. Corticosteroids reduce cytokine production through transcriptional regulation. On the other hand, pentoxifylline achieves a similar effect through the inhibition of phosphodiesterase. These two agents have relatively strong side effects; hence, they are mainly used for cases of severe AH. New agents are showing promise for treating AH patients. Many antioxidants are effective in treating alcohol-fed animals. The consequent depletion of antioxidants leads to elevated oxidative stress that contributes to both the genesis and progression of ALD in animal models. However, the effectiveness of antioxidant therapy in human patients with ALD remains obscure. TLR3 activation might be a novel therapeutic strategy for the treatment of ALD. Probiotics are also effective in ALD. *Lactobacillus* reduces endotoxemia and improves liver injury in the rat model of ALD [112]. Moreover, some clinical studies have indicated the effectiveness of probiotics treatment. However, despite these improvements, an effective treatment for ALD has not yet been established. New treatment methods are required for ALD in the near future.

9. Conclusion

Alcohol is one of the most common causes of chronic liver disease in the world [113]. There are numerous factors, such as inflammation, oxidative stress, innate immunity, or fibrosis, that result in the development and progression of ALD. The inflammatory cytokines or chemokines appear to play an important role in ALD. The progression of alcohol-induced liver injury involves some immune cells and hepatocytes through the release of cytokines, chemokines, and inflammasomes. Kupffer cells play an important role in the early stage of ALD, producing TNF-α through TLR4. Based on the understanding of the pathogenesis of ALD, TNF-α is a key to developing new approaches to treatment, which has advanced very little since the introduction of corticosteroid therapy. The development of targeted therapies for ALD is hampered by poor knowledge of the molecular mechanisms involved in its development, particularly in humans, and by the perception that it is an addictive and a self-inflicting disease. We consider that more studies are needed to increase the understanding of the pathogenesis of inflammatory cytokines in order to open new therapeutic avenues for ALD.

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Research Article

Intoxication by Intraperitoneal Injection or Oral Gavage Equally Potentiates Postburn Organ Damage and Inflammation

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The increasing prevalence of binge drinking and its association with trauma necessitate accurate animal models to examine the impact of intoxication on the response and outcome to injuries such as burn. While much research has focused on the effect of alcohol dose and duration on the subsequent inflammatory parameters following burn, little evidence exists on the effect of the route of alcohol administration. We examined the degree to which intoxication before burn injury causes systemic inflammation when ethanol is given by intraperitoneal (i.p.) injection or oral gavage. We found that intoxication potentiates postburn damage in the ileum, liver, and lungs of mice to an equivalent extent when either ethanol administration route is used. We also found a similar hematologic response and levels of circulating interleukin-6 (IL-6) when either ethanol paradigm achieved intoxication before burn. Furthermore, both i.p. and gavage resulted in similar blood alcohol concentrations at all time points tested. Overall, our data show an equal inflammatory response to burn injury when intoxication is achieved by either i.p. injection or oral gavage, suggesting that findings from studies using either ethanol paradigm are directly comparable.

1. Introduction

Ethanol is the most commonly abused substance in the United States and the third leading cause of preventable death [1], many of which are associated with unintentional injuries [2]. Binge drinking, defined as reaching a blood alcohol content of 0.08 [3], in particular, is an increasingly prevalent form of intoxication [4] and the characteristic drinking pattern of trauma patients [5]. As a central nervous system depressant, alcohol likely plays a causative role in many accidents but the diverse cellular effects of alcohol and its metabolites can also negatively alter the physiologic response to injury [6]. As a small neutral compound capable of freely traversing lipid membranes, alcohol can influence nearly every cell in the body with effects dependent on the amount and duration of exposure [7]. Even a single dose of alcohol in animals has been shown to worsen systemic inflammation after injuries, such as burns [8, 9]. Burns are a devastating injury with a complex natural history and high association with alcohol [10]. Nearly half of adult burn patients have a positive blood alcohol concentration (BAC) at the time of admission and this predisposes them to worsened clinical outcomes compared to patients with similar injuries not under the influence [11]. Specifically intoxicated patients were found to be twice as likely to acquire an infection, required more surgical procedures, had longer durations of stay in the intensive care unit, and generated more cost than their nonintoxicated counterparts [12]. Interestingly, these patients are not typically chronic alcoholics but are considered binge drinkers [13], consistent with the majority of alcohol consumption in the US [4]. With nearly 450,000 burns requiring medical attention each year in the American healthcare system [14], alcohol greatly contributes to the socioeconomic burden of this destructive injury as both

a causative agent and complicating factor in recovery. Despite the high prevalence and established consequences of binge intoxication at the time of burn injury, there are currently few differences in the treatment and management of burn patients with and without prior alcohol exposure. This may be due in part to the aforementioned dynamic natural history of burns as well as the complex and duration dependent effects of alcohol. In order to develop much needed targeted therapies, the effects of intoxication on the physiologic response to burn injury need to be studied and manipulated under controlled conditions. To this end, mouse models of binge ethanol exposure and burn have been in use for nearly 20 years and yielded insightful information into the mechanisms by which ethanol exacerbates the response to burn. Of note is the finding that ethanol can potentiate burn-induced damage in the intestine [15, 16], liver [17, 18], and lungs [19, 20] with increased serum interleukin-6 playing an important role in inflammation of these organs [21, 22]. The majority of these studies administer ethanol by gavage or i.p. injection to reach a desired BAC in mice. While presumably the presence and level of intoxication are the most important factors in these models, no one to date has investigated the impact of the route of ethanol administration in the context of burn. It is important to establish if results from historical experiments using i.p. injection and gavage are directly comparable as well as be aware of any unintentional confounding factors in future studies. Herein we examine the effects of ethanol, given by gavage or i.p. injection, on postburn inflammation and damage in the intestines, liver, and lungs of mice.

2. Materials and Methods

2.1. Mice. Male wild-type (C57BL/6) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and sacrificed at 8–10 weeks old. Mice were housed in sterile microisolator 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.

2.2. Murine Model of Ethanol and Burn Injury. A murine model of a single binge ethanol intoxication and burn injury was employed using either i.p. injection or oral gavage as described previously [23, 24]. Briefly, i.p. mice were given a single i.p. dose of 150 μL of 20% (v/v) ethanol solution (1.12 g/kg) or saline control. Gavaged mice were given a single dose of 300 μL of 10% (v/v) ethanol solution (1.12 g/kg) or water control. The mice were then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum was shaved, and they 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. The scald injury results in an insensate, full-thickness burn [25]. 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.

2.3. Blood Alcohol Concentration (BAC). Mice were sacrificed at 30 minutes, 1 hour, or 4 hours after a single dose of ethanol (1.12 g/kg) administered by either i.p. injection or gavage. Whole blood was collected via cardiac puncture, incubated at room temperature for 20 minutes and then centrifuged at 3000 rpm at 4°C for 20 minutes. Serum was isolated and BAC was measured using the GM7 Micro-Stat Analyzer (Analox, Lunenburg, MA).

2.4. Blood and Serum Measurements. At 24 hours after injury mice were euthanized, blood was collected via cardiac puncture, and 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 as described above and stored at −80°C. Serum aliquots were used to measure IL-6 by enzyme linked immunosorbent assay (ELISA) (BD Biosciences, Franklin Lakes, NJ) or liver transaminase levels using a DRI-CHEM 7000 (HESKA, Loveland, CO).

2.5. Histopathologic Examination of the Ileum and Liver. At 24 hours after injury mice were euthanized and the ileum, liver, and lungs were harvested. The ileum was fixed overnight in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H & E). The length of 5 individual villi in 5 fields of view (100x) was measured for a total of 25 measurements per animal. The average was considered representative of the villus length in the ileum and demonstrative images are presented herein. The whole liver was removed at the time of sacrifice, weighed, and normalized to total body weight.

2.6. Bacterial Translocation. Bacterial translocation was assessed as previously described [26]. Briefly, 3–5 mesenteric lymph nodes per mouse were removed, placed in cold RPMI, and kept on ice. Nodes were separated from connective tissue and homogenized with frosted glass slides. Homogenates were plated on tryptic soy agar and incubated at 37°C overnight.

2.7. Histopathologic Examination of the Lung. The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously [27], embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H & E). 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 [21]. Neutrophils were counted in a blinded fashion in 10 high power fields (400x).

2.8. KC Analysis of Lung Homogenates. The right middle lung lobe was snap-frozen in liquid nitrogen. The tissues were then homogenized in 1 mL of BioPlex cell lysis buffer according to manufacturer's instructions (BioRad, Hercules, CA). The

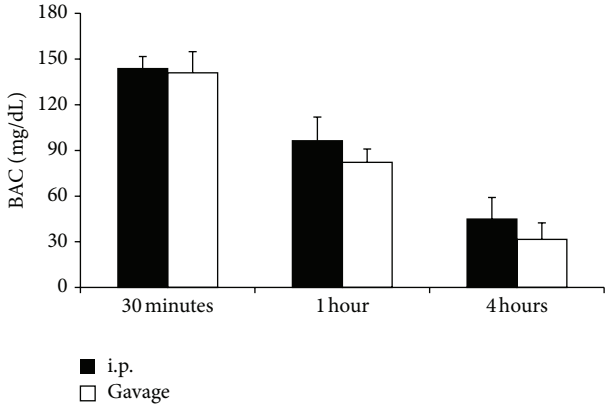

Figure 1: Blood alcohol concentration (BAC). Mice were administered 1.12 g/kg ethanol and the subsequent BAC measured at various time points. Data are presented at mean values ± SEM. $N = 6$–9 animals per group.

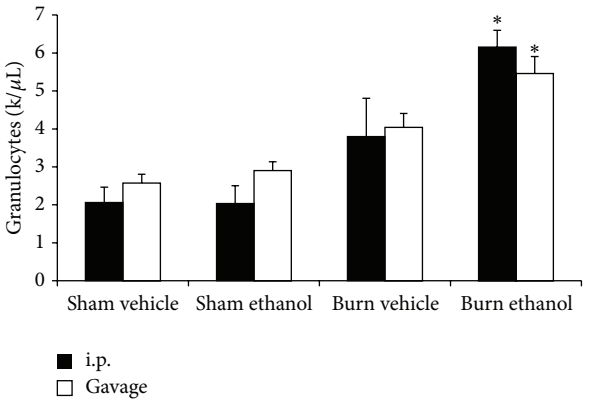

Figure 2: Circulating blood granulocytes 24 hours after injury. $^{*}P < 0.05$ compared to Sham groups. Data are presented at mean values ± SEM. $N = 3$–6 animals per group.

homogenates were filtered and analyzed for cytokine production using an ELISA for KC (BD Biosciences, Franklin Lakes, NJ). The results were normalized to total protein using the BioRad protein assay (BioRad, Hercules, CA).

2.9. Statistical Analysis. Statistical comparisons were made between i.p. and gavage animals in the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in 8 total groups analyzed. One-way analysis of variance was used to determine differences between treatment responses, and Tukey's post hoc test once significance was achieved ($P < 0.05$). Data are reported as mean values ± the standard error of the mean.

3. Results

3.1. Blood Alcohol Concentration Is Equal after I.P. Injection or Gavage. To determine if the route of ethanol administration impacted the kinetics of its absorption and clearance, the blood alcohol concentration (BAC) of mice was determined at 30 minutes, 1 hour, and 4 hours after a single dose of 1.12 g/kg ethanol by i.p. injection or gavage. Mice receiving ethanol by i.p. injection were found to have a BAC of 143 mg/dL at 30 minutes, which was reduced by 33% by 1 hour and 69% by 4 hours (Figure 1). Similarly, mice receiving ethanol by gavage demonstrated a BAC of 141 mg/dL at 30 minutes, which by 1 hour was decreased by 41% and by 77% at 4 hours (Figure 1). No significant difference between BAC in mice receiving ethanol via i.p. injection or gavage at each time point was found, suggesting that equivalent amounts of ethanol are absorbed into the bloodstream and are cleared at similar rates.

3.2. Intoxication by I.P. Injection or Gavage Increases Peripheral Blood Granulocytes after Burn. To examine if administration route effected the hematologic response to intoxication and burn, the number of circulating granulocytes was enumerated by an automated counter after burn or sham injury when preceded by ethanol given by i.p. injection or gavage. In gavaged mice, there was a 2-fold increase ($P < 0.05$) in blood granulocytes in intoxicated burned mice relative to sham injured mice regardless of prior intoxication status (Figure 2). Likewise in mice given an i.p. ethanol injection before burn, a 3-fold increase ($P < 0.05$) in blood granulocytes was found when compared to sham injured mice with and without prior intoxication (Figure 2). No significant differences were found between i.p. injected and gavaged mice within treatment groups suggesting that both routes of ethanol administration induce an equal neutrophilic leukocytosis after burn injury.

3.3. Serum IL-6 Is Elevated When Intoxication Precedes Burn Injury regardless of Administration Route. Circulating IL-6 levels were quantified by ELISA in all treatment groups of i.p. injected and gavaged mice. Burn injury alone increased the amount of serum IL-6 by greater than 25-fold above sham injured animals in both i.p. and gavage mice (Figure 3). When intoxication preceded the burn, a further 3- to 4-fold increase ($P < 0.05$) above burn alone was observed, regardless of the route of ethanol administration (Figure 3). No significant differences were found between i.p. and gavage mice within treatment groups suggesting that intoxication before burn injury increases serum IL-6 irrespective of the ethanol paradigm used.

3.4. Villus Blunting Is Similar in I.P. and Gavage Intoxicated Mice after Burn. We previously reported that intoxication by i.p. injection furthers the diminution of ileal villi after burn [26]. Consistent with our earlier observations, at 24 hours after burn (Figures 4(e)-4(f)), villi in the ileum were shortened in comparison to sham injured animals regardless of intoxication status (Figures 4(a)–4(d)). Furthermore when mice were intoxicated by i.p. injection (Figure 4(g)) or gavage (Figure 4(h)), villus blunting was pronounced beyond burn alone.

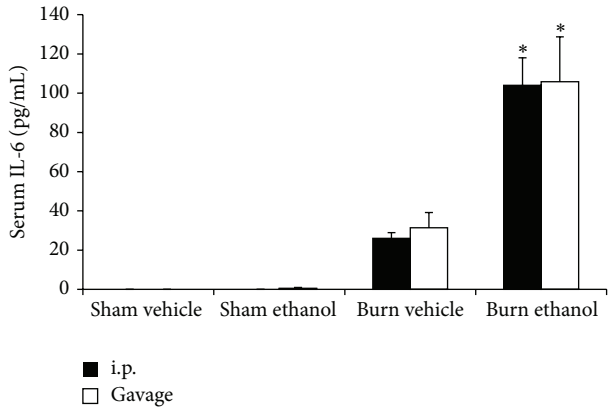

Figure 3: Serum IL-6 at 24 hours after injury. $^{*}P < 0.05$ compared to Sham groups. Data are presented at mean values ± SEM. $N = 4–8$ animals per group.

The average villus length in the ileum of burn injured mice was blunted by greater than 20% ($P < 0.05$) compared to sham injured animals regardless of intoxication status or administration method (Figure 5(a)). Antecedent intoxication by i.p. injection or gavage caused a further ~20% reduction ($P < 0.05$) compared to burn alone (Figure 5(a)), demonstrating that this increased intestinal damage is present to a similar extent whether intoxication is achieved by i.p. injection or gavage. This villus blunting corresponded to an increase in bacterial translocation to the mesenteric lymph nodes where intoxication increased the number of colony forming units by >400-fold over sham animals and 5-fold over burn alone (Figure 5(b)). No significant differences between i.p. and gavage mice were found within treatment groups suggesting that both routes of ethanol administration effect intestinal damage after burn injury in a similar manner.

3.5. I.P. or Gavage Intoxication Equally Exacerbates Hepatic Damage after Burn. Hepatic damage was measured by levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and by the liver weight to total body weight ratio. Burn alone increased serum ALT levels by greater than 6-fold compared to sham injured animals (Figure 6(a)). This increase was found irrespective of the presence or absence of ethanol or route of administration before sham injury. When i.p. or gavage mice were intoxicated at the time of burn, however, a greater than 12-fold elevation ($P < 0.05$) was observed over sham injured animals which corresponded to a ~2-fold increase ($P < 0.05$) above burn alone (Figure 6(a)). A similar pattern was found for serum AST with burn alone causing a greater than 5-fold increase relative to sham injured groups in both i.p. injected and gavaged mice with and without ethanol (Figure 6(b)). Intoxication in both i.p. and gavage mice at the time of burn increased serum AST an additional 2-fold ($P < 0.05$) which is a greater than 20-fold elevation ($P < 0.05$) over sham injured mice (Figure 6(b)).

Finally, the liver weight to total body weight ratio (LW : BW) was recorded as a measure of hepatic edema. No significant changes in LW:BW were found between Sham groups regardless of ethanol intoxication or administration route (Figure 7). Similarly, burn alone did not cause a significant change in LW : BW relative to sham injured mice. However when mice received ethanol by i.p. injection or gavage before burn, a ~47% increase ($P < 0.05$) above all other groups was observed. Taken together, the serum transaminase and LW : BW suggest that ethanol potentiates liver damage after burn injury irrespective of the route of intoxication.

3.6. I.P. or Gavage Administration of Ethanol Enhances Alveolar Wall Thickness after Burn. At 24 hours after intoxication and burn injury, there is a marked increase in the thickness of the alveolar wall and increased cellularity, which is more pronounced than after burn alone (Figures 8 and 9). The alveolar wall thickness and cellularity was quantified using imaging software to measure the area of lung tissue in 10 high power fields per animal which is reported as a percentage of the entire field of view. A significant increase in tissue area, corresponding to a relative decrease in air space, was found after burn injury, compared to sham animals ($P < 0.05$). Intoxication increased the tissue area after burn regardless of how it was achieved ($P < 0.05$), indicating a greater level of pulmonary congestion.

3.7. Neutrophil Accumulation and Pulmonary KC Levels Are Amplified after I.P. or Gavage Intoxication and Burn. Similar to previous studies [19, 20, 27], following the combined insult of i.p. ethanol injection and burn, there was a 20-fold increase in pulmonary neutrophils compared to sham animals ($P < 0.05$) and a 2-fold increase over burn alone ($P < 0.05$) (Figure 10(a)). This neutrophil accumulation in i.p. intoxicated animals corresponded to a 6-fold increase in KC compared to sham animals ($P < 0.05$) and a 2-fold elevation compared to burn alone (Figure 10(b)). When an equal amount of ethanol was given by gavage, similar results were observed with intoxicated burned mice having neutrophil numbers and KC levels that were 15- and 6-fold above sham animals ($P < 0.05$) and 2.5- and 2-fold above burn alone, respectively (Figure 10). No significant differences between i.p. and gavage mice within treatment groups were found suggesting that intoxication enhances post burn pulmonary neutrophil accumulation and KC regardless of administration route.

4. Discussion

The studies above indicate that in the context of burn injury in mice, intoxication at an equivalent BAC and duration exacerbates organ inflammation and damage to a similar extent whether given by oral gavage or i.p. injection. Equal BACs at the time of injury resulting in comparable amounts of organ damage are consistent with findings that suggest ethanol acts through worsening ischemic damage [28], altering cytokine networks [29, 30], and impairing immune responses [31]

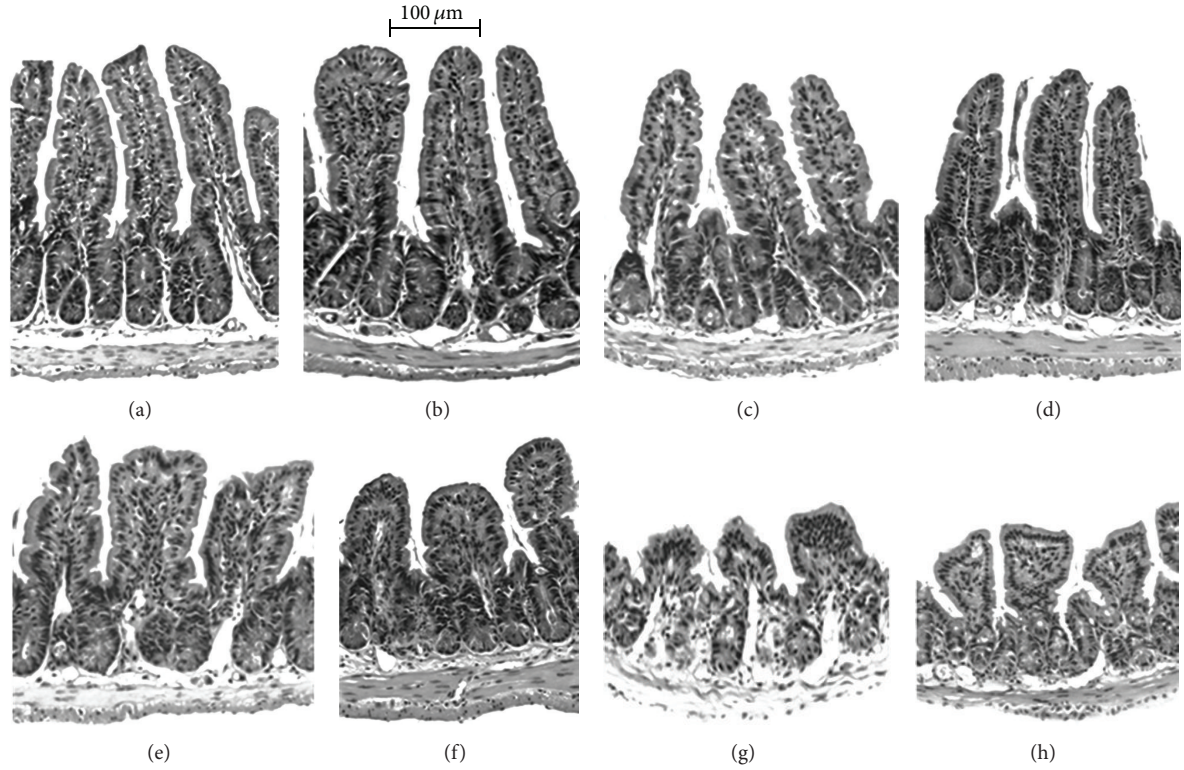

Figure 4: Histologic state of the ileum 24 hours after intoxication and burn injury. Sham injured mice receiving i.p. (a) and gavage (b) control, or i.p. (c) and gavage (d) ethanol have normal appearing villi. Burn injury alone receiving i.p. (e) and gavage (f) control demonstrate rounded and widened villi that are markedly blunted when combined with ethanol by i.p. injection (g) or gavage (h).

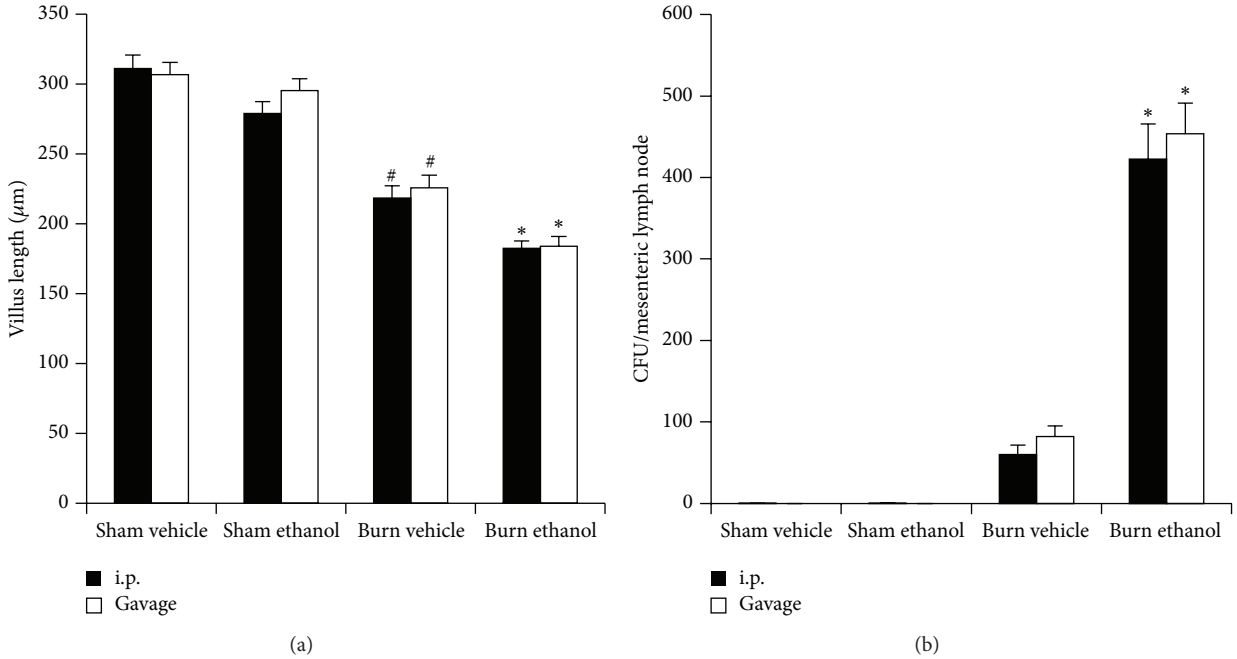

Figure 5: Villus length in the ileum (a) and bacterial load per mesenteric lymph node (b) 24 hours after injury. $^{*}P < 0.05$ compared to Sham and Burn Vehicle groups. $^{\#}P < 0.05$ compared to Sham groups. Data are presented at mean values ± SEM. $n = 4$–6 animals per group.

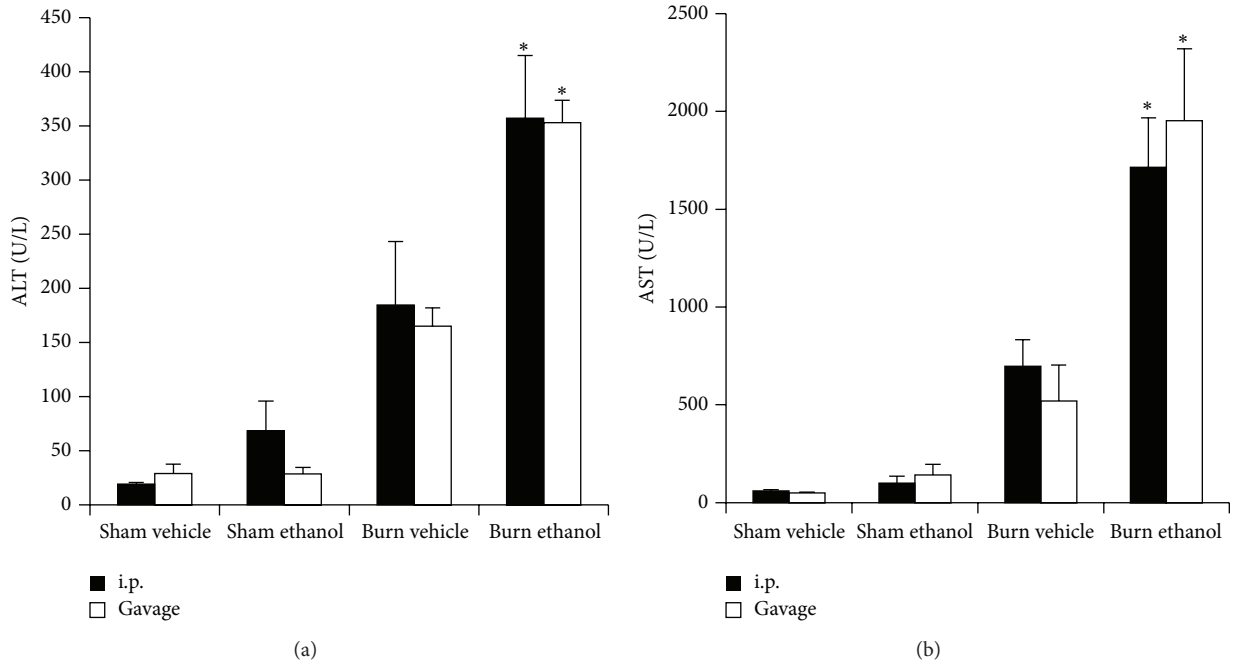

Figure 6: Serum alanine aminotransferase (ALT) (a) and serum aspartate aminotransferase (AST) (b) 24 hours after injury. $^{*}P < 0.05$ compared to Sham and Burn Vehicle groups. Data are presented at mean values ± SEM. $N = 3$–5 animals per group.

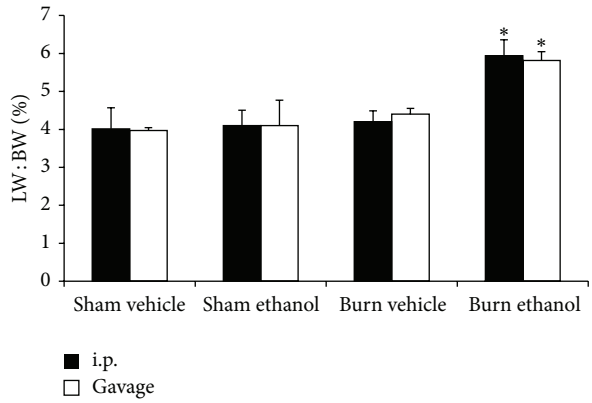

Figure 7: Liver weight (LW) to body weight (BW) ratio 24 hours after injury. $^{*}P < 0.05$ compared to Sham and Burn Vehicle groups. Data are presented at mean values ± SEM. $N = 4$–6 animals per group.

after burn injury and perhaps not through interactions at the site of absorption. The near infinite water solubility of ethanol allows for a quick distribution throughout the blood and we observed peak BACs near 30 minutes when ethanol was administered by oral gavage or i.p. injection. As seen in Figure 1, identical doses of ethanol by both paradigms resulted in nearly the same BAC at 30 minutes, 1 hour, and 4 hours after administration. The absorption time and BAC of the i.p. mice agree with our previously published studies [17, 26, 30] but the equivalency of BAC profiles between gavage and i.p. injection is in contrast with the work by Livy et al. [32] who concluded that in mice, ethanol given by gavage resulted in a lower BAC than an equivalent amount of ethanol given by i.p. injection. They proposed that this discrepancy may be due to metabolism by gastric alcohol dehydrogenase, which only occurs when ethanol traverses the gastrointestinal tract. While the reasons for the contrasting results are unclear, several possibilities, including differences in ethanol amount (3.8 g/kg versus 1.12 g/kg), the volume of ethanol administered (up to 0.41 mL versus 0.15 mL (i.p.)), and discrepancy between the vehicle used in i.p. injections (water versus saline) may have been contributing factors. Nevertheless, the mice used in our studies, which were given equal doses of ethanol by gavage and i.p. injection, demonstrated equivalent BAC profiles and an over exuberant inflammatory response after burn. A further discussion regarding considerations of i.p. and gavage ethanol administration can be found elsewhere as reviewed by D'Souza El-Guindy et al. [33].

A neutrophilic leukocytosis is seen in a variety of illnesses and conditions and is widely regarded an indicator of infection or inflammation. Trauma can also induce a leukocytosis where it is considered an acute phase marker and is clinically associated with increased morbidity and mortality risk [34]. We observed, in Figure 2, that intoxication by either paradigm induced a similar granulocytic leukocytosis at 24 hours after burn. The sequestration of these circulating neutrophils in end organs after injury is proposed as a major mechanism in the pathogenesis of multiple organ failure [35]. We and others have shown that intoxication at the time of burn leads to increased neutrophil infiltration into the gut, liver, and lungs of mice within 24 hours [8, 9, 36], Figure 10(a). Furthermore, prevention of neutrophil transmigration using ICAM knockout mice in this setting

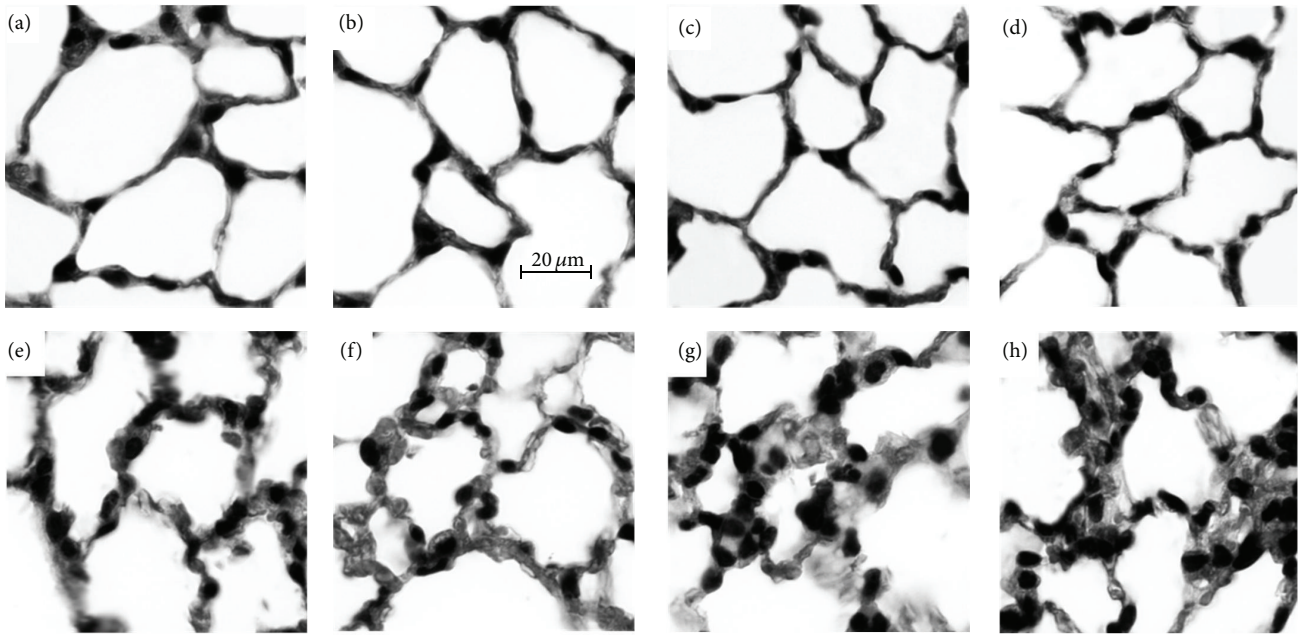

Figure 8: Histologic state of the lungs 24 hours after injury. Sham injured mice receiving i.p. (a) and gavage (b) control, or i.p. (c) and gavage (d) ethanol have normal appearing alveoli. Burn injury alone receiving i.p. (e) and gavage (f) control display an increase in alveolar wall thickness compared to sham injured animals (a–d). Intoxication by i.p. injection (g) or gavage (h) prior to burn results in further amplified alveolar wall thickness and cellularity.

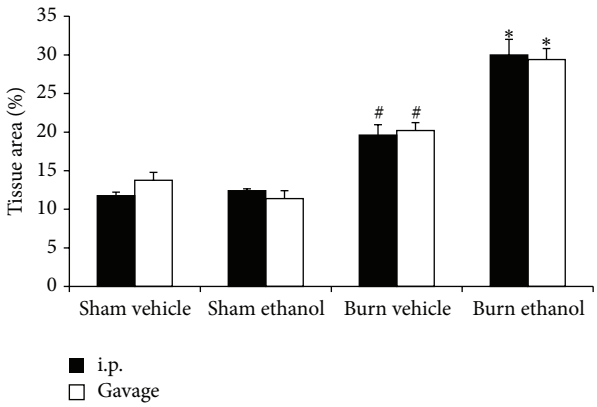

Figure 9: Quantification of pulmonary congestion 24 hours after injury. $^{*}P < 0.05$ compared to Sham and Burn Vehicle groups. $^{\#}P < 0.05$ compared to Sham groups. Data are presented at mean values ± SEM. $N = 4$–6 animals per group.

decreased pulmonary inflammation [20], highlighting the important role of neutrophil infiltration in this setting.

Circulating neutrophils migrate from the blood into tissues along a density gradient of chemoattractants, which in the mouse include KC. In mice, a burn injury increases pulmonary KC and ethanol has been shown to amplify this accumulation both in the absence [19–21] and presence of an intratracheal infection with *Pseudomonas aeruginosa* [30, 37]. We observed that both ethanol paradigms increase pulmonary KC equally after burn (Figure 10(b)) and this corresponded to increased neutrophil numbers in the lung (Figure 10(a)). The leukocytosis after intoxication and burn, together with an increase in neutrophil chemoattractants, likely plays a key role in the subsequent pulmonary inflammation and appears to be independent of the method of ethanol administration.

Elevated levels of circulating IL-6 also correlate with mortality risk in trauma patients [38] and are further increased when intoxication precedes burn injury [7, 17, 39]. We confirm our previous findings that burn alone increases serum IL-6 levels in mice and intoxication at the time of injury raises circulating IL-6 even further. We now report that this amplified IL-6 level when intoxication precedes burn injury is not affected by the route of ethanol administration (Figure 3). IL-6 in the setting of ethanol and burn has a causative role in intestinal damage [22] and pulmonary inflammation [21] though the source of systemic IL-6 is currently unknown. Of interest is the finding that the combination of intoxication and burn injury leads to greater bacterial translocation than either insult alone [26], which may incite a hepatic response, including IL-6 production.

Intestinal bacteria and lipopolysaccharide (LPS) that enter the portal system encounter Kupffer cells in the liver. This interaction between the intestinal microbiome and liver homeostasis is known as the "gut-liver axis" and plays a role in a myriad of diseases. Both burn and alcohol are known manipulators of the gut-liver axis and the combination of these insults has been shown to synergistically worsen hepatic damage in mice [18]. Clinically, liver function closely correlates to mortality risk after burn [40] and the importance of the gut-liver axis is highlighted by animal studies demonstrating improved outcomes after trauma when the gut is prophylactically sterilized with antibiotics [41, 42]. Increased liver damage and LPS stimulation may lead to hepatic production of excessive amounts of systemic IL-6,

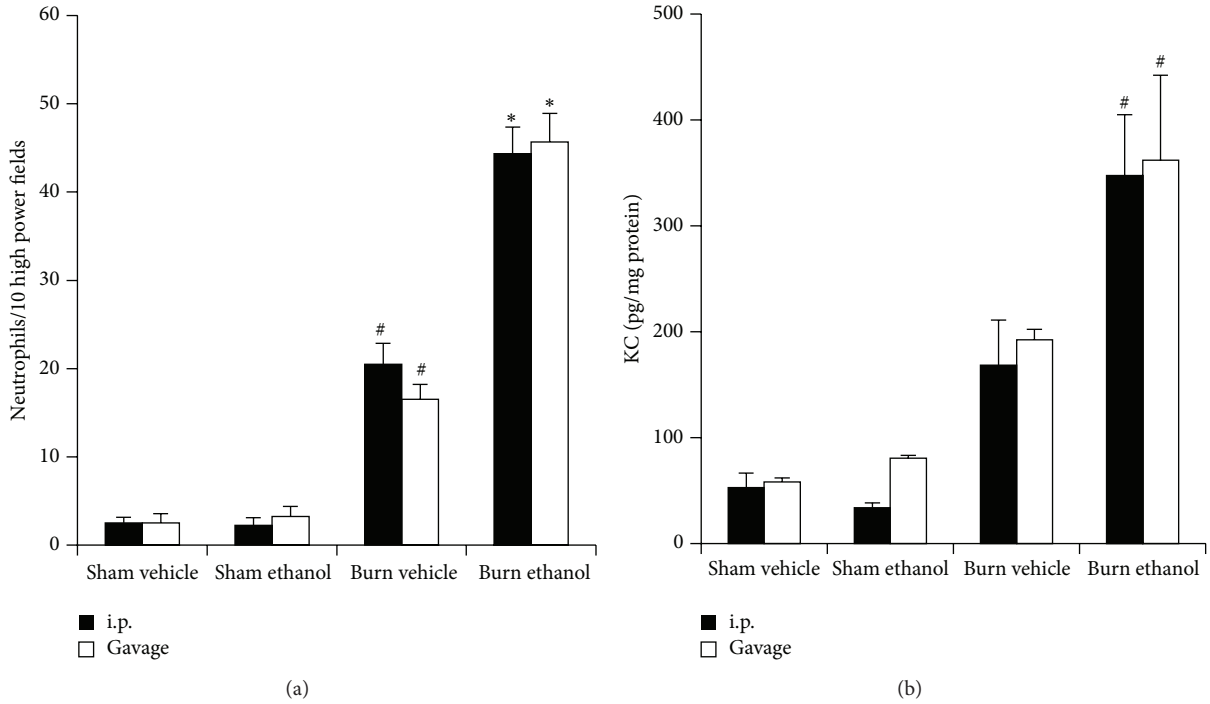

Figure 10: Pulmonary neutrophils in 10 high power (400x) fields of view (a) and pulmonary KC levels (b) 24 hours after injury. $^{\#}P < 0.05$ compared to Sham groups. $^{*}P < 0.05$ compared to Burn Vehicle groups. Data are presented at mean values ± SEM. $N = 4$–8 animals per group.

which as mentioned above, plays a causative role in the increased pulmonary inflammation of burned intoxicated mice. This is of clinical significance because multiple organ failure is common after a substantial injury and the lungs are among the first organs to fail.

We now report gavage or i.p. intoxication potentiated postburn intestinal damage as demonstrated by histology (Figure 4) and villus length (Figure 5(a)). Furthermore, intestinal damage corresponded to an increase in bacterial translocation (Figure 5(b)) and hepatic damage as assessed by serum transaminase levels (Figure 6) and hepatic weight (Figure 7). These findings were independent of the ethanol administration route in our model and support the idea of an altered gut-liver axis when intoxication is present at the time of burn injury. Of note is the rise in serum IL-6 levels (Figure 3) that mimic the pattern of damage observed in the liver (Figure 6).

Increased serum IL-6 is linked to poor survival in patients with acute respiratory distress syndrome (ARDS) [43]. ARDS is characterized by inflammation and edema in the lung parenchyma leading to impaired gas exchange. When examined by histology (Figure 8), the lungs of mice from both gavage and i.p. paradigms appear congested relative to all other treatment groups. When the amount of tissue relative to air space was quantified (Figure 9), the alveolar wall thickening and increased cellularity seen visually was found to be increased after burn and further increased with prior intoxication. This finding agrees with our previously reported work with i.p. injected mice [21] and is unaffected by the route of administration.

5. Conclusions

The socioeconomic impact and clinical relevance of intoxication at the time of burn injury merit in-depth investigation into the mechanisms for worsened outcome in these patients. Animal studies using mice offer controlled conditions, manipulatable genomes, and pharmacologic interventions not available in humans. An important variable is the level of intoxication achieved before burn and while historically animal studies have administered known amounts of ethanol by i.p. injection, oral gavage is considered a more physiologic method of intoxication. We now describe that postburn inflammation and damage in the ileum, liver, and lungs of mice are exacerbated to an equal extent when preceded by intoxication achieved by i.p. injection or gavage. Furthermore, the administration route had no impact on the hematologic changes observed when intoxication precedes burn. Taken together our data suggest that either i.p. injection or gavage is appropriate for studying the effects of ethanol on postburn inflammation and response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Asymmetric Dimethylarginine Blocks Nitric Oxide-Mediated Alcohol-Stimulated Cilia Beating

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The airway epithelium is exposed to alcohol during drinking through direct exhalation of volatized ethanol from the bronchial circulation. Alcohol exposure leads to a rapid increase in the cilia beat frequency (CBF) of bronchial epithelial cells followed by a chronic desensitization of cilia stimulatory responses. This effect is governed in part by the nitric oxide regulation of cyclic guanosine and adenosine monophosphate-dependent protein kinases (PKG and PKA) and is not fully understood. Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, is implicated in the pathogenesis of several pulmonary disorders. We hypothesized that the inhibition of nitric oxide synthase by ADMA blocks alcohol-stimulated increases in CBF. To test this hypothesis, ciliated primary bovine bronchial epithelial cells (BBEC) were preincubated with ADMA (100 μM) and stimulated with 100 mM ethanol. CBF was measured and PKA assayed. By 1 hr, ethanol activated PKA, resulting in elevated CBF. Both alcohol-induced PKA activation and CBF were inhibited in the presence of ADMA. ADMA alone had no effect on PKA activity or CBF. Using a mouse model overexpressing the ADMA-degrading enzyme, dimethylarginine dimethylaminohydrolase (DDAH), we examined PKA and CBF in precision-cut mouse lung slices. Alcohol-stimulated increases in lung slice PKA and CBF were temporally enhanced in the DDAH mice versus control mice.

1. Introduction

Alcohol use disorders are associated with increased lung infections including pneumonia and bronchitis [1]. While alcohol is an established risk factor for acute lung injury leading to sepsis [2], lung immunosuppression is also a common response to heavy and sustained alcohol use [3]. Chronic alcohol exposure also impairs lung innate host defense by desensitizing effective mucociliary clearance [1], rendering cilia unresponsive to stress. Collectively, this multifaceted impact of alcohol on the pathophysiology of lung function has been termed "alcoholic lung disease" [4].

Alcohol is a small, uncharged molecule that readily crosses the lipid bilayer of all cell membranes and easily enters the lungs, where as much as 15% of ingested alcohol can be excreted by the exhalation of volatilized alcohol through the airways. In contrast to dissolved oxygen and carbon dioxide, which traffic through the pulmonary circulation to and from the alveolar spaces, systemic arterial blood alcohol directly off-gases into the conducting airways via diffusion from the bronchial circulation [5]. Furthermore, higher localized concentrations of ethanol can be found in the airways of the lungs due to vapor condensation or "rain" effect of exhaled alcohol [1]. For these reasons, the ciliated airway epithelial cells lining the lungs are directly exposed to significant amounts of alcohol in persons who drink.

When ciliated airway epithelial cells are exposed to pathophysiologic concentrations of ethanol for extended periods

of time, a change occurs in the cells' ability to respond to external stimuli. Specifically, the normative cilia stimulatory response to inhaled particles of increased beat frequency becomes desensitized with sustained alcohol exposure [1]. This desensitization involves the uncoupling of downstream cyclic nucleotide-dependent protein kinase action on ciliary axonemal targets [6]. In contrast, brief alcohol exposure rapidly and transiently increases cilia beat frequency (CBF) of bronchial epithelial cells [7]. This alcohol effect on cilia is mediated by the nitric oxide (NO)-mediated regulation of cyclic adenosine monophosphate-dependent protein kinase (PKA) [1]. However, the exact mechanism that transitions the initial alcohol-induced cilia stimulatory response toward eventual cilia desensitization is not fully understood.

First demonstrated over 20 years ago [8], significant levels of ADMA are found in the lung [9] and specifically the bronchial epithelium [10]. Dysregulation of ADMA has been implicated in the pathogenesis of several pulmonary disorders such as asthma, fibrosis, pulmonary hypertension, and sepsis [11]. Previously, we have demonstrated that exogenously applied synthetic inhibitors of NOS are capable of blocking alcohol-stimulated PKA activation, and thereby preventing the stimulated CBF response [1, 12]. Therefore, we hypothesized that the action of an endogenously produced nitric oxide synthase inhibitor, ADMA, blocks alcohol-stimulated increases in CBF. The action of ADMA on NOS could therefore represent the mechanistic pathway in the alcohol-mediated transition of cilia from stimulatory to desensitized phenotype.

2. Materials and Methods

2.1. Mice. Wild type (WT) C57BL/6 and dimethylarginine dimethylaminohydrolase transgenic (DDAH-I) mice on the C57BL/6 strain that overexpress DDAH were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred and maintained in microisolator units in the UNMC specific pathogen-free animal facility. The transgene expression of hDDAH is observed in aorta, heart, and brain. Mice were allowed food and water *ad libitum* and were used experimentally at 6–12 weeks of age. All animals were used in accordance with National Institutes of Health guidelines and the University of Nebraska Medical Center Institutional Animal Care, and Use Committee approved the study.

2.2. Ex Vivo Mouse Tracheal Ring Model. Mice were sacrificed, and tracheae and lungs were removed. Each trachea was removed and maintained in sterile serum-free M199 containing penicillin/streptomycin (100 units/100 mg per mL) (Gibco) and fungizone (2 μg/mL) (Gibco) at room temperature. Tracheal rings were cut (width $\approx$ 0.5–1 mm) from the distal end of the trachea just proximal to the first bifurcation of the trachea into right and left mainstream bronchi. The rings were incubated in serum-free M199 (Gibco) for 30 min prior to measuring CBF determinations. After measuring baseline CBF, tracheal rings were incubated with experimental treatments for up to 6 hr at 37°C and 5% CO_2 then were allowed to equilibrate at 25°C for 10 min before CBF readings were recorded. In addition, the posttreatment epithelial cells from the remaining trachea were extracted with a sterile cell lifter (Fisher, Springfield, NJ) into cell lysis buffer as previously described [13]. The epithelial lysate was then immediately flash-frozen in liquid nitrogen for PKA activity assay.

2.3. Ex Vivo Mouse Lung Slice Model. Precision-cut lung (PCL) slices were made as previously described [14]. Briefly, C57BL/6 or DDAH-I mice were sacrificed and the lungs were inflated with low melting point agarose (Invitrogen, Carlsbad, CA). Chilled lungs were then sliced into 150 μm precision-cut slices (OTS 4500 Tissue slicer, Electron Microscopy Sciences, Hatfield, PA) followed by incubation at 37°C to remove the agarose. Slices (3–5 per well) were placed in 12-well tissue culture plates. After 5 days of incubation with daily change of media, slices were exposed for up to 1 hr with 100 mM ethanol, and CBF was measured. Lung slices were snap-frozen in liquid nitrogen for PKA activity assay. Data were normalized to the total amount of PCL protein contained in each well.

2.4. Ciliated Cell Culture. Ciliated mouse tracheal epithelial cells (MTEC) were cultured using an air-liquid interface system as previously described [15]. Primary bovine bronchial epithelial cells (BBEC) were obtained fresh from cow lungs (ConAgra Inc., Omaha, NE) and grown to confluent monolayers in culture as previously described [16].

2.5. Cell Viability Assay. An aliquot (50 μL) of supernatant media from BBEC monolayers, tracheal rings, MTEC, or PCL slices treated with ADMA, ethanol, or media alone was assayed for cell viability using a TOX-7 kit (Sigma) to measure lactate dehydrogenase (LDH) release, according to the manufacturer's instructions. As a positive control, confluent 60 mm dishes of BBEC cells were lysed and LDH was measured.

2.6. Immunohistochemistry for ADMA and DDAH. Mouse lungs were inflation-fixed through the trachea with 10% formaldehyde-PBS, processed, paraffin-embedded, and sectioned (5 μm). Mounted slides were either stained for ADMA or DDAH expression. Immunohistochemical detection of free and protein-bound ADMA was performed using a rabbit anti-ADMA antibody (1 : 1000; EMD Millipore, Billerica, MA) or rabbit anti-DDAH-I and DDAH-II antibodies (1 : 400; Santa Cruz Biotechnology, Dallas, TX). Sections were deparaffinized, hydrated, and washed with PBS. Before staining, endogenous peroxidase activity was inhibited using Peroxo-Block (Zymed Laboratories, South San Francisco, CA). Slides were incubated for 30 min with primary antibody and developed using an immunoperoxidase kit (Vector Laboratories, Burlingame, CA). Control sections were incubated with a nonspecific normal rabbit IgG (Sigma) followed by secondary antibody. All sections were counterstained with hematoxylin (Fisher Scientific). All slides were scanned by the Tissue Science Facility at the University of Nebraska Medical Center on a Ventana Coreo at 40x with a resolution of 0.2325 microns per pixel (Ventana, Tucson, Arizona).

2.7. Cilia Beat Frequency Assay. CBF was recorded from adhered cells, tracheal rings, and precision-cut lung slices in media-submerged cultures. The frequency of the beating cilia and the total number of motile points within a given field of view were determined using the Sisson-Ammons Video Analysis (SAVA) system [17].

2.8. PKA Activity Assay. Primary MTEC isolated from trachea directly or cultured on air-liquid interface as well as PCL slices was assayed for PKA activity. After experimental treatment conditions, culture supernatants were removed, 250 μL of cell lysis buffer was added, and cells or tissue was flash-frozen. Dishes were thawed and scraped into centrifuge tubes and kept on ice. The supernatant containing the cells was sonicated, and cells were centrifuged at 10,000 ×g at 4°C for 30 min. PKA activity was measured in the soluble fraction from the extracted cell or tissue sample as previously described [13]. Data was standardized to the total amount of cell protein assayed and expressed as pmol radiolabeled phosphate transferred onto a standard amount of heptapeptide substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly; Sigma) per minute of reaction time. Each unique condition was measured a minimum of 3 separate experiments.

2.9. Statistical Analysis. Replicate data from at least 3 separate experiments are presented as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) with Tukey multicomparison posttest was employed to compare responses between 3 or more groups. Differences between groups were accepted as significant using a 95% confidence interval ($P < 0.05$). In all analyses, GraphPad Prism (San Diego, CA; version 5.01) software was utilized to determine statistical significance.

3. Results

3.1. ADMA and DDAH Are Located in the Ciliated Cells of the Airway Epithelium. While the lung has been shown to have a high expression of DDAH-I [18] and DDAH-II [10], specific airway epithelial cell expression of ADMA has not been demonstrated. We hypothesized that ADMA and DDAH are preferentially expressed in ciliated airway cells. To test this hypothesis, tissue immunohistochemistry was performed on lung sections from mice to determine the presence and cellular location of both ADMA and DDAH. Sections were stained with primary antibodies to ADMA, DDAH-I, or DDAH-II and detected using a secondary immunoperoxidase-conjugated antibody detection reaction. Readily visible brown staining was observed in all lung sections corresponding to ADMA, DDAH-I, and DDAH-II in wild type mice (Figure 1). Staining of all 3 proteins was evident throughout the alveolar parenchyma but was particularly prominent in the epithelial cells lining the airways both in proximal and distal regions of the lung. Similarly, ADMA, DDAH-I, and DDAH-II were each detected in the lungs of transgenic DDAH-I expressing mice although the levels of ADMA appeared to be somewhat decreased, yet still present. All lung sections were counterstained with hematoxylin stain revealing visible purple stain in the absence of any positive brown staining due to primary antibody localization. As a control, lung tissue slices were incubated with a non-specific IgG followed by the same secondary antibody used above. These results demonstrate that the ciliated airway epithelium contains significant amounts of ADMA, DDAH-I, and DDAH-II.

3.2. Increasing ADMA Levels Alone Has No Baseline Effect on Cilia Beat. Because ADMA and DDAH were distinctly localized to the ciliated airway epithelium in the unstimulated state, we hypothesized that baseline unstimulated CBF is not NO-dependent so it would not be affected by changing ADMA levels. To test this hypothesis, we measured unstimulated CBF following exposure to supplemental ADMA. Mouse tracheal ring cilia beating was unaffected by exogenous treatment with 10 μM–10 mM ADMA tested over a 6 hr period (Figure 2). A small, but insignificant, decrease in CBF at 6 hr treatment was observed under conditions of nonphysiologic concentrations (10 mM) of ADMA. Similarly, baseline unstimulated CBF in tracheal rings from DDAH transgenic mice does not differ from that of wild type mice while beta agonist (procaterol; 10 nM) significantly stimulated CBF in both wild type and DDAH mice (data not shown). These data demonstrate that the increases in ADMA levels that are physiologically relevant do not alter cilia beating in the absence of any other cilia modulator.

3.3. ADMA Blocks Alcohol-Stimulated Increases in Both CBF and PKA Activity in Bovine Bronchial Epithelial Cells. Previously, we have shown that short-term alcohol treatment of ciliated bronchial epithelial cells rapidly and transiently stimulates increased cilia beating in a nitric oxide-dependent manner [1]. Although increasing ADMA by itself has no effect on CBF (as shown in Figure 2), we hypothesized that elevated ADMA would block the alcohol/NO-induced stimulation of CBF. Ciliary beat in primary cultures of ciliated bovine bronchial epithelial cells (BBEC) was significantly stimulated by 1 hr alcohol (100 mM; Figure 3(a)). Pretreatment of BBEC with 100 μM ADMA for 30 min prior to alcohol stimulation completely blocked alcohol-induced increases in CBF. This inhibition of alcohol-stimulated CBF was comparable to that of the NOS inhibitor, NG-Monomethyl-L-arginine (L-NMMA). Because alcohol-stimulated NO production is required for alcohol-stimulated PKA activation and subsequent increases in CBF [1], we hypothesized that ADMA blocks PKA activation by alcohol. To test this hypothesis, we measured PKA activity in alcohol-exposed BBEC in the presence or absence of ADMA. While 1 hr alcohol (100 mM) treatment significantly elevated BBEC PKA, pretreatment with 100 μM ADMA or 10 μM L-NMMA for 30 min prior to alcohol stimulation blocked ethanol-stimulated increases in PKA (Figure 3(b)). These data show that ADMA prevents alcohol-stimulated and NO-mediated increases in PKA-stimulated CBF.

3.4. Alcohol Stimulation of Tracheal Epithelial CBF Is Enhanced in Mice Expressing an ADMA-Degrading Enzyme.

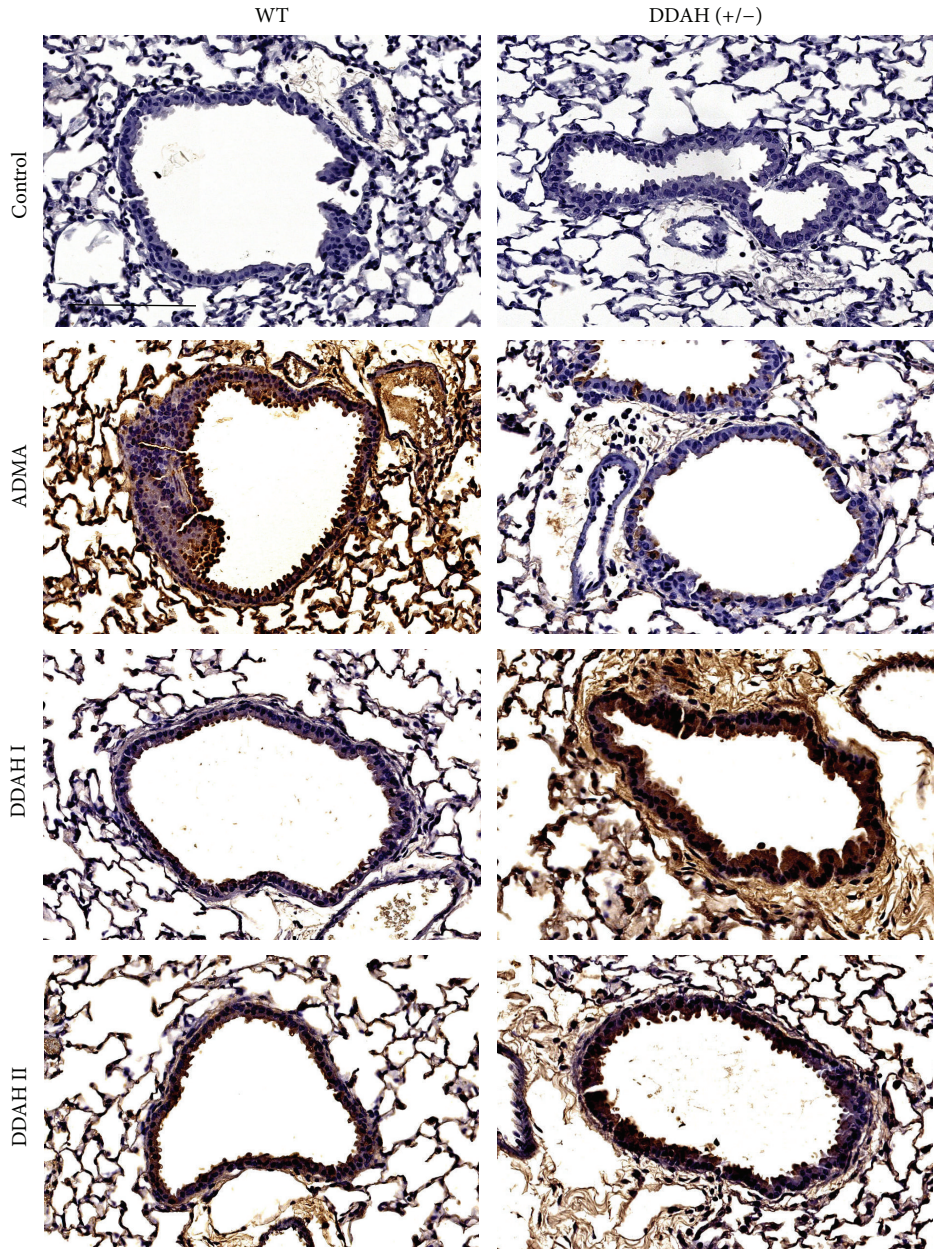

Figure 1: ADMA and DDAH are located in the ciliated cells of the airway epithelium. Free and bound ADMA, DDAH-I, and DDAH-II were immunolocalized in wild type (WT) and DDAH-I overexpressing [DDAH (+/−)] mouse lung tissue sections and visualized by immunoperoxidase stain. Controls consist of non-specific IgG primary antibody. Original magnification is 40x. Bar represents 100 μm.

Because ADMA is degraded by the action of dimethylaminohydrolase (DDAH) into L-citrulline, we hypothesized that overexpression of DDAH enhances alcohol stimulation of CBF. To test this hypothesis, we determined the impact of elevated DDAH levels on alcohol-stimulated NO action with regard to PKA activation and CBF in ciliated epithelium from mouse tracheal rings derived from transgenic mice overexpressing DDAH-I, ADMA-degrading enzyme. Compared to wild type mice, alcohol more rapidly stimulated CBF in the tracheal cilia of DDAH overexpressing mice. Alcohol-stimulated CBF in the DDAH mice at 15–35 min compared to 40–50 min in the wild type mice (Figure 4). Furthermore, the peak magnitude of enhanced CBF for DDAH mice at 30 min was significantly increased over the peak magnitude of wild type mice at 1 hr (data not shown). These results show that high levels of DDAH enhance the alcohol-stimulated cilia response by facilitating potential NOS activation in response to alcohol.

3.5. Alcohol-Stimulated Increases in Lung Slice Airway CBF and PKA Are Enhanced in DDAH Mice. Previously, we reported a precision-cut lung slice model for measuring CBF changes [15]. Similar to tracheal ring CBF, alcohol rapidly (1 hr) stimulates increases in mouse lung slice airway CBF

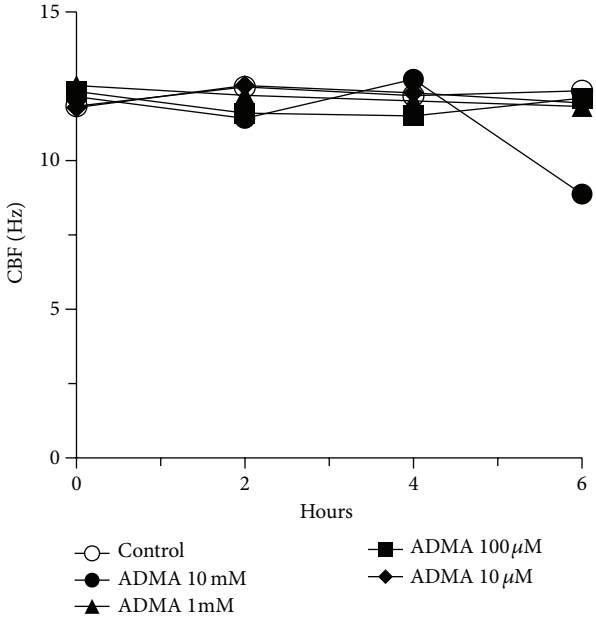

Figure 2: ADMA blocks alcohol-stimulated increases in both CBF and PKA activity in bovine bronchial epithelial cells. Cilia beat frequency (CBF) in ciliated mouse tracheal rings treated with 10 μM–10 mM asymmetric dimethylarginine (ADMA) for up to 6 hr. ADMA had no effect on baseline CBF versus control media at any time point. Bars represent SEM of triplicate independent experiments (n = 3 mice) measuring at least 10 separate fields per experiment.

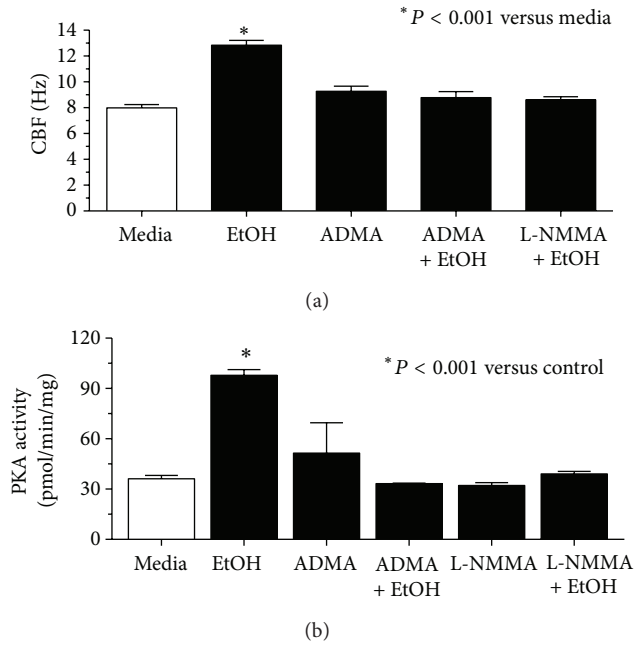

Figure 3: ADMA blocks alcohol-stimulated increases in both CBF and PKA activity in bovine bronchial epithelial cells. (a) Cilia beat frequency (CBF) in primary bovine bronchial epithelium treated with 100 mM alcohol (EtOH). Pretreatment (30 min) with ADMA (100 μM) blocked 1 hr alcohol stimulation of CBF ($P < 0.001$) versus control media. This inhibition was comparable to that observed with the NOS blocker (L-NMMA; 10 μM). (b) ADMA pretreatment blocks alcohol-stimulated increases in PKA activity in ciliated bovine epithelium ($^*P < 0.001$). Bars represent SEM of triplicate separate experiments (n = 3).

(Figure 5). We hypothesized that lung slices made from DDAH overexpressing mice would have enhanced CBF and PKA responses to alcohol. Indeed, CBF from DDAH overexpressing mice not only responds to 100 mM alcohol with stimulated increases in CBF, but also demonstrates a significant ($P < 0.01$) increase in CBF response versus wild type lung slices treated with alcohol. As with tracheal rings, the time of maximal CBF in lung slices is enhanced in DDAH mice as compared to wild type mice (30 min versus 1 hr). Similar to isolated BBEC, lung slice PKA was significantly ($P < 0.05$) increased by 1 hr treatment with 100 mM alcohol in wild type mice (Figure 6). In lung slices from DDAH mice, the time of PKA activation was significantly enhanced as compared to alcohol-stimulated wild type lung slices at 15 and 30 min. It was not until 1 hr that alcohol-stimulated PKA activity in wild type mice became equivalent to that of DDAH mice. These data demonstrate that airway epithelial-localized DDAH can function to enhance a NO-mediated cilia stimulation response (both in terms of increased PKA activity and CBF) in the preserved structure of the lung slice as well as the individual bronchial epithelial cell. Collectively, these data demonstrate that the action of ADMA regulates CBF in concert with the action of DDAH to affect the transitional impact of alcohol on cilia from initial CBF stimulation toward the eventual uncoupling of NO-mediated cilia stimulation observed with chronic alcohol desensitization of mucociliary clearance.

4. Discussion

Our findings demonstrate that ADMA, an endogenous inhibitor of NO, is highly expressed in the lung and is particularly localized to the ciliated airway epithelium. Pretreatment with ADMA blocks alcohol-stimulated increases in PKA activity and cilia beating in *in vitro* exposed bovine cell cultures. Mice that overexpress DDAH, an endogenous enzyme that degrades ADMA, demonstrate an enhanced PKA and CBF response in their tracheal ring ciliated epithelium to acute alcohol treatment. Similarly, mice that overexpress DDAH demonstrate an enhanced PKA and CBF response in their lung slices to acute alcohol treatment. These data suggest that ADMA is an endogenous regulator for the action of NO in response to alcohol exposure in the airways. ADMA may represent a potential pathway by which alcohol-elevated NO levels are reduced, PKA activity decreased, and stimulated cilia beating returned to baseline levels.

Alcohol uniquely requires both NO [7] and cyclic nucleotide elevation [13] in order to stimulate CBF (Figure 7). An alcohol-sensitive soluble adenylyl cyclase [12] and guanylyl cyclase are all colocalized to what is termed the ciliary metabolon [19]. Without the generation of NO [1] and the activation of PKG [1], alcohol cannot activate PKA, a necessary kinase for ethanol-stimulated increases in CBF

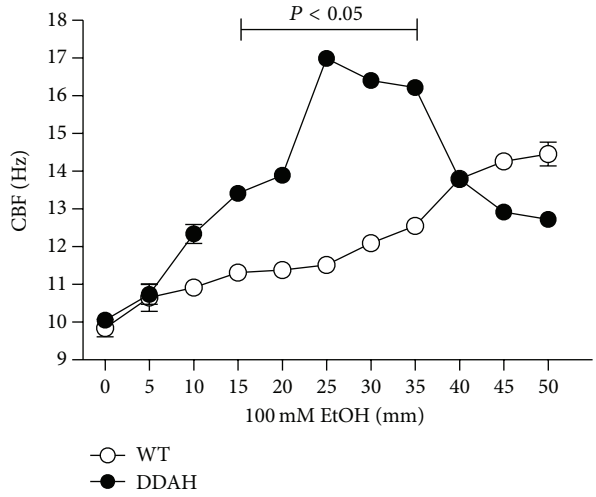

Figure 4: Alcohol stimulation of tracheal epithelial CBF is enhanced in mice expressing an ADMA-degrading enzyme. Tracheal rings from mice overexpressing the ADMA-degrading enzyme dimethylaminohydrolase (DDAH) were treated with 100 mM alcohol (EtOH) and their CBF compared to wild type (WT) mice. While the magnitude of maximal alcohol-stimulated CBF increases did not differ between mice, the time to maximal CBF was significantly decreased in the DDAH mice versus WT mice treated with alcohol ($^*P < 0.05$). No significant differences in CBF were observed in control, media-incubated tracheal rings. Bars represent SEM of triplicate separate experiments ($n = 3$).

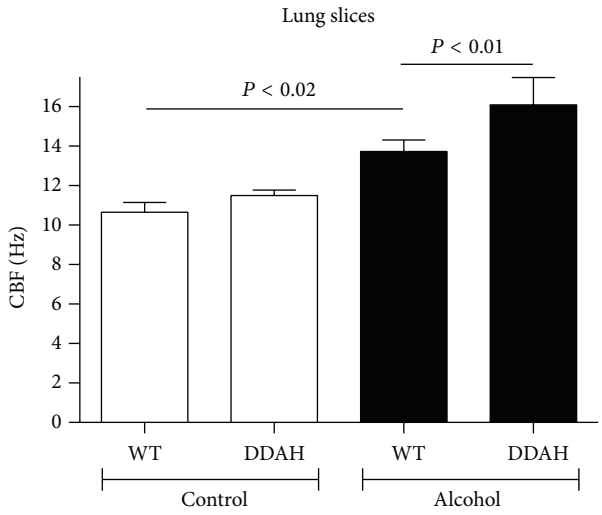

Figure 5: Alcohol-stimulated increases in lung slice airway CBF are enhanced in DDAH mice. Precision-cut lung slices from both wild type (WT) and dimethylaminohydrolase (DDAH) expressing mice were treated with alcohol, and their maximal CBF stimulation peaks were recorded. Alcohol (EtOH; 100 mM) significantly stimulated CBF in slices from both mice. Alcohol-stimulated CBF was significantly enhanced in DDAH (30 min) versus WT lung slices (1 hr). Bars represent SEM of triplicate separate experiments ($n = 3$).

[6]. Recently, it was demonstrated that brief alcohol treatment leads to heat shock protein 90 (HSP90) phosphorylation and binding to eNOS in the apical region of the ciliated airway epithelium [20]. This binding and chaperone function of HSP90 may serve to activate eNOS in the ciliated cell. The action of ADMA may serve as an "off switch" to this eNOS activation, thus leading to the eventual return to baseline CBF levels observed shortly after alcohol treatment. Indeed, evidence exists that ADMA is capable of blocking HSP90 [21]. ADMA as a negative regulator of NO-activated CBF is supported by the inhibitory action of exogenous ADMA on alcohol-stimulated CBF and the enhancement of alcohol-stimulated CBF by the inhibitor of ADMA, DDAH. Both ADMA and DDAH are highly expressed in the airway epithelium.

The lung has been established as a major source for ADMA [9], and DDAH2 has been localized in the bronchial epithelium of mice [10, 18]. Arginine methylation of cellular proteins is catalyzed by protein arginine methyltransferases (PRMT). The expression of PRMT isoforms in the lung has been demonstrated to be primarily localized to the bronchial and alveolar epithelium [22]. It has been proposed that a delicate balance between ADMA-metabolizing enzymes is disturbed in bronchial epithelium during acute airway injury, potentially causing increased nitrosative stress in the form of ADMA-induced peroxynitrite production [10]. ADMA levels have been reported to be decreased in the plasma of individuals with alcohol use disorders in a recent paper by Frieling et al. [23]. However, in many disease states associated with alcohol abuse (such as hepatitis and cirrhosis), plasma ADMA levels have been demonstrated to be elevated in alcohol abuse [24, 25]. Thus, there is no clear consensus on the systemic ADMA levels after chronic alcohol consumption.

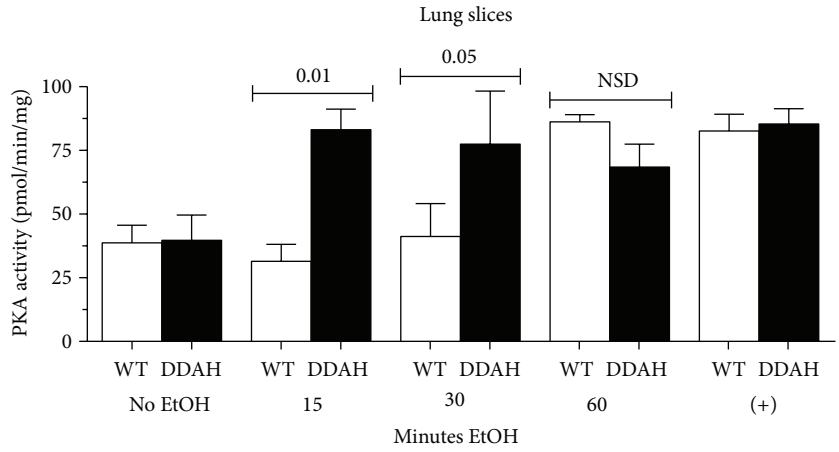

Figure 6: Alcohol-stimulated increases in lung slice PKA activity are enhanced in DDAH mice. Precision-cut lung slices from both wild type (WT) and dimethylaminohydrolase (DDAH) expressing mice were treated with alcohol, and their maximal PKA activity was recorded. Alcohol (EtOH; 100 mM) significantly stimulated PKA in slices from both mice at 1 hr. Alcohol-stimulated PKA was earlier in DDAH (15–30 min) versus WT lung slices (1 hr). Positive control (+) represents a 30 min treatment with 10 μM 8Br-cAMP. Bars represent SEM of triplicate separate experiments ($n = 3$).

There have been no specific lung tissue measurements of ADMA after alcohol consumption in humans reported. Acute consumption of alcohol results in elevated lung NO in the lung. Studies have not been reported in the alcoholic human lung, but chronic alcohol feeding results in the uncoupling of this NO response in rodents as ADMA levels are elevated, and stimulated NO levels are decreased [26, 27].

By itself, ADMA has been shown to have no effect on cilia beating in rat tracheal epithelium at concentrations up to 1 mM [28]. Superphysiologic concentrations (10 mM) resulted in a small, nonsignificant decrease in CBF. In addition, ADMA alone did not alter IL-8, RANTES, or TNF release from BEAS-2B. Our studies examining the effects of ADMA on mouse tracheal epithelial explant rings are in agreement with those of Galal et al. as we furthermore observed small decreases in CBF at the 10 mM ADMA dose [28]. These findings support the concept that ADMA does not impact cilia bioreactivity in the absence of NOS modulation by another agent such as alcohol. While nebivolol-induced degradation of ADMA levels through the elevation of DDAH-2 expression has been reported in endothelial cells [29], we found that nebivolol does not impact baseline CBF, nor does it enhance cilia responsiveness to alcohol (data not shown). This may be due to differences in cell type expression of DDAH-2 in response to nebivolol as transgenic mice overexpressing DDAH clearly enhance the alcohol stimulation of CBF.

ADMA has already been implicated in the pathogenesis of lung disease. Elevated ADMA exacerbates airway inflammation [18] and modifies lung function [30] in mouse models. Lung ADMA was elevated in a mouse model of allergic asthma, and the exogenous administration of inhaled ADMA to normal mice resulted in airway hyperresponsiveness to methacholine challenge, suggesting that ADMA is increased in asthma [31]. ADMA levels in the exhaled breath condensate of asthmatic children were significantly higher compared to those of healthy controls regardless of whether asthmatic children were on inhaled steroid treatment [32]. Alternately, plasma levels of ADMA were found to be significantly lower in allergic pediatric mild asthmatic patients compared to healthy subjects [33]. Statins may function to modulate asthmatic lung injury via the modulation of ADMA. Using an alveolar epithelial cell model, ADMA and inducible nitric oxide synthase were reduced by simvastatin, but eNOS was increased [34]. Increased severity of lung injury after *P. aeruginosa* sepsis was associated with elevated ADMA concentrations [35]. Impaired mucociliary clearance due to elevated ADMA may be an additional mechanism contributing to the severity of acute lung injury in *P. aeruginosa* sepsis.

Although elevated ADMA does not appear to impair or slow CBF as evidenced by our data, ADMA may function to slow cilia in concert with a cilia toxin in an opposite manner to how DDAH enhances CBF only under conditions of a NOS activating agent such as alcohol. For example, Wu et al. [36] showed that ADMA increased protein kinase C (PKC) while DDAH decreased PKC expression in response to ischemia/reperfusion injury with the opposite effects on NO levels in the lung. Recently, we have identified that cilia *slowing* is actively regulated through PKC epsilon in response to many agents that are known to have a cilia slowing effect [15]. Perhaps, in the presence of elevated ADMA, an enhanced cilia slowing response would be produced under injury conditions by a PKC epsilon-activating cilia toxin. Thus, the enhancement of proper innate defense against inhaled pathogens might focus on therapeutic agents that either decrease ADMA or increase DDAH in the airway epithelium.

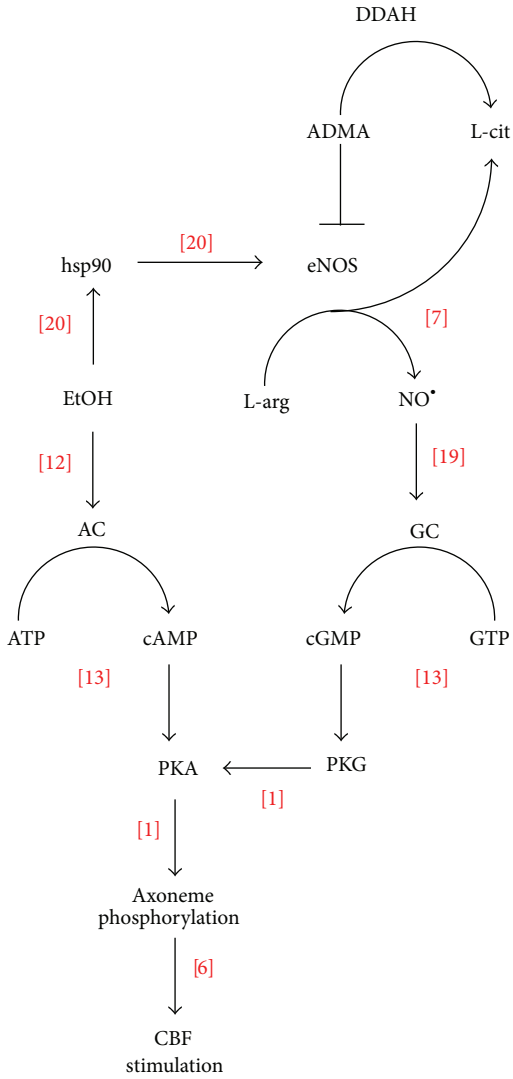

Figure 7: Proposed model of ADMA action on alcohol-stimulated cilia beating. Alcohol (EtOH) rapidly and transiently stimulates cilia beat frequency (CBF) by both activating the alcohol-sensitive adenylyl cyclase isoform 7 (AC) and increasing the phosphorylation of heat shock protein 90 (hsp90) leading to the increased binding of hsp90 and the activation of nitric oxide synthase (eNOS). Nitric oxide ($NO^{\bullet}$) activates soluble guanylyl cyclase (GC) leading to a cGMP-dependent protein kinase (PKG) activation required for alcohol-induced activation of cAMP-dependent protein kinase (PKA). Asymmetric dimethylarginine (ADMA) functions to negatively regulate alcohol stimulation of eNOS, except under the conditions of elevated dimethylarginine dimethylaminohydrolase (DDAH) whereby ADMA is converted to L-citrulline (L-cit).

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Clinical Study

Association of Serum Adiponectin, Leptin, and Resistin Concentrations with the Severity of Liver Dysfunction and the Disease Complications in Alcoholic Liver Disease

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Background and aims. There is growing evidence that white adipose tissue is an important contributor in the pathogenesis of alcoholic liver disease (ALD). We investigated serum concentrations of total adiponectin (Acrp30), leptin, and resistin in patients with chronic alcohol abuse and different grades of liver dysfunction, as well as ALD complications. *Materials and Methods*. One hundred forty-seven consecutive inpatients with ALD were prospectively recruited. The evaluation of plasma adipokine levels was performed using immunoenzymatic ELISA tests. Multivariable logistic regression was applied in order to select independent predictors of advanced liver dysfunction and the disease complications. *Results*. Acrp30 and resistin levels were significantly higher in patients with ALD than in controls. Lower leptin levels in females with ALD compared to controls, but no significant differences in leptin concentrations in males, were found. High serum Acrp30 level revealed an independent association with advanced liver dysfunction, as well as the development of ALD complications, that is, ascites and hepatic encephalopathy. *Conclusion*. Gender-related differences in serum leptin concentrations may influence the ALD course, different in females compared with males. Serum Acrp30 level may serve as a potential prognostic indicator for patients with ALD.

1. Introduction

White adipose tissue (WAT) represents an active endocrine organ that regulates body fat mass and energy balance. There is increasing evidence that WAT-derived adipokines may contribute to hepatic damage associated with fatty infiltration, inflammation, and fibrosis [1, 2]. Adiponectin, leptin, and resistin are the best described molecules in this class. Adiponectin is secreted exclusively from adipose tissue and circulates in different isoforms: trimers, of low molecular weight (LMW), hexamers (trimer dimer) of medium molecular weight (MMW), and multimeric high molecular weight (HMW) isoforms [3]. Leptin is expressed mainly by adipose tissue, although low levels have been detected in the placenta, skeletal muscle, gastric and mammary epithelium, and the brain [3]. Although resistin is secreted by human adipocytes, the most significant source appears to be blood mononuclear cells [3].

Plasma concentrations of adipokines have been investigated in patients with different liver disorders, that is, nonalcoholic fatty liver disease [4–6], type 1 autoimmune hepatitis [7], and viral hepatitis B [8] and C [9]. Alcoholic liver disease (ALD) is another one where adipokines may play pivotal role and represent a link between inflammation and metabolic state. However, the published data on the underlying pathophysiological mechanisms are ambiguous.

Therefore, the objective of the present study was to determine serum concentrations of total adiponectin (Acrp30), leptin, and resistin in patients with chronic alcohol abuse and different grades of liver dysfunction. We also investigated the

adipokine correlation with traditional indicators of inflammation, liver laboratory parameters, and ALD complications. Furthermore, on the basis of above obtained results, we tried to select noninvasive predictors of the disease severity and ALD outcome. The prospective study was conducted in the Department of Gastroenterology of Medical University in Lublin, Poland.

2. Material and Methods

One hundred forty-seven consecutive adult inpatients (pts) with ALD admitted to hospital due to the disease decompensation were prospectively recruited. The control group consisted of 30 healthy volunteers matched to the study group in terms of age and sex and recruited mostly among academics and trainees of Department of Gastroenterology, Medical University of Lublin.

The diagnosis of ALD was based on clinical criteria such as a detailed patient history, typical symptoms and physical findings of chronic liver disease, laboratory values (elevated serum aminotransferases activity (normal range: ALT < 31 IU/L; AST < 34 IU/L), the AST/ALT ratio higher than 2), and imaging studies, in the setting of excessive alcohol intake (i.e., alcohol consumption exceeding 40 g/d for male and 20 g/d for female pts for a minimum of 6 months). In order to confirm alcohol misuse, the Alcohol Use Disorders Identification Test-Consumption (AUDIT-C) questionnaire was used [10]. An AUDIT score of ≥ 8 for men up to age 60, or ≥ 4 for women, or men over age 60 was considered a positive screening test. Positive AUDIT-C, in addition to the amount of alcohol consumption, was an inclusion criterion. Based on the AUDIT-C score, the alcohol consumption status was determined in both studied groups. Two individuals from the control group, who pledged total abstinence, were categorized as abstainers and eighteen who self-reported their consumption as no more than 20 g ethanol per day (10–16 drinks per month; 1 drink refers to 10 g of pure alcohol) as light drinkers. Quantity of drinking among risky, current drinkers from the ALD group ranged as follows: in females 40 g/d to more than 100 g/d and in males 50 g/d to more than 100 g/d.

According to the study protocol, patients signed informed consent, completed an anamnesis (their medical history), and they answered the AUDIT-C questionnaire.

Patients entering the study did not consume alcoholic beverages at least 24 hours prior to obtaining blood samples for laboratory tests. No one was treated with corticosteroids or pentoxifylline at the time of qualification. All data necessary for further analysis (i.e., the major demographic variables included age, gender, alcohol intake and the period of alcohol abuse, education, family history of alcoholism, employment status, treatment of ALD before admission, and comorbidities) were recorded, and the planned procedures including blood sampling were performed within 48 hours after hospital admission. Blood samples from all patients were collected at 07:30 am after a minimum 8-hour overnight fast in order to avoid the circadian and feeding impact on serum adipokine fluctuations. Basic laboratory tests included determination of

(1) liver function parameters (alanine aminotransferase-ALT, aspartate aminotransferase-AST, alkaline phosphatase-AP, gamma-glutamyl transpeptidase-GGT, total bilirubin-Tbil, albumin, INR, and prothrombin time);

(2) complete blood count (hemoglobin-Hgb, erythrocytes-RBC, platelets-PLT, and leukocytes-WBC);

(3) parameters of renal function (creatinine-CREA, serum sodium level-Na);

(4) the traditional markers of inflammation: neutrophils, neutrophil to lymphocytes count rate (NLR) and the level of C-reactive protein-CRP);

(5) indicators of other etiology of chronic liver disease, that is, HBV and HCV infection (HBsAg, anti-HBc class IgM and IgG antibodies; anti-HCV antibodies), antinuclear, antismooth muscle, antimitochondrial antibodies, and markers of Wilson's disease and hemochromatosis as appropriate).

Based on the lab results, the baseline severity of liver dysfunction was determined according to the Child-Turcotte-Pugh (CTP) [11] and the Model of End-Stage Liver Disease (MELD) [12] criteria. For the score calculation, internet available calculators were used, that is, http://www.mayoclinic.org and http://potts-uk.com/livercalculator.html.

Patients enrolled into the study were divided into subgroups according to:

(1) gender;

(2) age: ≥ 50 and < 50 years old;

(3) the severity of liver dysfunction according to the CTP (classes A, B, C) and MELD (≥ 20 or < 20) scores;

(4) the presence of ALD complications at the time of hospital admission, that is, ascites, hepatic encephalopathy (HE), oesophageal varices, cholestasis, and renal dysfunction.

Pts with any other severe diseases, that is, uncontrolled diabetes, heart failure, pulmonary insufficiency or malignancy at the time of inclusion were excluded.

Symptoms of overt hepatic encephalopathy (HE) were classified according to West-Heaven criteria [13].

Cholestasis was defined based on the recommendations of the European Association for the Study of the Liver (EASL), that is, alkaline phosphatase (AP) greater than 1.5 times above the upper limit of normal (ULN) and the activity of γ-glutamyl transpeptidase (GGT), more than three times the ULN [14]. Ultrasonography of the abdomen was performed in order to confirm the presence of ascites and to rule out other causes of cholestasis (e.g., choledochal cyst and gallstones). Tests for antimitochondrial antibodies (AMAs) to exclude the diagnosis of primary biliary cirrhosis (PBC) were done, and drugs hepatotoxicity was ruled out.

Gastroscopy was performed in order to identify esophageal varices. Renal dysfunction was determined on

Table 1: Baseline characteristics of the study population*.

	ALD group $n = 147$	Control group $n = 30$	P
Age (years)			
Males	49.84 ± 11.53	44.31 ± 10.23	0.09
Females	48.82 ± 9.94	43.11 ± 8.43	0.10
Gender			
Males	107 (72.8%)	17 (56.7%)	0.12
Females	40 (27.2%)	13 (43.3%)	

*ALD: alcoholic liver disease.

the basis of elevated levels of serum creatinine (above the upper limit of normal, that is, 1.3 mg/dL).

In some cases, a CT scan was also performed when any doubts about the nature of the pathology existed.

Body mass index (BMI) and/or the waist-to-hip ratio (WHR) are commonly used for evaluation of body composition and fat mass. However, we did not apply those indicators due to their bias and inaccuracy of fat content assessment in ascitic cirrhotics. Ascites was present in 89 of 147 (60.5%) subjects included in the survey. Alcoholics with decompensated liver disease have both high prevalence of malnutrition and high BMI values due to fluid retention at the same time. Furthermore, some of our patients could not stand at the time of hospital admission, so the measure of their weight and height was impossible. The applicability of these constants for an estimation of body fat mass has often been questioned [15, 16].

The evaluation of plasma levels of selected adipokines (total adiponectin-Acrp30, leptin, and resistin) was performed with immunoenzymatic ELISA (enzyme linked immunosorbent assay) tests using commercially available kits (Quantikine ELISA kit, R & D Systems, USA). The study was conducted according to the procedure recommended by the producer and described in the attached materials. Measurements were made using VictorTM3 Reader (PerkinElmer, USA).

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the institutional review board of Medical University of Lublin. All subjects signed an informed consent form prior to the investigation.

3. Statistical Analysis

Statistical analysis was performed using the Statistica 10 software package (StatSoft, Poland). The distribution of the data in the groups was preliminarily evaluated by Kolmogorov and Smirnov test. The analysis showed a skewed distribution of all values; therefore, continuous variables were described as median with interquartile range, its 95% confidence interval (95% CI), and compared using Mann-Whitney U-test. Categorical variables are presented as numbers and percentage. Differences between categorical variables were assessed by Fisher's exact test or the χ^2 test with Yates correction for continuity, as appropriate. The differences of studied adipokine levels between CTP classes were analyzed using Kruskal-Wallis and multiple comparisons post hoc tests. Spearman's rank correlation test was used for the assessment of association between parameters of liver function, traditional indicators of inflammation, and studied biomarker serum levels. The receiver operating curves (ROCs) for significant adipokines, that is, Acrp30 and resistin were constructed to assess their areas under the curve (AUCs) and the best threshold values for predicting ALD complications. The method of DeLong et al. [17] for the calculation of the standard error of the AUC was used. The Youden index and its associated cut-off point were estimated for each adipokine [18]. AUCs of significant variables were compared to assess their accuracy in predicting the severity of liver dysfunction and the development of ALD complications. Multivariable logistic regression was applied in order to select independent predictors of advanced liver dysfunction and the occurrence of the disease complications. A two-sided P value of less than 0.05 was considered to be associated with statistical significance.

4. Results

The survey population included 147 patients (107 males (72.8%) and 40 females (27.2%)). Their mean age was 49.56 ± 11.85 (range 26 to 74). The baseline characteristics of the study cohort are shown in Tables 1 and 2.

Initially we performed an assessment of the adipokine serum levels in patients with ALD in comparison with healthy controls. The results indicated that serum Acrp30 and resistin levels were significantly higher in patients with ALD as compared to healthy controls. They are presented in Table 3.

The next step was to compare the levels of studied biomarkers inside the ALD and control group according to gender. Serum Acrp30 ($P = 0.004$) and leptin ($P = 0.03$) concentrations were significantly higher in females compared to males from the control group. On the other hand, none of studied adipokines showed a significant difference according to gender in ALD group. Furthermore, significantly higher levels of Acrp30 in both sexes were found in ALD patients as compared to the control group. Serum leptin levels in females with ALD were significantly lower compared to controls. There were no significant differences in serum leptin levels in men in both groups. Resistin serum levels were significantly higher in both sexes in ALD patients as compared to the control group. The results are presented in Table 4.

Since aging alters body fat mass and its function, we evaluated serum adipokine levels in two age subgroups: ≥ 50 and < 50 years old. As expected, serum leptin concentration was significantly higher in the older patients in comparison with the younger subgroup. There were no age-related differences in serum levels of two other adipokines. The results are presented in Table 5.

It was found that only the level of Acrp30 among studied adipokines significantly increased with the severity of liver dysfunction classified according to both CTP and MELD scores. The results are presented in Tables 6 and 7.

Table 2: Characteristics of patients with ALD based on their gender*.

	ALD group (n = 147)						P
	Females (n = 40)			Males (n = 107)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Age years	51.00	48.03–54.96	45.00–56.00	51.00	48.00–52.49	40.00–60.00	0.19
ALT IU/L	39.50	28.03–44.93	23.00–47.00	56.00	50.00–69.00	35.25–84.00	**0.004**
ASP IU/L	100.50	78.45–114.90	66.00–120.00	110.00	78.51–131.00	64.50–189.00	0.72
AP IU/L	118.50	111.68–156.27	105.00–179.00	129.00	118.00–148.00	79.00–223.00	0.62
GGT IU/L	415.00	174.00–543.00	172.00–772.00	359.00	200.50–504.88	93.00–1066.00	**0.020**
T-Bil mg/dL	4.20	3.51–5.27	3.30–8.40	3.00	1.75–4.00	1.10–8.10	0.70
Alb g/dL	3.10	2.70–3.29	2.63–3.50	3.20	3.00–3.30	2.73–3.61	0.12
INR	1.45	1.39–1.64	1.31–1.71	1.21	1.16–1.30	1.07–1.43	**0.034**
Crea mg/dL	0.80	0.70–0.80	0.70–1.00	0.90	0.90–1.00	0.80–1.10	0.51
Na mEq/L	139.00	136.03–140.96	134.00–141.00	138.00	136.51–139.00	134.00–140.00	0.38
Hgb g/dL	11.20	10.34–11.50	9.70–12.00	12.10	11.60–12.70	10.30–13.50	<**0.001**
RBC $\times 10^6$ kom/uL	3.17	3.08–3.50	2.86–3.52	3.86	3.57–3.97	3.15–4.11	<**0.001**
PLT $\times 10^3$ kom/uL	135.50	114.38–137.96	97.00–251.00	136.00	116.00–166.46	80.00–202.00	0.81
WBC $\times 10^3$ kom/uL	8.12	5.42–11.63	4.89–13.04	7.12	6.30–8.28	5.01–10.80	0.75
NEUT $\times 10^3$ kom/uL	8.44	3.20–8.97	2.57–13.51	5.02	4.19–6.10	2.91–7.92	**0.053**
NLR	4.38	2.34–4.52	2.34–7.63	3.47	3.26–4.45	2.13–6.04	0.12
CRP mg/L	17.33	16.19–33.14	5.98–42.17	17.53	13.40–21.30	5.01–43.00	0.58
mDF	17.35	12.00–22.96	9.00–28.00	9.00	6.00–12.00	4.00–16.74	0.21
MELD	17.50	15.03–18.00	12.00–20.00	15.00	14.00–16.00	11.00–17.00	**0.047**
CTP	9.50	9.00-10.00	8.00–10.00	7.00	7.00–8.00	7.00–9.00	<**0.001**

* Alb: albumin (normal range (NR) 3.2–4.8); ALT: alanine aminotransferase (NR) < 31); AP: alkaline phosphatase (NR 45–129); AST: aspartate aminotransferase (NR < 34); Crea: creatinine (NR 0.5–1.1); CRP: C-reactive protein (NR 0.0–5.0); CTP-Child-Turcotte-Pugh score; GGT: gamma-glutamyl transpeptidase (NR < 50.0); Hgb: hemoglobin (NR 14.0–18.0); INR: International Normalized Ratio (NR 0.8–1.2); MELD: Model for End-Stage Liver Disease; Na: sodium (NR 136–145); NEUT: neutrophils (NR 1.8–7.7); NLR: neutrophil to lymphocyte ratio; PLT: platelets (NR 130–400); 25–75 P: percentiles, RBC: red blood cells (NR 4.5–6.1); T-Bil: total bilirubin (NR 0.3–1.2); WBC: white blood cells (NR 4.8–10.8).

Table 3: Comparison of serum adipokine levels in ALD patients and the control group*.

	Adipokines						P
	ALD group (n = 147)			Control group (n = 30)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	18.69	16.86–24.03	13.17–44.94	6.38	4.83–8.19	4.06–8.68	<**0.0001**
Leptin ng/mL	8.80	6.80–10.41	2.75–13.96	8.76	5.32–16.94	5.13–17.31	0.36
Resistin ng/mL	16.91	15.27–18.84	10.63–29.01	8.87	8.36–10.71	8.36–11.41	**0.0001**

* Acrp30: total adiponectin; CI: confidence interval, 25–75 P: percentiles.

Table 4: Comparison of serum adipokine levels in females and males with ALD and the control group.

	Females						P
	ALD group (n = 40)			Control group (n = 13)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	17.93	15.76–30.09	13.06–48.37	7.78	6.35–24.81	6.71–24.81	**0.02**
Leptin ng/mL	6.80	3.30–11.00	2.55–13.00	16.64	5.13–42.10	5.13–42.10	**0.009**
Resistin ng/mL	16.18	11.42–17.05	8.89–19.22	9.88	6.30–10.73	6.30–10.45	**0.01**
	Males						P
	ALD group (n = 107)			Control group (n = 17)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	18.89	16.93–24.83	13.17–43.94	4.06	3.59–6.38	3.59–6.38	<**0.0001**
Leptin ng/mL	9.45	7.82–10.88	3.40–14.96	8.57	1.79–9.17	1.79–8.97	0.17
Resistin ng/mL	17.69	15.36–21.58	11.08–31.17	8.87	8.36–15.12	8.36–15.12	**0.005**

Table 5: Comparison of serum adipokine levels according to the age of patients with ALD.

	Adipokines in ALD group						P
	Age ≥ 50 (n = 83)			Age < 50 (n = 64)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	23.11	16.20–40.14	11.49–70.52	18.07	16.60–23.42	14.13–32.25	0.32
Leptin ng/mL	10.69	8.51–12.33	3.03–15.57	6.57	4.77–8.81	2.67–10.88	**0.02**
Resistin ng/mL	17.03	16.36–19.04	13.48–29.69	13.82	11.05–21.20	8.50–28.62	0.13

Table 6: Serum adipokine levels in patients with ALD according to the CTP class.

	CTP class									P
	Class A (n = 30)			Class B (n = 73)			Class C (n = 44)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	15.76	8.97–22.50	7.80–29.60	23.33	17.06–29.15	14.67–45.78	23.11	16.73–95.85	14.92–156.63	**0.02**
Leptin ng/mL	8.45	3.03–10.39	2.00–11.88	9.88	7.84–10.94	5.25–14.01	5.28	3.04–12.95	2.13–16.15	0.34
Resistin ng/mL	14.40	9.39–27.59	8.47–32.33	16.99	14.80–25.32	10.84–31.44	16.77	14.57–18.89	13.33–21.26	0.59

Table 7: Serum adipokine levels in patients with ALD according to the MELD score.

	MELD						P
	<20 points (n = 117)			≥20 points (n = 30)			
	Median	95% CI	25–5 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	17.93	15.97–23.33	12.49–42.04	37.60	19.29–157.63	17.06–161.19	**0.01**
Leptin ng/mL	8.95	6.80–10.88	2.69–14.52	7.56	3.21–10.39	2.91–12.41	0.51
Resistin ng/mL	16.64	14.40–18.17	10.23–28.08	19.36	14.57–27.92	13.37–63.53	0.08

The analysis of the association of adipokine serum levels and liver function parameters showed a significant, positive correlation between leptin and both aminotransferases and bilirubin serum levels in males. A positive correlation of leptin with albumin serum levels was observed in females. There was an inverse correlation of Acrp30 and resistin with serum albumin levels and a positive correlation of Acrp30 with AP and bilirubin levels in men. The results of analysis are shown in Table 8.

We found a significant positive correlation of serum resistin level and both the white blood cells count and CRP level. Serum leptin concentrations showed a weak inverse correlation with the white blood cells count. The results of the above analysis are presented in Table 9.

The next step of the study was carried out to compare the level of studied adipokines in ALD patients divided according to the presence of the disease complications. For adipokines which serum concentrations differed significantly in subgroups of patients selected according to the severity of liver dysfunction (MELD ≥ 20) and the complications of ALD, the areas under the curve (AUCs) were checked, and the diagnostic accuracy of studied variables for the association with complications of the disease was compared. Serum Acrp30 levels were identified as an independent predictor of advanced liver dysfunction (MELD ≥ 20) and the development of ascites and HE. Serum resistin levels lost their significance for renal dysfunction when adjusted for other variables. None of the studied adipokines was independently associated with cholestasis and esophageal varices. Above results are summarized in Tables 10, 11, and 12.

5. Discussion

Excessive and chronic alcohol consumption leads to inflammation in adipose tissue, insulin resistance, and hepatic steatosis [19]. Therefore, the exploration of the pathogenic mechanisms of ALD should include the role of adipose tissue secretion and adipokines. In addition, alcohol abuse is associated with impaired energy intake and expenditure, as well as increased catabolism. All above mentioned processes are modulated by adipokines. The recent study of Zhong et al. [20] demonstrated a significant loss of white adipose tissue (WAT) in a mouse model of alcoholic steatosis. It suggests that WAT dysfunction can directly impact hepatic lipid homeostasis by reverse triglyceride transport. Clinical studies have shown that lower fat mass is associated with higher liver fat content in alcoholics [21]. Those reports prompted us to design a study in order to explain the association of serum adipokine concentrations with the severity of liver dysfunction and ALD complications. Results obtained in the present study confirmed the crucial role of WAT endocrine secretion in the pathogenesis of ALD. Significantly higher levels of two adipokines, Acrp30 and resistin were found in patients with ALD compared to the control subjects. Unexpectedly, there were no differences in leptin levels in both studied groups (Table 3).

The analysis of data by gender showed significantly higher levels of Acrp30 and leptin in females from the control group in comparison with the level of both adipokines in males (Table 4). Our results are consistent with other reports regarding gender-related differences of serum adipokine

Table 8: Analysis of the correlation between serum adipokine levels and liver function parameters.

	Females ($n = 40$)		Males ($n = 107$)		Resistin
	Acrp30	Leptin	Acrp30	Leptin	
ALT					
*Rho	−0.08	0.30	−0.14	0.25	−0.15
P	0.62	**0.06**	0.14	**0.01**	0.06
ASP					
Rho	−0.14	0.12	−0.13	0.25	−0.11
P	0.40	0.46	0.18	**0.01**	0.20
AP					
Rho	−0.07	−0.14	0.21	−0.15	−0.01
P	0.67	0.42	**0.04**	0.15	0.92
GTP					
Rho	0.03	0.02	0.08	0.16	−0.13
P	0.87	0.92	0.44	0.34	0.14
Albumin					
Rho	−0.20	0.38	−0.27	0.13	−0.17
P	0.22	**0.02**	**0.007**	0.18	**0.04**
T-Bilirubin					
Rho	0.14	0.05	0.23	0.27	−0.03
P	0.37	0.76	**0.02**	**0.006**	0.71
INR					
Rho	0.23	−0.28	0.14	0.01	0.09
P	0.15	0.07	0.15	0.95	0.29

*Rho: Spearman's rank correlation coefficient.

Table 9: Analysis of a correlation between serum adipokine levels and traditional markers of inflammation.

	WBC	Neutrophils	NLR	CRP
Acrp30				
*Rho	−0.05	−0.07	−0.13	−0.00
P	0.52	0.40	0.11	0.98
Leptin				
Rho	−0.17	−0.13	−0.07	0.00
P	**0.04**	0.13	0.41	0.96
Resistin				
Rho	0.23	0.14	0.11	0.31
P	**0.004**	0.09	0.17	**0.0002**

*Rho: Spearman's rank correlation coefficient.

concentrations [22]. However, no significant difference in the level of three studied adipokines in relation to sex in the ALD group was observed. In contrast, a significantly lower leptin level was found in females with ALD in comparison with healthy controls. The result corresponds to the above mentioned Zhong et al. report [20] indicating possible reduction of the body fat content. When comparing the severity of liver dysfunction in both sexes, females showed significantly higher scores of the CTP and MELD (Table 2). Hypoleptinemic states are associated with increased risk of infection [23, 24]. Therefore it may alter the course of ALD in both sexes.

We are tempted to speculate that metabolic alterations caused by ethanol in the course of ALD, by modulating secretion of leptin, might be responsible for different clinical presentation of the disease in females and males. It has been already reported by Röjdmark et al. [25] that ingestion of moderate amounts of alcohol had an inhibitory effect on leptin secretion in normal subjects. The effect might be direct rather than indirect, since several factors known to affect leptin were not influenced by alcohol in their study. Furthermore, leptin levels increase during abstinence, and this may be related to a reduction of dopaminergic action in mesolimbic system [26]. Gender-related differences observed in our study are also consistent with the results obtained by Dammann et al. [27] who observed no significant effect of acute moderate alcohol intake on leptin levels in healthy male volunteers.

Previous reports concerning leptin levels in human alcoholics are inconclusive. The data were highly divergent and dependent on the population studied. Some of them pointed out increased [28, 29], and others lowered leptin concentrations in the peripheral blood [30].

Greco et al. [31] also observed higher serum leptin levels in healthy females compared to healthy males in the control group. However, unlike in the present work, they reported elevated levels of leptin in women with alcoholic cirrhosis child class C. The above mentioned study revealed a significant reduction in leptin serum levels in posthepatitis cirrhotic patients. The discrepancy between our results could be likely explained by evaluation of patients in different stages

Table 10: Serum adipokine levels in patients with ALD according to the presence of the disease complications.

	Ascites						P
	Absent ($n = 58$)			Present ($n = 89$)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	16.94	14.78–23.04	10.16–29.62	23.33	17.93–37.81	14.55–74.20	**0.013**
Leptin ng/mL	10.47	8.42–11.89	6.34–15.13	6.80	4.40–9.88	2.20–13.32	**0.054**
Resistin ng/mL	17.15	11.62–24.88	9.33–32.27	16.75	15.13–18.54	11.36–28.08	0.70
	Hepatic encephalopathy						P
	Absent ($n = 127$)			Present ($n = 20$)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	17.93	16.08–23.38	11.76–41.82	55.65	23.11–164.21	22.62–164.55	**0.003**
Leptin ng/mL	8.82	6.80–10.49	2.75–14.27	6.42	3.06–12.63	2.76–13.33	0.54
Resistin ng/mL	16.37	14.39–18.91	10.30–29.69	18.17	16.03–20.86	15.83–21.36	0.55
	Oesophageal varices						P
	Absent ($n = 60$)			Present ($n = 87$)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	17.55	15.20–23.11	11.76–31.42	23.61	17.91–33.14	14.68–51.66	0.16
Leptin ng/mL	8.88	3.66–11.88	2.00–13.94	8.38	5.97–10.13	3.23–13.56	0.69
Resistin ng/mL	18.73	14.40–22.34	9.09–27.24	15.83	13.46–17.53	11.14–28.31	0.59
	Cholestasis						P
	Absent ($n = 117$)			Present ($n = 30$)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	18.22	16.33–29.60	10.60–68.81	20.10	14.13–25.37	11.76–41.45	0.77
Leptin ng/mL	8.94	7.03–10.69	3.84–15.14	5.23	2.27–10.92	2.00–11.90	0.07
Resistin ng/mL	15.29	13.37–16.99	9.28–26.81	19.74	17.11–31.21	13.46–33.87	**0.02**
	Renal dysfunction						P
	Creatinine < 1.3 mg/dL ($n = 125$)			Creatinine ≥ 1.3 mg/dL ($n = 22$)			
	Median	95% CI	25–75 P	Median	95% CI	25–75 P	
Acrp30 μg/mL	18.22	16.65–24.07	11.50–44.27	22.63	17.03–46.19	16.33–161.19	0.12
Leptin ng/mL	9.23	6.80–10.91	3.11–15.00	7.56	2.00–9.08	2.00–10.09	0.18
Resistin ng/mL	16.18	13.46–17.87	10.23–26.08	29.22	16.98–39.77	16.75–63.53	**0.001**

of ALD. The level of leptin in our cohort was assessed during an acute phase of liver disease with coexistence of signs of hypermetabolism and systemic inflammatory activation. In contrast to present report, the above-cited study was performed in patients with a stable chronic phase of the disorder.

De Timary et al. [32] proposed a dual model for regulation of energy intake in alcohol-dependent subjects. They showed that alcohol accelerates metabolism and decreases fat mass and leptin levels in individuals consuming above 12.5 kcal/kg/day of alcohol (lower leptin level was observed in females with ALD in our study). For individuals consuming below 12.5 kcal/kg/day of alcohol, alcohol intake is compensated for by a decrease in nonalcoholic nutrient intakes, probably due to changes in metabolic and satiety factors.

On the other hand, Campillo et al. [28] observed an increase in leptin levels in the blood after nutritional therapy for patients with alcoholic cirrhosis in parallel to the liver function improvement.

Similar profile of serum adipokine concentrations that is elevated Acrp30 and resistin, and decreased leptin was reported in patients with inflammatory bowel disease [33].

We investigated the levels of adipokines in subgroups of ALD patients with varying severity of hepatic dysfunction defined according to the criteria of the CTP and MELD. We found that serum Acrp30 levels significantly rose with the degree of liver function impairment (Tables 6 and 7). Multivariable analysis confirmed its independent impact on the severity of liver dysfunction (MELD ≥ 20) (Table 12). It is in contrast to nonalcoholic fatty liver disease (NAFLD), where serum adiponectin concentrations were reported to be low and negatively related to necroinflammatory injury [34, 35]. On the other hand, a great body of evidence suggests a beneficial and protective effect of Acrp30 in liver injury in mice [36, 37]. Its administration reduced ROS production stimulated by LPS and expression of Tumor Necrosis Factor α (TNF α) in Kupffer cells [38]. The mechanism by which adiponectin levels and action differ between humans and rodents is still unknown.

Correlations were found in men between serum Acrp30 levels and the parameters of liver function: positive with the level of bilirubin, inverse with albumin level (Table 8). These results are consistent with previous indices of positive Acrp30 association with the severity of liver dysfunction

Table 11: Comparison of the diagnostic accuracy (AUCs) of single variables in the diagnosis of advanced liver dysfunction (MELD ≥ 20) and ALD complications. (univariable analysis)*.

Complication of ALD	Variable	*P* value	AUC (95% CI)	SE
MELD ≥ 20	Acrp30	0.002	0.652 (0.569–0.728)	0.060
	CRP	0.004	0.609 (0.527–0.686)	0.058
	RBC	0.003	0.675 (0.596–0.747)	0.055
	WBC	0.0003	0.656 (0.577–0.730)	0.061
	Ascites	0.003	0.652 (0.572–0.725)	0.050
	HE	<0.0001	0.666 (0.587–0.739)	0.058
Ascites	Acrp30	0.015	0.621 (0.537–0.700)	0.046
	Albumin	<0.0001	0.819 (0.748–0.877)	0.036
	ALT	0.0001	0.710 (0.633–0.779)	0.041
	AST	0.003	0.606 (0.526–0.683)	0.047
	INR	<0.0001	0.808 (0.739–0.866)	0.036
	RBC	0.002	0.663 (0.584–0.736)	0.044
	WBC	0.008	0.597 (0.517–0.674)	0.045
HE	Acrp30	0.002	0.704 (0.623–0.776)	0.071
	AP	0.006	0.652 (0.567–0.730)	0.071
	albumin	0.005	0.686 (0.605–0.759)	0.055
	T-bilirubin	0.0001	0.770 (0.697–0.833)	0.048
	INR	0.0001	0.737 (0.661–0.804)	0.059
	PLT	0.035	0.633 (0.553–0.708)	0.057
	Ascites	0.012	0.646 (0.567–0.720)	0.056
Renal dysfunction	Resistin	0.001	0.721 (0.641–0.792)	0.060
	Albumin	0.034	0.654 (0.572–0.729)	0.059
	AST	0.042	0.601 (0.521–0.678)	0.062
	AP	0.030	0.677 (0.593–0.753)	0.068
	Na	0.012	0.588 (0.508–0.666)	0.080
	CRP	0.001	0.714 (0.636–0.784)	0.060
	WBC	0.011	0.689 (0.611–0.760)	0.053
	RBC	0.031	0.688 (0.610–0.759)	0.059

*AUC: area under the ROC curve; SE: standard error; CI: confidence interval.

Table 12: Independent predictors of advanced liver dysfunction (MELD ≥ 20) and ALD complications (multivariable analysis)*.

Complication of ALD	Variable	*P* value	Adjusted OR (95% CI)	AUC (95% CI)	SE
MELD ≥ 20	Acrp30	0.001	1.013 (1.005–1.022)	0.873 (0.807–0.923)	0.041
	CRP	0.004	1.017 (1.005–1.029)		
	WBC	0.030	1.119 (1.011–1.238)		
	HE	0.0009	8.184 (2.373–28.224)		
Ascites	Acrp30	0.050	1.009 (1.000–1.019)	0.902 (0.840–0.946)	0.028
	albumin	0.0001	0.095 (0.030–0.300)		
	ALT	0.003	0.978 (0.964–0.993)		
	INR	0.003	22.137 (2.780–176.287)		
	RBC	0.021	2.962 (1.177–7.452)		
HE	Acrp30	0.010	1.011 (1.003–1.019)	0.830 (0.757–0.889)	0.048
	albumin	0.050	0.323 (0.105–0.998)		
	T-bilirubin	0.001	1.116 (1.045–1.193)		

*AUC: area under the ROC curve, CI: confidence interval, OR: Odds ratio, SE: standard error, HE: hepatic encephalopathy.

(CTP and MELD). In addition, a weak inverse correlation between serum resistin concentrations and the level of albumin in ALD group was observed.

Furthermore, the results of our study indicate the presence of adipokine association with major complications of the disease (Table 10). Again Acrp30 seems to play a pivotal role. Its serum level was significantly elevated in patients with ascites and encephalopathy. Multivariable analysis confirmed independent impact of Acrp30 on both complications (Table 12).

The borderline statistical significance ($P = 0,054$) of leptin level differences in subgroups with and without ascites was found and might suggest its negative influence on the evolution of ALD (Table 10). Nevertheless, the multivariable analysis failed to prove its impact on the development of the above complication. As observed in our study, also Brennan et al. [39] did not find any significant association of leptin concentration with morbidity and mortality for cardiovascular disease in women with diabetes. Conversely, reports of other authors indicate that leptin deficiency may enhance the sensitivity to the toxic effects of inflammatory factors, including endotoxin and TNF α [40–42].

Serum resistin levels were significantly increased in the subgroups with symptoms of cholestasis and renal dysfunction (Table 9). Nevertheless, they lost their significance when adjusted for other studied variables. The presence of elevated levels of resistin was previously reported in the course of chronic kidney disease [43–45]. On the other hand, Menzaghi et al. [46] demonstrated that resistin may play an important role in modulating the kidney function in healthy subjects. In the present study, the multivariable analysis failed to confirm its independent effect on both the above-mentioned complications.

Suggestions about the prognostic value of adiponectin and resistin in the assessment of severity and outcome of inflammatory diseases and cancer appeared in several recent publications [47–51]. We measured the plasma levels of total adiponectin (Acrp30), which has been reported to be more useful than HMW for assessing mortality risk. The high plasma concentration of Acrp30 was an independent prognostic predictor in chronic heart failure patients with normal BMI [52]. Higher concentrations of total adiponectin were also associated with heart failure and mortality among patients with existing ischemic heart disease [53].

The development of ALD is believed to combine metabolic and inflammatory activity, so the assessment of the relationship between serum levels of selected adipokines and inflammatory markers was of vast interest. We speculate that significantly increased serum levels of Acrp30 and resistin in the studied cohort may be an indicator of systemic inflammatory activation in the course of the disease. The data from the literature suggest that the levels of both adipokines rise in chronic inflammation [54–57]. As described elsewhere, leptin also affects immune and inflammatory functions. We found its weak inverse correlation with the white blood cells count.

On the other hand, an increase in Acrp30 concentration in the blood of patients with ALD may reflect the defense mechanism rather than inflammatory response due to its known anti-inflammatory properties [58]. Furthermore, the level of Acrp30 in the studied group did not show any correlation with the traditional parameters of inflammation (Table 9). The results correspond to the general belief about its anti-inflammatory potential. It was reported that Acrp30 deficiency leads to persistent subclinical inflammation in the course of obesity, nonalcoholic fatty liver disease (NAFLD), ischemic heart disease, and type 2 diabetes [59–63].

In contrast, increased levels of serum adiponectin have been observed in inflammatory and/or autoimmune disorders which show no association with obesity and positive energy balance. Those include rheumatoid arthritis, lupus erythematosus, and inflammatory bowel diseases [64, 65]. It appears that secretion of Acrp30 may be differently regulated depending on the pathogenesis of underlying disease, in particular on its association with energy balance (overweight or obesity).

Furthermore, Behre [66] hypothesized that in subjects with an energy deficit in the course of anorexia, cachexia, type 1 diabetes, and renal failure, when the blood level of adiponectin increased, it might have a protective role and adapt the body during fasting. The results obtained in the present study are consistent with such an interpretation of the facts. The patients with ALD, regardless of their gender, had significantly elevated plasma levels of Acrp30 compared with the control group (Table 4). It may be explained by malnutrition frequently occurring in the course of the disease.

Proinflammatory properties of resistin in our study were confirmed by a positive correlation of its serum concentration with the white blood cells count and CRP level (Table 9). Yoshino et al. [57] reported that the plasma levels of resistin and leptin positively correlated with the CRP level in patients with rheumatoid arthritis. Similar observations in patients with Kawasaki disease come from Kemmotsu et al. report [67]. The level of CRP was significantly associated with an increased level of resistin in that study. The results of Trzeciak-Ryczek et al. [68] from *in vitro* studies revealed that macrophage stimulation with lipopolysaccharide or proinflammatory cytokines (IL-1, IL-6 and TNF) considerablly increased resistin production during infection. The presented data support the hypothesis that resistin may become a new marker of inflammation.

Our study has some limitations. It was a single-center trial, and it should be emphasized that due to the insufficient sample size, our results before their generalization need to be confirmed in prospective, multicenter studies. Such validation may help to eliminate possible errors resulting from research techniques and subjective differences in the selection of the study population. Another limitation of the study was lack of estimation of the body fat content by objective and validated measures. The newest diagnostic option is dual-energy X-ray absorptiometry (DEXA), which may be used as a gold standard with high accuracy [69]. Nevertheless, the procedure was unavailable during our study. Furthermore, the control group of healthy abstainers and light drinkers (≤ 20 g/day) was used as the reference category for alcohol consumption in the study. More often disease-free drinkers are included as a comparison cohort to control for the possible effect of alcohol consumption.

Alcohol intake was self-reported in our study. Participants had to determine the amount of alcohol consumed in drinks per day, week, and/or month. Since these values (i.e., the volume and alcohol concentration of a beverage) are not easy to estimate, the real ethanol intake may lack precision [70]. A study by Stockwell and Stirling [71] also showed that most people are not able to accurately assess the volume and power of a drink. In countries like Poland, alcohol intake patterns vary considerably by regions and beverage type, so researchers should pay special attention to careful assessment of drink types and sizes for accurate alcohol consumption estimation.

In conclusion, the results obtained in the present study confirmed the active participation of WAT in the pathogenesis of ALD. We speculate that gender-related differences in serum leptin concentrations may influence the ALD course, different in females compared to males. It seems that, beyond their metabolic influence, adipokines may play a role of inflammatory modulators. An independent association of the high serum Acrp30 level with advanced liver dysfunction, as well as the development of ALD complications (i.e., ascites and hepatic encephalopathy) may indicate its potential role as an ALD prognostic indicator. In addition, due to the inverse serum concentration, Acrp30 may become a relevant marker for differentiation between ALD and NAFLD. The association of resistin levels with renal dysfunction in ALD patients should be further elucidated.

Good availability of serum biomarkers makes them a promising, minimally invasive diagnostic tool for the possible widespread use in the clinical practice. It appears that the determination of molecules involved in different stages of the disease evolution may help to generate a reliable diagnostic algorithm in the future. Whether combination therapy with adipokines may have a role in ALD patients is being greatly anticipated. The modulation of their secretion might be considered as a tempting therapeutic procedure but needs to be investigated in detail.

Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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Research Article

Heavy Ethanol Intoxication Increases Proinflammatory Cytokines and Aggravates Hemorrhagic Shock-Induced Organ Damage in Rats

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Hemorrhagic shock (HS) following acute alcohol intoxication can increase proinflammatory cytokine production and induce marked immunosuppression. We investigated the effects of ethanol on physiopathology and cytokine levels following HS in acutely alcohol-intoxicated rats. Rats received an intravenous injection of 5 g/kg ethanol over 3 h followed by HS induced by withdrawal of 40% of total blood volume from a femoral arterial catheter over 30 min. Mean arterial pressure (MAP) and heart rate (HR) were monitored continuously for 48 h after the start of blood withdrawal. Biochemical parameters, including hemoglobin, ethanol, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine (Cre), lactic dehydrogenase (LDH), and creatine phosphokinase (CPK), were measured at 30 min before induction of HS and 0, 1, 3, 6, 9, 12, 18, 24, and 48 h after HS. Serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were measured at 1 and 12 h after HS. The liver, kidneys, and lungs were removed for pathology at 48 h later. HS significantly increased HR, blood GOT, GPT, BUN, Cre, LDH, CPK, TNF-α, and IL-6 levels and decreased hemoglobin and MAP in rats. Acute ethanol intoxication further increased serum levels of GOT, GPT, BUN, Cre, LDH, CPK, TNF-α and IL-6 elevation following HS. Acutely intoxicated rats exacerbated the histopathologic changes in the liver, kidneys, and lungs following HS.

1. Introduction

Traumatic injury is a leading cause of death and disability worldwide, and hemorrhagic shock (HS) is responsible for up to 40% of trauma deaths [1]. HS can lead to hemodynamic instability, decrease in oxygen delivery, and induce tissue hypoperfusion, leading to cellular hypoxia, organ damage, and death [2, 3]. After HS, nuclear factor-κB (NF-κB) is activated causing the expression of several proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or interleukin-6 (IL-6), and this series of events can, in turn, lead to multiple organ dysfunction [2–5].

Acute alcohol intoxication is a significant risk factor for traumatic injury and causes higher morbidity or mortality rates in patients with HS [6, 7]. Acute alcohol intoxication is a clinically harmful condition that commonly follows the ingestion of a large amount of alcohol [8]. Not only risk of injury is increased by alcohol use, acute intoxication negatively affects severity of trauma-related immune compromise and recovery from trauma-related hospitalization [9].

Following HS, but also acute alcohol intoxication increases proinflammatory cytokine production and induces marked immunosuppression [6]. Hospital emergency rooms regularly see patients with HS as a result of accidents occurring while in a state of acute alcohol intoxication. This study used a rat model to explore how acute alcohol intoxication affects recovery from HS on cytokines (TNF-α, and IL-6) and damage to organs (liver, kidney, and lung).

2. Materials and Methods

2.1. Preparation of Animals. Thirty-two male Wistar-Kyoto rats weighing 260–300 grams were purchased from the National Animal Center (Taipei, Taiwan). They were housed in the university Animal Center in a controlled environment at a temperature of 22 ± 1°C with a 12-hour light/dark cycle. Food and water were provided *ad libitum*. The Animal Care and Use Committee of Tzu Chi University approved the experimental protocol.

The animals were anesthetized with ether inhalation for about 15 min. During the period of anesthesia, a polyethylene catheter (PE-50) was inserted into the femoral artery to collect blood samples and was connected to a pressure transducer (Gould Instruments, Cleveland, OH, USA) to record arterial pressure (AP) and heart rate (HR) on a polygraph recorder (Power Lab, AD Instruments, Mountain View, CA, USA). Another PE-50 catheter was inserted into the femoral vein for intravenous administration of drugs or fluid. The operation was completed within 15 min, leaving a small wound (less than 0.5 cm^2). After the operation, the animals were placed in a conscious rat metabolic cage (Shingshieying Instruments, Hualien, Taiwan). Rats awoke soon after the operation, and acute alcohol intoxication was induced 24 h later, with the rats in a conscious state [10–12].

2.2. Acute Alcohol Intoxication. After PE-50 catheters were inserted into the femoral artery and femoral vein in rats 24 h later. Acute alcohol intoxication in rats was given 5 g/kg ethanol in normal saline mixed to 4 mL intravenously over 3 h [13].

2.3. Hemorrhagic Shock. HS was induced by drawing blood from the femoral arterial catheter into a 10 mL syringe after acute alcohol intoxication. An infusion pump controlled the withdrawal rate to mimic a typical bleeding event. The amount withdrawn was 40% of total blood volume (6 mL/100 gm BW + 0.77 mL) over a period of 30 min [14]. The HS procedure was followed by resuscitation with 0.5 mL normal saline at 0, 1, 3, 6, 9, 12, 18, 24, and 48 h after HS. After blood withdrawal, the animals were continuously observed for 48 h and sacrificed later for pathological study [10–12].

2.4. Experimental Design. Animals were randomly divided into four groups. Rats in the Ethanol group ($n = 8$), were given 5 g/kg ethanol in normal saline mixed to 4 mL intravenously over 3 h and were not subjected to HS [14]. Rats in the HS group ($n = 8$) received an intravenous drip of 4 mL normal saline for 3 h followed by induction of HS. The Ethanol + HS group ($n = 8$) were given 5 g/kg ethanol in normal saline mixed to 4 mL intravenously over 3 h after which HS was immediately induced. In the Vehicle group ($n = 8$), rats received an intravenous drip of 4 mL normal saline for 3 h and were not subjected to HS (Figure 1).

2.5. Blood Sample Analysis. Arterial blood samples were obtained to determine baseline values before heparinization. Heparin (2 IU/gm BW) in 1 mL normal saline was injected via the catheter into rats over 20 min [10–12]. Arterial blood samples (0.5 mL) were collected for measurement of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine (Cre), lactic dehydrogenase (LDH), creatine phosphokinase (CPK), and ethanol at 3 h before induction of HS, and at 0, 1, 3, 6, 9, 12, 18, 24, and 48 h following HS, while an equal volume of 0.5 mL normal saline was used for fluid resuscitation. Blood samples of about 0.1 mL for hemoglobin (Sysmex K-1000, Sysmex American, Mundelein, IL, USA) and of 0.4 mL blood samples were immediately centrifuged at 3,000 g for 10 min. The serum was decanted and separated into two parts; one part was stored at 4°C within 1 h after collection for biochemical analysis. We measured serum levels of GOT, GPT, BUN, Cre, LDH, CPK, and ethanol with an autoanalyzer (COBAS C111, Roche Diagnostics, Basel, Switzerland) to obtain various biochemical data. The other part of the serum collected at 1 h after HS was stored at −80°C for later measurement of TNF-α and IL-6 concentrations [10–12].

2.6. TNF-α and IL-6 Measurement by ELISA. TNF-α and IL-6 concentrations in the blood samples were measured separately 1 and 12 hours after induction of HS by antibody enzyme-linked immunosorbent assay (ELISA) using commercial antibody pairs, recombinant standards, and a biotin-streptavidin-peroxidase detection system (Endogen, Rockford, IL, USA) as previously described [10–12]. Blood samples were collected in serum separator tubes. All reagents, samples, and working standards were brought to room temperature and prepared according to the manufacturer's directions. Reactions were quantified by optical density using an automated ELISA reader (Sunrise, Tecan Co., Grödingen, Austria) at 450/540 nm wavelengths.

2.7. Histological Examination. Rats were sacrificed 48 hours after induction of HS and the livers, kidneys, and lungs were immediately removed. Livers, kidneys, and lungs tissues specimens were fixed overnight in 4% buffered formaldehyde, processed using standard methods and stained with hematoxylin and eosin (H & E). One observer who was blinded to the group assignment performed the tissue analysis. The severity of liver injury observed in the tissue sections was scored as follows: 0, no evidence or minimal evidence of injury; 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and 3, severe necrosis with disintegration of the hepatic cords, hemorrhage, and neutrophil infiltration [10–12]. The severity of renal tubular

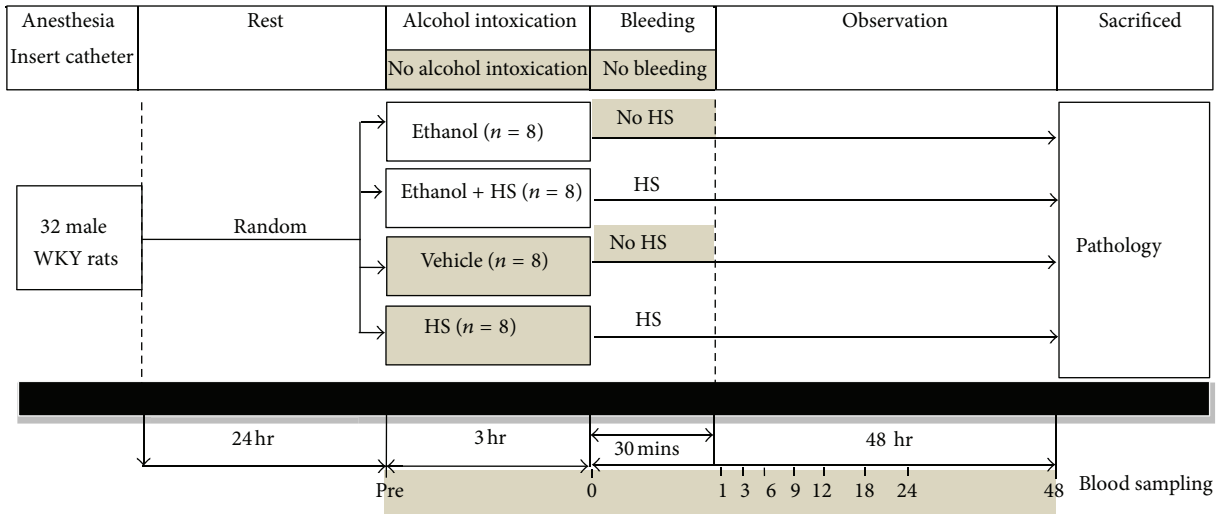

Figure 1: Timeline of hemorrhage and blood sampling protocols for this experiment.

injury was scored by estimating the percentage of tubules in the cortex or the outer medulla that showed epithelial necrosis or had luminal necrotic debris, tubular dilation, and hemorrhage: 0, none; 1, <5%; 2, 5 to 25%; 3, 25 to 75%; and 4, >75% [10–12]. Lung injury was scored as follows: 0, no evidence; 1, mild injury; 2, moderate injury; 3, severe injury with lung edema, interstitial inflammatory cell infiltration, and hemorrhage [10–12]. All evaluations were made on five fields per section and five sections per organ.

2.8. Statistical Analysis. Data were expressed as mean ± SD. Statistical comparisons between different groups at corresponding time points were made by repeated measures of two-way ANOVA followed by a *post hoc* test (Bonferroni's method). Histological scores were analyzed by the Kruskal-Wallis test followed by the Dunn's test. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Mean Arterial Pressure (MAP) and Heart Rate (HR). All rats were alive during the first 48 h of the study. The rats' mean arterial pressure (MAP) decreased rapidly after withdrawal of 40% of total blood volume from the femoral arterial catheter. MAP stayed relatively low during the 48 h after induction of HS (Figure 2(a)). Compared with the Vehicle group which was not subjected to HS, the Ethanol + HS group had decreased MAP at 0, 1, 3, 6, 9, 12, 18, 24, and 48 h after HS ($^{*}P < 0.05$; Figure 2(a)). MAP was not significantly different between the Ethanol group and the Vehicle group (Figure 2(a)). Moreover, no significant difference was observed in MAP after HS when comparing the HS group with the Ethanol + HS group (Figure 2(a)). Heart rate (HR) was significantly increased during HS (Figure 2(b)). The HS group had increased tachycardia at 6, 9, 12, 18, 24, and 48 h after HS compared with the Vehicle group ($^{*}P < 0.05$; Figure 2(b)). No significant difference in HR was observed after HS when we compared the HS group to the Ethanol + HS group (Figure 2(b)).

3.2. Serum Ethanol Level and Hemoglobin. Serum ethanol levels were significantly elevated after ethanol intravenous drip in the Ethanol group and the Ethanol + HS group, with the peak at 0 h, then gradually decreasing to normal at 24 h (Figure 2(c)). Compared with the Vehicle group, the Ethanol group had higher serum ethanol levels at 0, 1, 3, 6, 9, 12, 18, and 24 h after HS ($^{*}P < 0.05$; Figure 2(c)). HS did not affect serum ethanol levels when we compared the Ethanol only group with the Ethanol + HS group (Figure 2(c)). Hemoglobin gradually decreased after induction of HS (Figure 2(d)). Compared with the Vehicle group, the HS group showed decreased hemoglobin at 1, 3, 6, 9, 12, 18, 24, and 48 h after HS ($^{*}P < 0.05$; Figure 2(d)), but compared with the Vehicle group, the Ethanol group had increased hemoglobin at 0, 1, 3, and 6 h after HS ($^{+}P < 0.05$; Figure 2(d)). Compared with the HS only group, the Ethanol + HS group showed increased hemoglobin at 0 h and decreased hemoglobin at 18, 24, and 48 h after HS ($^{\#}P < 0.05$; Figure 2(d)). After ethanol intoxication following HS, hemoglobin increased first and then decreased compared with the HS only group.

3.3. Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT). GOT and GPT are measurements of liver function. GOT gradually increased at 18, 24, and 48 h after induction of HS ($^{*}P < 0.05$; Figure 3(a)). Compared with the Vehicle group, the Ethanol group showed higher GOT levels at 18, 24, and 48 h after HS ($^{+}P < 0.05$; Figure 3(a)). Compared with the HS only group, the Ethanol + HS group had higher levels of GOT at 1, 3, 9, 12, 18, 24, and 48 h ($^{\#}P < 0.05$; Figure 3(a)). We observed no statistically significant difference in serum GOT in the Ethanol group compared with the HS group (Figure 3(a)). GPT gradually increased after induction of HS ($^{*}P < 0.05$; Figure 3(b)). Compared with the Vehicle group, the Ethanol only group

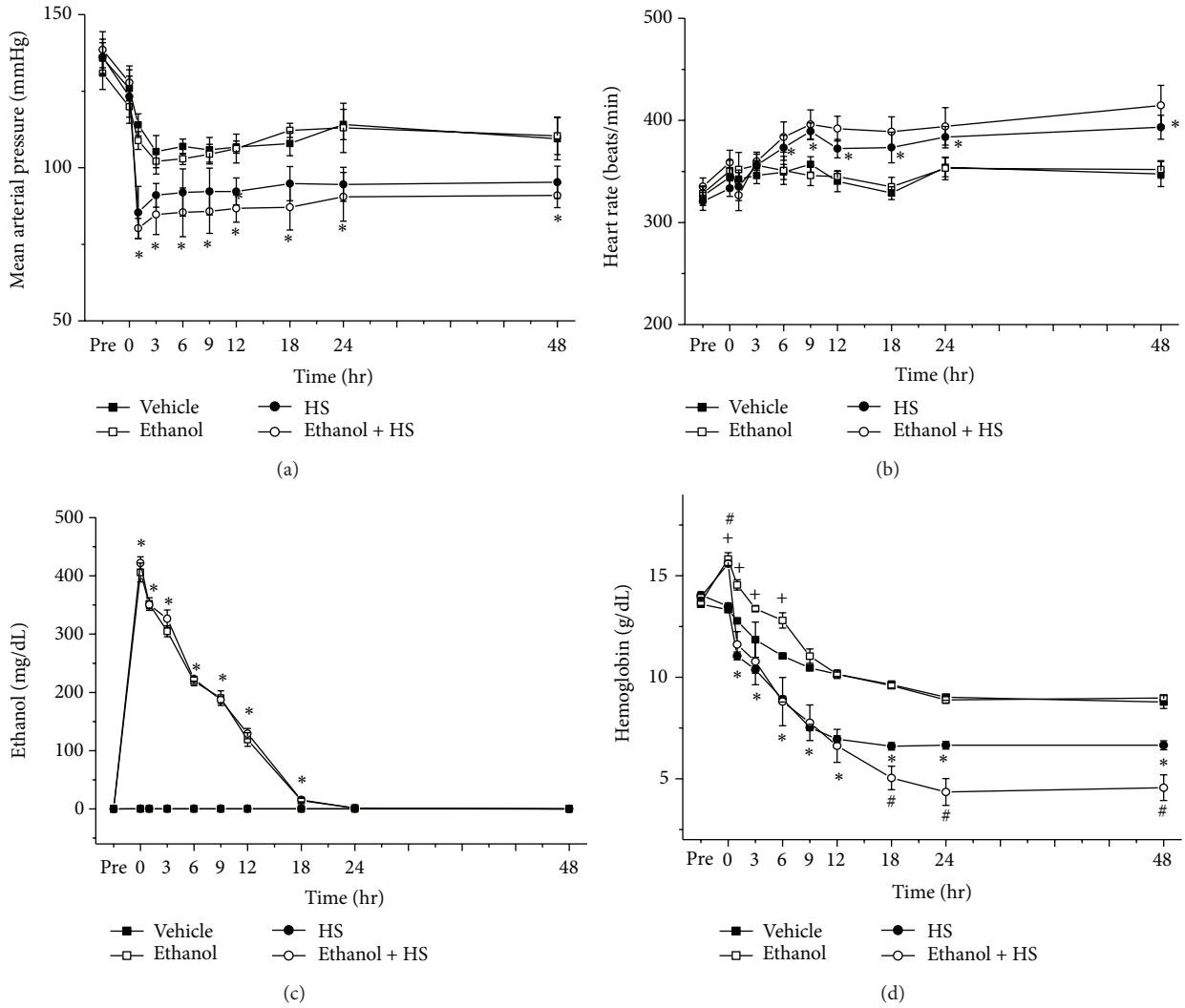

Figure 2: Changes in (a) mean arterial pressure, (b) heart rate, (c) serum ethanol concentration, and (d) hemoglobin following hemorrhagic shock in rats. $^{*}P < 0.05$ for the HS group compared with the Vehicle group. $^{+}P < 0.05$ for the Ethanol group compared with the Vehicle group. $^{\#}P < 0.05$ for the Ethanol + HS group compared with the HS group.

had increased GPT at 48 h after HS ($^{+}P < 0.05$; Figure 3(b)). Compared with the HS group, the Ethanol + HS group had even higher GPT at 3 and 48 h ($^{\#}P < 0.05$; Figure 3(b)).

3.4. Blood Urea Nitrogen (BUN), and Creatinine (Cre). BUN and Cre are measured of kidney function. HS increased blood BUN at 1, 3, 6, 9, 18, 24, and 48 h ($^{*}P < 0.05$; Figure 4(a)). Compared with the Vehicle group, the Ethanol group had increased BUN at 1, 3, 6, and 9 h after HS ($^{+}P < 0.05$; Figure 4(a)). Compared with the HS group, the Ethanol + HS group had even higher BUN at 1, 3, 6, 9, 12, 18, and 48 h ($^{\#}P < 0.05$; Figure 4(a)). No statistically significant difference was observed in serum BUN when comparing the Ethanol group with the HS group (Figure 4(a)). Serum Cre increased rapidly after induction of HS. The serum Cre values increased at 1, 3, 6, 9, 12, and 48 h after HS compared with the Vehicle group ($^{*}P < 0.05$; Figure 4(b)). But compared with the HS group, the Ethanol + HS group had even higher Cre at 1, 3, 6, 12, 18, 24, and 48 h ($^{\#}P < 0.05$; Figure 4(b)).

3.5. Lactic Dehydrogenase (LDH) and Creatine Phosphokinase (CPK). The Ethanol group had increased LDH at 0 and 1 h compared with the Vehicle group ($^{+}P < 0.05$; Figure 5(a)). The Ethanol + HS group had increased LDH at 0, 1, 18, 24, and 48 h compared with the HS group ($^{\#}P < 0.05$; Figure 5(a)). HS increased blood CPK at 3, 6, 9, 12, and 18 h ($^{*}P < 0.05$; Figure 5(b)). Compared with the Vehicle group, the Ethanol group showed increased CPK at 0 and 1 h ($^{+}P < 0.05$; Figure 5(b)). Compared with the HS group, the Ethanol + HS group had still further increased CPK at 0, 1, 3, 6, 9, 12, 18, 24, and 48 h ($^{\#}P < 0.05$; Figure 5(b)).

3.6. Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6). HS greatly elevated serum TNF-α compared with the Vehicle group at 1 and 12 h ($^{*}P < 0.05$; Figure 6(a)). Prior administration of ethanol significantly increased the serum TNF-α at 1 and 12 h after induction of HS ($^{\#}P < 0.05$; Figure 6(a)). HS increased serum IL-6, compared with the Vehicle group at 1 and 12 h after induction of HS ($^{*}P < 0.05$;

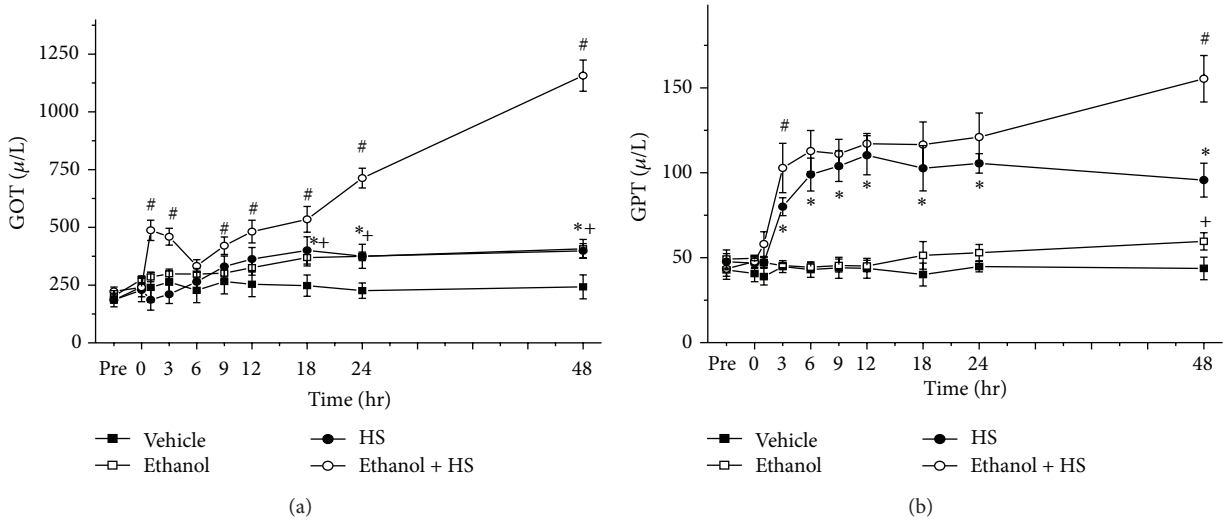

Figure 3: Changes in serum (a) glutamic oxaloacetic transaminase (GOT) and (b) glutamic pyruvic transaminase (GPT) after hemorrhagic shock in rats. $^{*}P < 0.05$ for the HS group compared with the Vehicle group. $^{+}P < 0.05$ for the Ethanol group compared with the Vehicle group. $^{\#}P < 0.05$ for the Ethanol + HS group compared with the HS group.

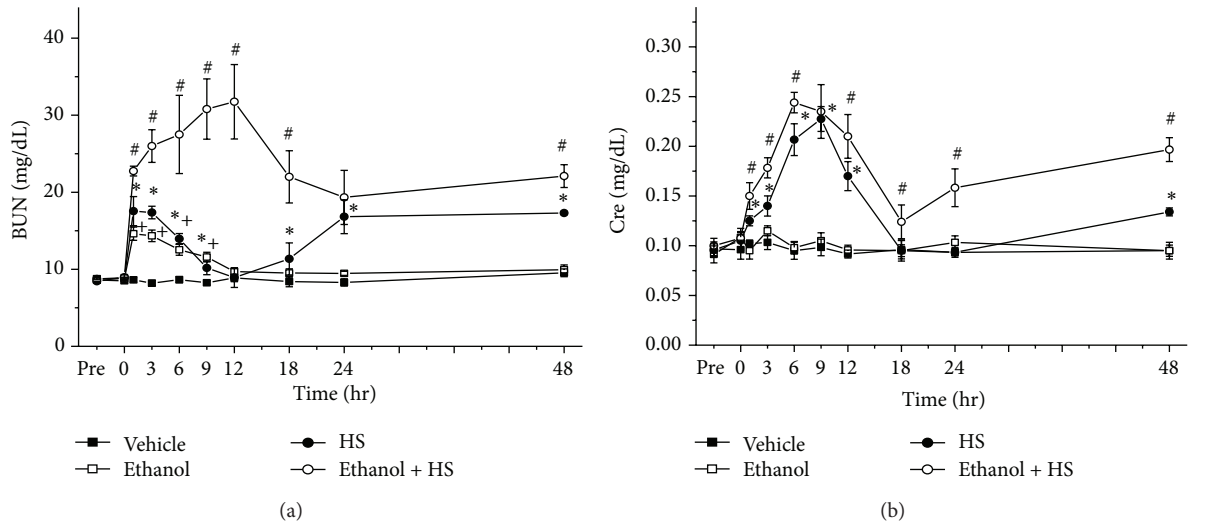

Figure 4: Changes in serum (a) blood urea nitrogen (BUN) and (b) creatinine (Cre) after hemorrhagic shock in rats. $^{*}P < 0.05$ for the HS group compared with the Vehicle group. $^{+}P < 0.05$ for the Ethanol group compared with the Vehicle group. $^{\#}P < 0.05$ for the Ethanol + HS group compared with the HS group.

Figure 6(b)). Compared with the HS only group, the Ethanol + HS group had even higher IL-6 at 1 and 12 h after induction of HS ($^{\#}P < 0.05$; Figure 6(b)).

3.7. Histopathology of Liver, Kidney, and Lung. Histopathologic analysis of H & E-stained tissue sections from the liver, kidneys, and lungs after HS revealed hepatocyte necrosis and leukocytes infiltration in the liver (Figure 7(g)), tubular cell swelling, nuclear loss, tubular dilatation, and brush border loss in the kidney (Figure 7(h)). Pulmonary edema, hemorrhage, and interstitial polymorphonuclear (PMN) inflammatory cells infiltration in the lung were observed after HS (Figure 7(i)). Compared with the HS group, the Ethanol + HS group had greater histopathologic changes and hemorrhage in the liver, kidney, and lung (Figures 7(j), 7(k), and 7(l)). Compared with the HS group, the Ethanol + HS group had increased injury scores of the liver, kidney, and lung ($^{\#}P < 0.05$; Figures 7(m), 7(n), and 7(o)).

4. Discussion

This study found that intravenous heavy ethanol increased serum TNF-α and IL-6 levels after HS and aggravated HS-induced organ damage (liver, kidney, and lung) in rats.

The prevalence of alcohol-related visits to U.S. trauma centers ranged from 26.2% to 62.5% [15]. Symptoms are

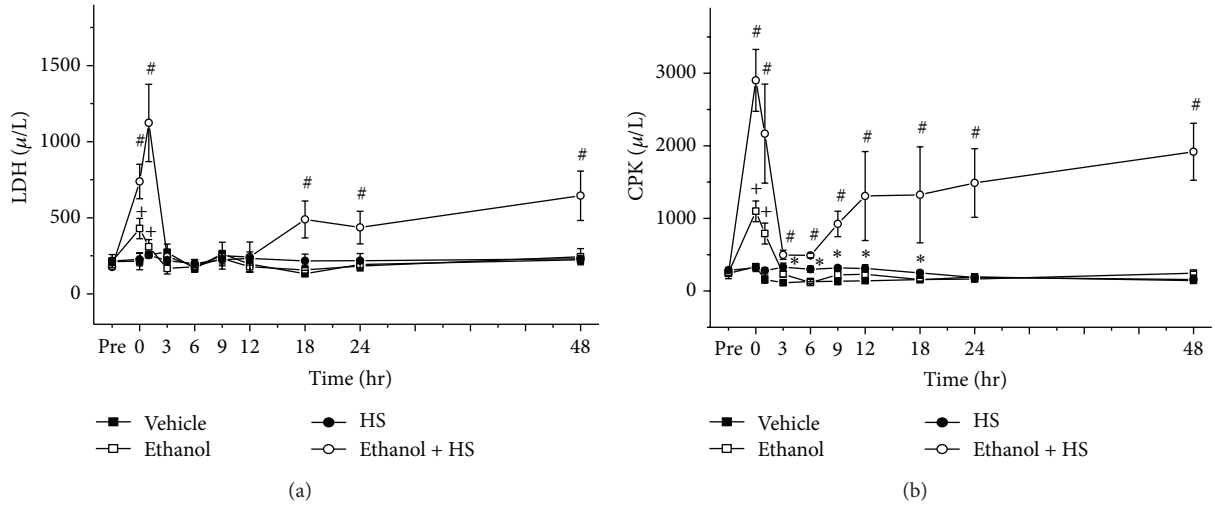

Figure 5: Changes in serum (a) lactic dehydrogenase (LDH), and (b) creatine phosphokinase (CPK) after hemorrhagic shock in rats. $^{*}P < 0.05$ for the HS group compared with the Vehicle group. $^{+}P < 0.05$ for the Ethanol group compared with the Vehicle group. $^{\#}P < 0.05$ for the Ethanol + HS group compared with the HS group.

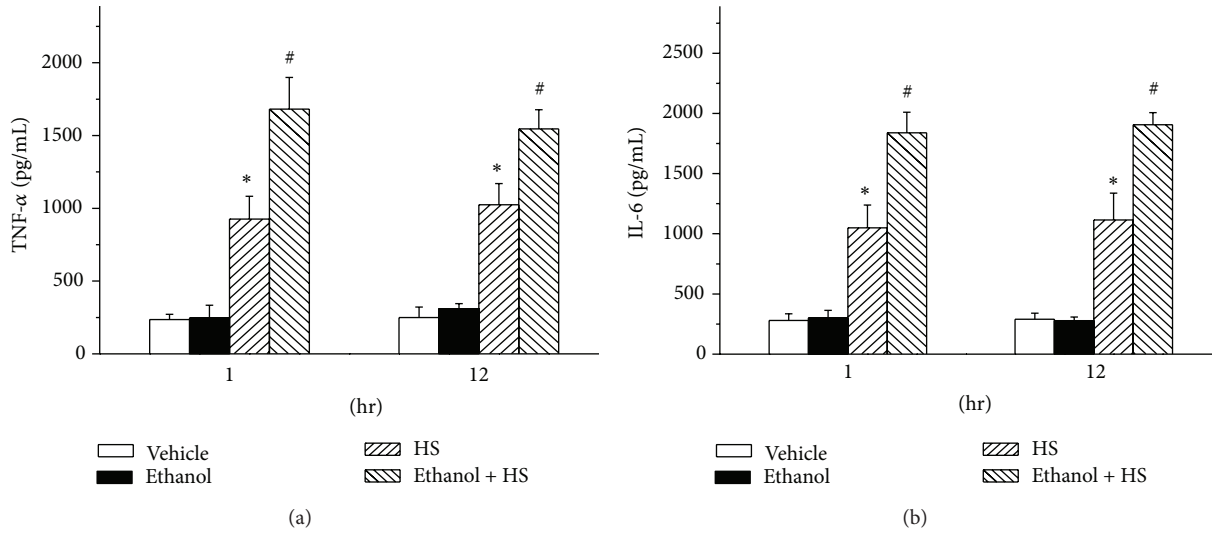

Figure 6: Changes in serum (a) tumor necrosis factor-α (TNF-α), and (b) interleukin-6 (IL-6) after hemorrhagic shock in rats. $^{*}P < 0.05$ for the HS group compared with the Vehicle group. $^{\#}P < 0.05$ for the Ethanol + HS group compared with the HS group.

usually related to blood alcohol concentration. At a blood alcohol concentration higher than 300 mg/dL, there is an increased risk of respiratory depression and cardiac arrest. Death attributable to acute alcohol intoxication generally occurs at a blood alcohol concentration higher than 500 mg/dL, although the lethal dose can vary [6]. In this study, blood alcohol concentration before HS was about 405.87 ± 16.5 mg/dL—similar to concentrations observed in humans after binge drinking.

Alcohol intoxication aggravates traumatic injury-related hemodynamic instability [16]. Low MAP at the time of arrival into the emergency department has been reported to be a predictor of poor patient outcome from traumatic injury and blood loss [17]. Alcohol intoxication may impair the ability of blunt trauma patients to compensate for acute blood loss, making them more likely to be hypotensive on admission and increasing their need for packed red blood cells and intravenous fluids [18]. Our study noted heavy ethanol intoxication after hemorrhage had lower MAP and tachycardia than ethanol group. But there was no significant difference in MAP and HR between the HS group and the Ethanol + HS group in this study. We observed that after ethanol intoxication, hemoglobin increased first, which may be due to the higher osmolality of ethanol induced hemoconcentration. However, hemoglobin decreased at 18, 24, and 48 h following hemorrhage in acute alcohol intoxicated rats compared with the HS only group. This might be because acute alcohol intoxication affected tissue hemorrhage

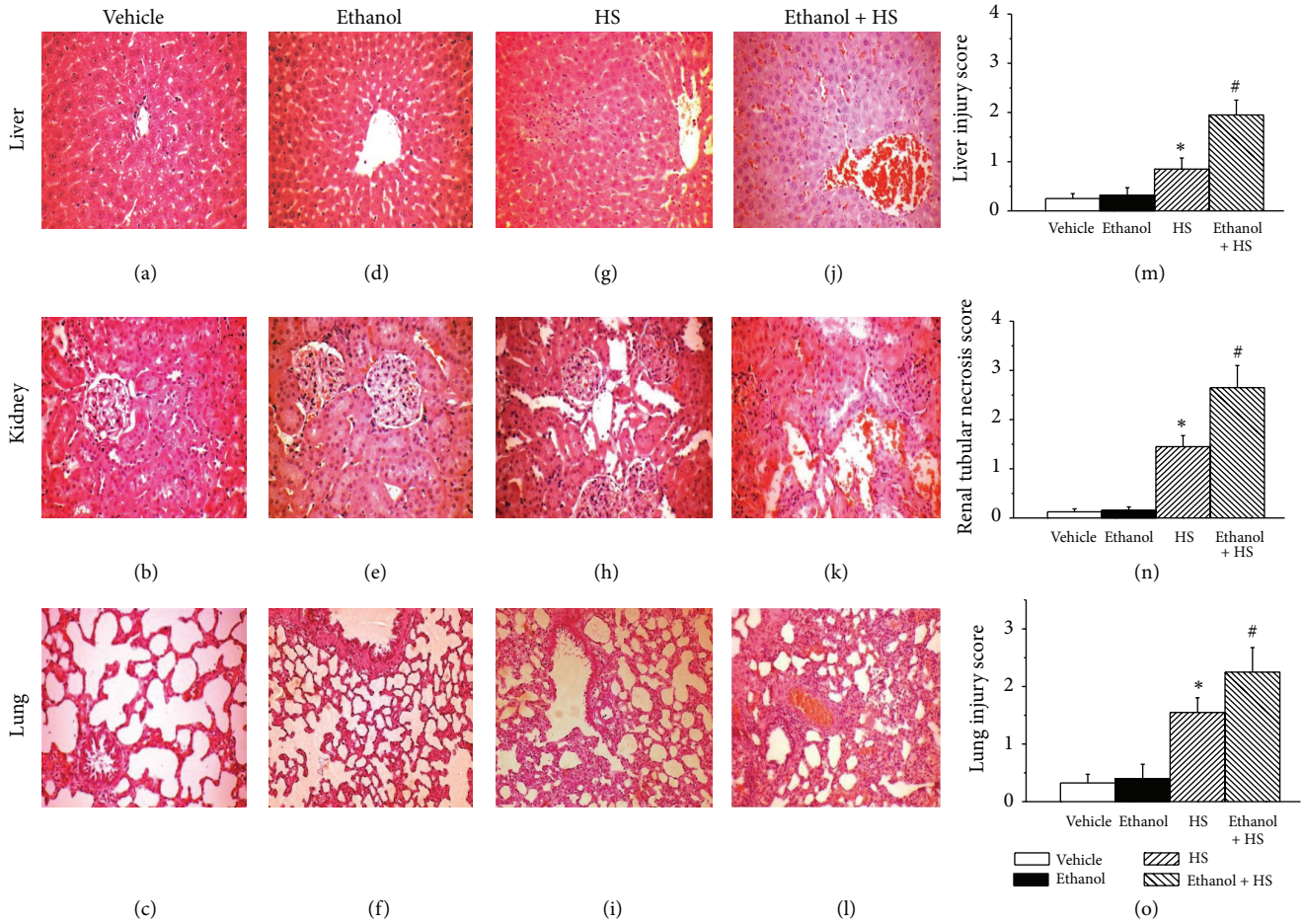

FIGURE 7: Histopathologic changes in the liver, kidneys, and lung after hemorrhagic shock in rats. Histologic sections from the Vehicle group ((a), (b), (c)), Ethanol group ((d), (e), and (f)), HS group ((g), (h), and (i)), and Ethanol + HS group ((j), (k), and (l)), stained with hematoxylin and eosin (liver, kidneys, lungs: magnification ×200). Photomicrographs (a), (d), (g), and (j) are liver sections; (b), (e), (h), and (k) are kidney sections; (c), (f), (i), and (l) are lung sections. Histopathologic injury score in liver (m), kidney (n), and lung (o) after hemorrhagic shock in rats. $^{*}P < 0.05$ for the HS group compared with the Vehicle group. $^{\#}P < 0.05$ for the Ethanol + HS group compared with the HS group.

following HS. Pathology examination proved that the Ethanol + HS group had more tissue hemorrhage in the liver, kidney and lung.

The liver is particularly at risk for alcohol-related damage because it receives portal blood directly from the intestinal tract and thus experiences the highest concentration of alcohol presented to any organ [19]. In addition, ethanol metabolism in the liver produces potentially harmful toxic metabolites such as acetaldehyde, acetate, and reactive oxygen species [20]. Increased serum TNF-α and IL-6 concentrations have frequently been found in alcoholic liver cirrhosis patients [21]. Alcohol intoxicated rodents, present with lower blood pressure at the time of injury, have decreased tolerance to blood loss and have impaired blood pressure recovery during fluid resuscitation. The accentuated hypotension leads to tissue hypoperfusion, which enhances susceptibility to tissue injury reflected in greater elevation in circulating liver function [22]. Our study also observed that acute ethanol intoxication aggravated liver damage by greater elevation of GOT, GPT, and the histopathologic analysis of liver revealed more hepatocyte necrosis and leukocytes infiltration following hemorrhage in acute alcohol intoxicated rats.

Alcohol consumption increased malondialdehyde levels, superoxide dismutase, and catalase activity significantly in alcohol intoxicated rats [23]. Alcohol intoxicated rats result in a greater reduction of blood flow to the kidney than that seen in nonintoxicated rats after HS [24]. The accentuated hypotension leads to tissue hypoperfusion, which enhances susceptibility to tissue injury reflected in greater elevation in circulating renal function [23]. Our study found that acute ethanol intoxication induced greater renal damage after HS in rats by elevated serum BUN, Cre and exacerbated the histopathologic changes in kidneys following HS in rats.

Alcohol exposure of the host can predispose to pneumonia infection [9]. Acute alcohol intoxication exacerbates the HS-induced increase in lung proinflammatory cytokine TNF-α expression in rats [25]. In other studies, alcohol-treated mice had worse clinical outcomes, deteriorated pulmonary structure, and increased levels of IL-6 compared with the nonalcohol treated mice [26]. Our study found

that heavy ethanol intoxication aggravated lung damage including pulmonary edema, hemorrhage, and interstitial PMN inflammatory cell infiltration in the lung following hemorrhage.

In response to HS, NF-κB is involved in apoptosis and the inflammatory cascade [5]. The organism provokes release of proinflammatory cytokines (TNF-α and IL-6) into surrounding tissues, thereby causing tissue damage and organ failure [2, 3]. TNF-α and IL-6 peak early after HS and continue elevating during HS because tissue hypoperfusion persist [10–12]. Treatment with anti- TNF-α antibodies reduced organ injury and improved survival in rats after HS [27]. Inhibition of the synthesis of IL-6 may exert beneficial effects on HS [28]. Acute alcohol intoxication can increase proinflammatory cytokine production and induce marked immunosuppression after HS [6]. Our study found that intravenous injection of heavy ethanol increased serum TNF-α and IL-6 production after HS in rats. Altered inflammatory cell and adaptive immune responses after alcohol consumption result in increased of infections and other organ-specific immune-mediated effects [9].

Sex differences in alcohol drinking is somewhat equivocal in rodent studies: one study noted female rodents tend to drink more alcohol than males [29] and other study noted adolescent males have been reported to drink more than females [30], whereas others suggest that there is no sex difference [31, 32]. Increases in pubertal hormones, including gonadal and stress hormones, are a prominent developmental feature of adolescence and could contribute to the progression of sex differences in alcohol drinking patterns during puberty [33]. Our study used adult male Wistar-Kyoto rats and found increased serum TNF-α and IL-6 levels after HS and aggravated HS-induced organ damage. Further studies are required to investigate sex differences in alcohol drinking behavior and/or the influence of pubertal hormone changes on the effects of ethanol on physiopathology and cytokine levels following HS in acutely alcohol-intoxicated rats.

Recent study noted that rats were given a single oral dose of ethanol (5 g/kg, 30%) increased survival after HS and decreased HS-induced liver injury [34]. However, the blood ethanol concentration in this study is unknown and only given a single oral dose of ethanol. Our study, blood alcohol concentration before HS was similar to concentrations observed in humans after binge drinking. Acute ethanol intoxication leads to a dysregulation of the hemodynamic, neuroendocrine, inflammatory, and immune responses to hemorrhage. This disruption of the normal neuroendocrine counterregulatory response impairs hemodynamic stability and recovery, contributing to compromised tissue perfusion and increased end-organ injury [6, 22]. Intravenous heavy ethanol injection of this study also noted increased serum TNF-α and IL-6 levels after HS and aggravated HS-induced organ damage in rats.

5. Conclusion

Acute ethanol intoxication increased serum TNF-α and IL-6 levels following HS, along with aggravating HS-induced organ damage in rats.

Conflict of Interests

The authors report no conflict of interests related to this study.

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