Original Paper

Ethanol Decreases Inflammatory Response in Human Lung Epithelial Cells by Inhibiting the Canonical NF- κ B-Pathway

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Key Words

Inflammation • Trauma • Alcohol • Intoxication • NF- κ B • Canonical • Non-canonical • Lung

Abstract

Background/Aims: Alcohol (ethanol, EtOH) as significant contributor to traumatic injury is linked to suppressed inflammatory response, thereby influencing clinical outcomes. Alcohol-induced immune-suppression during acute inflammation (trauma) was linked to nuclear factor-kappaB (NF- κ B). Here, we analyzed alcohol's effects and mechanisms underlying its influence on NF- κ B-signaling during acute inflammation in human lung epithelial cells.

Methods: A549-cells were stimulated with interleukin (IL)-1 β , or sera from trauma patients (TP) or healthy volunteers, with positive/negative blood alcohol concentrations (BAC), and subsequently exposed to EtOH (170 Mm, 1h). IL-6-release and neutrophil adhesion to A549 were analyzed. Specific siRNA-NIK mediated downregulation of non-canonical, and IKK-NBD-inhibition of canonical NF- κ B signaling were performed. Nuclear levels of activated p50 and p52 NF- κ B-subunits were detected using TransAm ELISA. **Results:** Both stimuli significantly induced IL-6-release (39.79 ± 4.70 vs. 0.58 ± 0.8 pg/ml) and neutrophil adhesion (132.30 ± 8.80 vs. 100% control, $p < 0.05$) to A549-cells. EtOH significantly decreased IL-6-release (22.90 ± 5.40 , $p < 0.05$) and neutrophil adherence vs. controls ($105.40 \pm 14.5\%$, $p < 0.05$). IL-1 β -induced significant activation of canonical/p50 and non-canonical/p52 pathways. EtOH significantly reduced p50 (34.90 ± 23.70 vs. 197.70 ± 36.43 , $p < 0.05$) not p52 activation. Inhibition of canonical pathway was further increased by EtOH (less p50-activation), while p52 remained unaltered. Inhibition of non-canonical pathway was unchanged by EtOH. **Conclusion:** Here, alcohol's anti-inflammatory effects are mediated via decreasing nuclear levels of activated p50-subunit and canonical NF- κ B signaling pathway.

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Introduction

Trauma is the leading cause of mortality in young people worldwide, which is characterized by early death occurring due to fatal injuries and late post-injury mortality, predominantly

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caused by infectious complications [1, 2]. Chest trauma as the most relevant injury pattern (about 60%) next to traumatic brain injuries (TBI) in trauma patients significantly affects patient morbidity [3]. In addition, the risk behavior including alcohol use is common among youth [4]. Nearly half of all trauma patients who are admitted to emergency departments, have positive blood alcohol concentration (BAC) [5, 6]. Next to increasing the risk of injuries, alcohol use is associated with altered inflammatory response upon trauma. While chronic alcohol use was shown to correlate with negative outcomes, acute alcohol use has been demonstrated to positively influence the post-injury clinical course, or to have no effects on diverse outcome parameters in trauma patients [7, 8]. Notably in alcohol-intoxicated TBI patients lower incidence of early coagulopathy, pneumonia and mortality were reported [9, 10]. In an *in vivo* animal model of haemorrhagic shock, our group found significant anti-inflammatory influence of alcohol on trauma-induced inflammation, which was associated with improved survival [11].

Severe injury is associated with an early onset of the systemic inflammatory response syndrome (SIRS), which is occasionally leading to multi-organ dysfunction. SIRS is characterized by increased levels of pro-inflammatory cytokines e.g. Interleukin (IL)-1 β and activation of neutrophils [12, 13]. In several *in vivo* models and in clinical trials, acute alcohol use has been described to significantly impact the immune response by suppressing the production of pro-inflammatory mediators such as tumor necrosis factor (TNF)- α , IL-1 β or IL-6 [14-16]. In severely injured TP, we found significantly lower leukocyte numbers as well as lower systemic IL-6 levels in patients with positive BAC (>0.5‰) compared with patients with BAC levels below <0.05‰ [8]. Szabo et al. described significantly reduced TNF- α and IL-1 β release from human blood monocytes in an *ex vivo* model of bacterial stimulation by acute exposure to alcohol [17]. Additional studies have shown that the chemotactic activity of neutrophils as well as their infiltration into tissues were decreased after alcohol use in models of acute inflammation [18]. In several trauma models, alcohol's mode of action has been attributed to the deactivation of the transcription factor NF- κ B, which is triggering the systemic and local propagation of inflammation [19, 20].

NF- κ B is a dimer composed of members of the Relish (Rel)-family, which contains 5 subunits: p50, p52, p65 (RelA), cRel, RelB. Those subunits homo- or heterodimerize in several combinations with differential stability [21]. In its inactive form, NF- κ B is associated with inhibitor proteins (I κ B) and is located in the cytoplasm. Upon phosphorylation of the inhibitor protein by a complex of three I κ B protein kinases (IKKs), activated NF- κ B translocates into the nucleus regulating the gene expression of e.g. cytokines, chemokines, adhesion molecules, receptors involved in antigen presentation or neutrophil transmigration [22, 23]. Two main pathways have been described for NF- κ B signaling, the canonical, or classical, and the non-canonical, or alternative, pathway. In the canonical pathway, the NF- κ B dimer, most commonly RelA(p65)/p50, is maintained in the cytosol by interaction with an I κ B-molecule. Binding of a ligand like IL-1 β results in activation of an IKK-complex, which leads to a phosphorylation and consecutive degradation of the I κ B, thereby inducing nuclear NF- κ B translocation [24, 25]. The IKK-complex contains two regulatory subunits, so called NEMO. On the other hand, in the non-canonical pathway, receptor binding activates the NF- κ B-inducing kinase NIK, resulting in phosphorylation of IKK α , which subsequently phosphorylates the p100/RelB-complex. This leads to liberation of the RelB/p52-dimer, which then translocates into the nucleus regulating gene expression [24-26].

Regarding the specific mechanism underlying alcohol's effects on NF- κ B signaling, acute alcohol decreased monocyte production of IL-1 β and DNA binding of the RelA/p50-dimer upon stimulation [17, 20]. Mandrekar et al. described an up-regulation of the p50/p50 NF- κ B homodimer upon acute exposure of human blood monocytes to alcohol [27]. Since p50/p50 homodimers have been demonstrated to have immunosuppressive properties inhibiting NF- κ B transcriptional activity, this may be one possible explanation for alcohol induced reduction of pro-inflammatory cytokines [28, 29]. Alcohol exposure of lean as well as obese mice reduced both, the nuclear protein level and the DNA-binding activity of p65 NF- κ B [30]. Equally important, other studies indicate that chronic alcohol use will activate p65 [31, 32].

Taken together, there is evidence that alcohol affects NF- κ B signaling, thereby exerting anti-inflammatory effects. We hypothesized that acute alcohol use will inhibit the canonical NF- κ B pathway. Therefore, the IL-6 secretory capacity and binding of neutrophils to human lung epithelial cells after their acute exposure to alcohol were evaluated. Using specific inhibitors for either canonical or non-canonical NF- κ B signaling, the underlying mechanism was investigated.

Materials and Methods

Ethics

The study was performed in the University Hospital Frankfurt of the Goethe-University with institutional ethics committee approval (312/10) in accordance with the Declaration of Helsinki and following STROBE-guidelines [33]. All included subjects signed the written informed consent forms themselves or informed consent was obtained from the nominated legally authorized representative consented on the behalf of participants as approved by the ethical committee.

Patients and blood samples

Forty trauma patients with a history of acute blunt trauma and an Injury Severity Score (ISS) ≥ 16 between 18 and 80 years of age were included. Patients with chronic history of alcohol use or known pre-existing immunological disorders, immunosuppressive and anti-coagulant medication, burns, concomitant acute myocardial infarction, thromboembolic events were excluded. Blood alcohol concentration (BAC) was measured immediately on admission to the emergency department in all patients included in this study. Twenty patients with a mean BAC of $1.54 \pm 0.10\%$, and 20 patients without any BAC were included. Fifteen healthy volunteers (22 - 57 years of age, 7 female and 8 male) were included.

Blood samples were obtained after admission of the patient to the emergency department (ED) for routine BAC determination as well as for subsequent plasma storage for stimulatory experiments. Blood samples were obtained in pre-chilled serum tubes (S-Manovette® Z-Gel tubes, Sarstedt, Nümbrecht, Germany) and kept on ice. After centrifuging at $2000 \times g$ for 15 minutes at 4°C , the obtained sera were pooled. For the experiments with serum containing EtOH, the pooled samples were spiked. While on the one hand, EtOH was not added, on the other hand, EtOH was added to serum to reach an alcohol concentration of 1.5% , before experimentation. These samples served as HV with positive BAC as reference for the TP with positive BAC and a mean alcohol concentration of 1.5% , also. For experimentations A549 cells were cultured in media containing 20% of plasma.

Cell culture and isolation of neutrophils

A549 human lung epithelial cells (Cell Line Services, Heidelberg, Germany) were cultured at 37°C under 5% CO_2 in RPMI-1640 medium (Seromed, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco, Karlsruhe, Germany) and 20 mM HEPES buffer (Sigma, Steinheim, Germany). Culture media were replaced each second or third day.

The isolation of polymorphonuclear leukocytes (PMN) from healthy volunteers was performed by density-gradient centrifugation (density: $1.113 \pm 0.001 \text{ g}/\text{ml}$, Polymorphprep, Axis-Shield, Oslo, Norway) according to manufacturer's instructions. Briefly, 4 ml Polymorphprep were carefully covered with 4 ml heparinized whole blood from HV and centrifuged for 30 minutes. Then, the PMN cell fraction was washed with phosphate buffered saline without Ca^{2+} and Mg^{2+} (Invitrogen). Supernatant was removed and PMN were cultured in RPMI 1640 with supplements as described above. PMN viability was determined by the trypan blue exclusion assay. Only cell cultures with a purity of $>95\%$ were utilized for experimental use.

Cell stimulation with IL-1 β or sera from HV/TP ($\pm$ BAC) and consecutive EtOH exposure

The concentrations of IL-1 β as well as alcohol (EtOH) are based on previous work to allow better comparison of data [34-36]. A549 cells were stimulated with recombinant human IL-1 β (1 ng/ml) (R&D Systems, Wiesbaden, Germany) or sera from TP or HV with positive or negative BAC for 24 h. 170 mM EtOH (corresponding to 1 vol vol $^{-1}$ per cent or 7.9 mg EtOH mL $^{-1}$, respectively) were added for 1 h afterwards in order to study the effects of an acute exposure to alcohol. The schematic timeline of the experimental design is shown in Fig. 1.

Blocking and knockdown studies

After stimulation with IL-1 β for and before exposure to EtOH, A549 cells were incubated overnight with 36 μ g/ml the NEMO „binding domain“ (NBD) inhibitor IKK-NBD Peptide (Enzo Life Sciences GmbH, Lörrach, Germany). IKK-NBD specifically blocks the interaction of NEMO with the I κ B kinase complex, thereby inhibiting the canonical NF- κ B signaling. Control cells were incubated with the corresponding peptide control inhibitor IKK-NBD Control Peptide (Enzo) or cell culture medium alone.

Additionally, A549 cells were transfected with small interfering RNA (siRNA) directed against NIK (NIK siRNA 10 μ M, Santa Cruz Biotechnology, Inc, Heidelberg, Germany) with a siRNA/transfection reagent overnight according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc). Briefly, cells were transfected at a confluency of 70-80%. Optimum transfection was achieved in RPMI1640 medium supplemented with a siRNA/transfection reagent ratio of 1:1 (8 μ l siRNA and 8 μ l transfection reagent in 1 ml transfection medium). Nontreated cells and cells transfected with the control siRNA (10 μ M, Santa Cruz Biotechnology, Inc.) served as controls. The transfection efficiency was evaluated by qRT-PCR after RNA isolation, which were carried out as described before [37] using gene-specific primers for human Mitogen-activated protein kinase kinase 14 (NM_003954.4 UniGene# Hs.404183, Cat# PPH00332F) and human GAPDH (NM_002046, UniGene# Hs.592355, Cat# PPH00150E; SABiosciences, SuperArray, Frederick, MD, USA) as reference gene. After transfection of the cells, those were exposed to EtOH as indicated above.

In order to confirm the findings, the experiments with both IKK-NBD inhibitor and siRNA against NIK simultaneously were performed. Incubation times as indicated above were applied.

Quantification of IL-6 release

A549 cells were incubated as described above and at each time point culture supernatants were harvested. The concentrations of IL-6 were determined using Diaclone IL6 ELISA Set (Diaclone, France) according to manufacturer's instructions with Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

Monolayer adhesion assay

In order to analyze neutrophil adhesion to pre-treated A549 cells, cells were transferred into 24-well multiplates (Sarsted, Nümbrecht, Germany) in RPMI-1640 medium with supplements. When a confluency of ~80% was reached, A549 cells were stimulated with IL-1 β or sera from TP or HV for 24 h and treated with 170 mM EtOH for one hour. Freshly isolated PMN were counted and adjusted to 5×10^4 vital cells / well, and then carefully added to the A549 monolayers. After an incubation for 15 min at 37°C under 5% CO₂, non-adherent PMN were washed off 3x by using pre-warmed (37°C) RPMI-1640 medium. The remaining PMN were fixed using 1% glutaraldehyde. Adherent PMN were counted in five different fields of a defined size (5 x 0.25 mm²) by using a phase contrast microscope (x10 objective). The mean cellular adhesion rate was calculated as the ratio to unstimulated controls in percent. The assay was performed as described previously [35].

Nuclear extraction and TransAm Transcription factor ELISAs for p50 und p52

Nuclear fractions of pre-treated A549 human lung epithelial cells were obtained using the Nuclear Extract Kit (Active Motif, La Hulpe, Belgium) according to manufacturer's instructions. Obtained protein concentrations were colorimetrically determined after adding methylene blue using Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

Fig. 1. Schematic timeline of the experimental design. A549 lung epithelial cells were stimulated with either IL-1 β (1 ng/ml) or sera from trauma patients and healthy volunteers, respectively, for 24 h. Before exposure to alcohol (ethanol, EtOH), cells were incubated with the NEMO „binding domain“ (NBD) inhibitor IKK-NBD Peptide, or cells were transfected with small interfering RNA (siRNA) directed against NIK (NIK siRNA, overnight, ON). Then cells were exposed to EtOH (170 mM) for one hour. After the incubation, analyses were performed.

Nuclear extracts were stored at -80°C . For the experiments, nuclear fractions were analysed for activated p50 and p52 levels using the TransAm NF- κ B Family Kit (Active Motif) and following manufacturer's instructions. The colorimetric change was measured using the Infinite M200 microplate reader in order to analyze nuclear levels of the activated NF- κ B subunits. Positive controls, provided within the kit (Active Motif) with known RNA concentration were used to calculate the levels of activation, and were used as reference. Then, the activation rates were referred to the unstimulated control set as 100%.

Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA) was used to perform the statistical analyses. Normality distribution was assessed by Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors P value. Then, according to the normality distribution of each data set, one-way ANOVA followed by Tukey post-hoc was applied, unless otherwise stated. A p value below 0.05 was considered significant. Data are given as mean $\pm$ standard deviation (SD). All experiments were performed 4-5 times.

Results

IL-6 release after IL-1 β stimulation or treatment with sera from HV or TP ($\pm$ BAC) and subsequent EtOH exposure of A549 human lung epithelial cells

In order to analyse the influence of EtOH on the secretory potential of pro-inflammatory IL-6 by A549 cells, cells were stimulated with IL-1 β and consecutively exposed to EtOH for one hour. IL-6 release from stimulated cells was significantly increased compared to unstimulated controls (39.8 ± 4.7 vs. 0.58 ± 0.8 , $p < 0.05$, Fig. 2A). Exposure to EtOH significantly decreased IL-6 release to 22.9 ± 5.4 compared with stimulated control ($p < 0.05$, Fig. 2A).

In order to determine the pro-inflammatory state after severe trauma more accurately, A549 cells were stimulated with sera from TP, who were either positive or negative for alcohol upon admission to ED ($\pm$ BAC). Treatment with sera from TP significantly enhanced IL-6 production in comparison to exposure to sera from HV ($p < 0.05$, Fig. 2B). This effect was observed for sera with positive BAC as well as for sera with negative BAC. There were no significant differences in IL-6 release when comparing BAC-positive sera with BAC-negative sera from HV as well as from TP. Consecutive treatment with EtOH did not change the serum-induced IL-6 release after stimulation with BAC-positive sera from HV (Fig. 2B). No significant differences could be observed for EtOH exposure after stimulation with sera from TP as well as with BAC- sera from HV.

PMN adhesion to A549 cells upon EtOH exposure after IL-1 β stimulation or treatment with sera from HV or TP ($\pm$ BAC)

In order to investigate the influence of alcohol on PMN adhesion capacity, A549 human

Fig. 2. Effects of alcohol exposure on IL-6 release upon (A) IL-1 β stimulation, or (B) stimulation with sera from trauma patients (TP) or healthy volunteers (HV). A: A549 lung epithelial cells were stimulated with IL-1 β (1 ng/ml) and then exposed to alcohol (ethanol, EtOH, 170 mM) for one hour. After the incubation period, supernatants were analyzed for IL-6 concentrations. B: Cells were stimulated sera from TP or HV, respectively, who were either positive or negative for alcohol (1.5%, BAC and no BAC, respectively) for 24 h as described in material and methods. Then, cells were exposed to EtOH for one hour, and supernatants were analyzed for IL-6. A: $n = 5$, B: $n = 4$. The data are presented as mean $\pm$ SD. *: $p < 0.05$ vs. all other groups; #: $p < 0.05$ vs. all HV groups.

Fig. 3. Effects of alcohol exposure on the adhesiveness of neutrophils to A549 cells upon (A) IL-1 β stimulation, or (B) stimulation with sera from trauma patients (TP) or healthy volunteers (HV). A: A549 lung epithelial cells were stimulated with IL-1 β (1 ng/ml) and then exposed to alcohol (ethanol, EtOH, 170 mM) for one hour. Isolated human neutrophils

were added and the adhesion capacity was determined. $n = 5$, *: $p < 0.05$. B: Cells were stimulated sera from TP or HV, respectively, who were either positive or negative for alcohol (1.5‰, BAC and no BAC, respectively) for 24 h as described in material and methods. Then, cells were exposed to EtOH for one hour and isolated human neutrophils were added, and the adhesion capacity was determined. The data are presented as mean $\pm$ SD. $n = 5$, *: $p < 0.05$; #: $p < 0.05$ vs. all.

lung epithelial cells were exposed to EtOH after stimulation with either IL-1 β or sera from HV and TP, respectively. PMN adhesion was significantly enhanced after IL-1 β stimulation compared with unstimulated control cells (132.3 ± 8.8 vs. 100%, $p < 0.05$, Fig. 3A). Consecutive treatment with EtOH afterwards significantly diminished PMN adhesion compared with stimulated cells (105.4 ± 14.5 vs. $132.3 \pm 8.8\%$, $p < 0.05$, Fig. 3A).

Stimulating A549 cells with sera from TP significantly increased PMN adhesion to A549 cells compared with stimulating cells with sera from HV (60.7 ± 9.7 vs. 36.2 ± 3.8 cells per high power field, $p < 0.05$, Fig. 3B). Comparable data were found for sera with positive as well as with negative BAC, although the effect was more distinct by using sera with negative BAC. Moreover, the difference in increased PMN adhesion rates in the experiments applying BAC-negative sera was significant as compared with applying BAC-positive sera in TP. Subsequent exposure to EtOH after stimulation with sera significantly decreased PMN adhesion rates in experiments applying HV sera as well as TP sera for stimulation of A549 cells. Summarized, EtOH present in the sera from intoxicated TP is associated with decreasing adhesion capacity of PMN to lung epithelial cells.

Influence of EtOH exposure of IL-1 β stimulated A549 cells on nuclear p50 and p52 levels with or without previous inhibition of specific NF- κ B pathways

In order to differentiate, which pathway of NF- κ B signaling is influenced by EtOH, nuclear levels of activated p50 as a keystone of the canonical, and of activated p52 as important element of the non-canonical NF- κ B pathway, were analyzed upon IL-1 β and consecutive EtOH exposure.

First, the impact of an isolated EtOH application was evaluated (Fig. 4A). EtOH treatment of A549 cells significantly increased nuclear p50 levels compared to untreated cells (305.2 ± 96.6 vs. 100%, $p < 0.05$, Fig. 4A). Nuclear levels of p52 remained unaltered by EtOH treatment of the cells in this experiment.

Stimulation with IL-1 β significantly increased nuclear levels of p50 as compared with unstimulated controls. Subsequent treatment with EtOH significantly diminished IL-1 β -induced nuclear p50 levels (34.9 ± 23.7 , $p < 0.05$, Fig. 4B). Stimulation with IL-1 β significantly increased nuclear levels of p52 as compared with unstimulated controls (371.9 ± 93.8 vs. 100%, $p < 0.05$, Fig. 4B). Subsequent treatment with EtOH did not alter IL-1 β -induced nuclear p52 levels (330.0 ± 50.0 Fig. 4B).

To further investigate the specific impact of EtOH on NF- κ B regulation, inhibition of the canonical (IKK-NBD) or the non-canonical (siRNA for NIK) pathway was performed. For

Fig. 4. Effects of alcohol exposure on p50 and p52 (A) and upon IL-1 β stimulation (B). A549 lung epithelial cells were stimulated with IL-1 β (1 ng/ml) and then exposed to alcohol (ethanol, EtOH, 170 mM) for one hour. After the incubation period, nuclear protein fractions were analyzed for p50 and p52 NF- κ B

activation as described in material and methods. In A, A549 were not stimulated with IL-1 β , but exposed to EtOH. The data are presented as mean $\pm$ SD. $n = 5$; *: $p < 0.05$; #: $p < 0.05$ vs. unstimulated control group in p52 experiments.

this experiment all cells were stimulated with IL-1 β in order to induced p50 as well as p52 activation as shown in Fig. 4B.

Inhibiting the canonical pathway of NF- κ B signaling by adding the specific IKK-NBD inhibitor resulted in significantly lower p50 levels upon IL-1 β stimulation (88.3 ± 10.4 vs. 179.8 ± 26.4 , $p < 0.05$, Fig. 5A). Additional exposure to EtOH further diminished the p50 activation as compared to IKK-NBD-inhibited cells, which were not exposed to EtOH (8.5 ± 8.2 , $p < 0.05$, Fig. 5A). Inhibiting the canonical pathway of NF- κ B signaling by adding the specific IKK-NBD inhibitor did not change the IL-1 β -induced p52 activation (279.5 ± 66.3 vs. 291.4 ± 50.6 , Fig. 5A). Additional exposure to EtOH did neither change the p52 activation as compared to IKK-NBD-inhibited cells, which were not exposed to EtOH (287.7 ± 17.4 , Fig. 5A).

Transfecting A549 cells with the specific siRNA against NIK resulted in significantly lower gene expression rates of NIK compared cells, which were transfected with control siRNA (53.6 ± 18.73 vs. 99.80 ± 1.5 , $p < 0.05$, $n = 5$, data not shown; Mann-Whitney U test). Inhibiting the non-canonical pathway of NF- κ B signaling by adding the specific siRNA against NIK did not markedly change the p50 activation upon IL-1 β stimulation (154.0 ± 28.1 vs. 156.5 ± 27.1 , $p < 0.05$, Fig. 5B). Additional exposure to EtOH diminished significantly the p50 activation as compared to siRNA-inhibited cells, which were not exposed to EtOH (77.7 ± 27.4 , $p < 0.05$, Fig. 5B). Inhibiting the non-canonical pathway of NF- κ B signaling by siRNA against NIK significantly reduced the IL-1 β -induced p52 activation compared with the IL-1 β not inhibited cells (58.4 ± 20.0 vs. 301.4 ± 42.9 , $p < 0.05$, Fig. 5B). Additional exposure to EtOH did not change the p52 activation as compared to siRNA-inhibited cells, which were not exposed to EtOH (76.7 ± 13.6 , Fig. 5B).

Inhibiting both, the canonical and the non-canonical pathway of NF- κ B signaling by adding the specific IKK-NBD inhibitor and specific siRNA against NIK, simultaneously, resulted in significantly lower p50 levels as well as p52 upon IL-1 β stimulation ($p < 0.05$, Fig. 5C).

Discussion

In the present study, we aimed at investigating the mechanism underlying alcohol's influence on specific NF- κ B signaling during the inflammatory response of human lung epithelial cells. Stimulating cells with either IL-1 β or sera from trauma patients induced a strong release of IL-6 as well as increased PMN adhesion to epithelial cells. Consecutive

Fig. 5. Effects of alcohol exposure on p50 and p52 upon (A) inhibition with the NEMO „binding domain“ (NBD) inhibitor IKK-NBD, (B) transfection with small interfering RNA (siRNA) directed against NIK (NIK siRNA), or (C) both, IKK-NBD and siRNA application. A549 lung epithelial cells were stimulated with IL-1 β (1 ng/ml). Then, either IKK-NBD or siRNA NIK were applied. Afterwards, cells were exposed to alcohol (ethanol, EtOH, 170 mM) for one hour, and nuclear protein fractions were analyzed for p50 and p52 NF- κ B activation as described in material and methods. The data are presented as mean $\pm$ SD. A: n = 4, B-C: n = 5, *: p < 0.05.

treatment with alcohol markedly decreased both, IL-6 release and PMN adherence. Regarding NF- κ B signaling, IL-1 β induced a significant activation of both, the canonical (p50) and non-canonical (p65) pathway. Stratification of alcohol's impact has shown that alcohol was able to significantly reduce p50, representing the canonical, but not p52 activation, representing the non-canonical pathway of NF- κ B signaling. Suppressing the canonical pathway by using a specific IKK-NBD inhibitor has synergistically with alcohol further reduced p50 activation, while p52 remained unaltered. On the other hand, specific inhibition of the non-canonical pathway by siRNA was not further changed by alcohol. Summarized, the anti-inflammatory effects of the acute alcohol use are apparently mediated *via* decreasing nuclear levels of activated p50 subunit involving the canonical NF- κ B signaling pathway in this model (Fig. 6).

In the acute period following severe trauma, levels of pro-inflammatory cytokines including IL-6, IL-1 β and others are increased above normal levels during the SIRS [13, 14]. Importantly, as critical players in the biological host-defense response to trauma, PMN become activated and are involved in the pathogenesis of infectious post-injury complications [38]. Moreover, trauma related acute lung injury involves rapid infiltration of immune cells with enzymatic degradation of matrix proteins, e.g. plasmin, causing loss of lung barrier function [39]. Therefore, in the present study, human lung epithelial cells were chosen as a model for the acute induced inflammation. In order to better reproduce the inflammatory state after severe trauma, in our cell culture model sera from TP with or without positive BAC were used for stimulation as well.

Sera from alcohol intoxicated trauma patients have shown a PMN modulating effect by reducing their adhesion to A549 cells. However, while the sera were stimulatory with regard to the PMN adhesion, they were not markedly altering the IL-6 secretory

Fig. 6. Alcohol (ethanol, EtOH) inhibits the canonical NF- κ B activation. IL-1 β induces both the activation of canonical and non-canonical NF- κ B signaling. Upon inhibition with the NEMO „binding domain“ (NBD) inhibitor IKK-NBD Peptide, degradation of the I κ B- α complex and subsequent p50 activation is prevented. Upon transfection with small interfering RNA (siRNA) directed against NIK (NIK siRNA), degradation of the IKK- α complex and subsequent p52 activation is prevented. EtOH inhibits the activation of p50, and therefore the canonical NF- κ B signaling.

potential of A549 cells. Here, the stimulatory and pro-inflammatory state upon trauma has been confirmed as in line with several clinical studies reporting elevated levels of pro-inflammatory cytokines after traumatic injury. Serum levels of IL-6 and IL-8 were shown to be significantly higher in patients suffering from blunt trauma in comparison to healthy controls [40]. Gebhard et al. as well as Mimasaka et al. found elevated levels of IL-6 as well as IL-8 in trauma patients, which correlated with injury severity [14, 41].

Summarized, several experimental and clinical studies demonstrated beneficial effects of alcohol, which were associated with lower rates of pneumonia after TBI or even improved mortality rates after trauma or haemorrhagic shock [9, 11, 16]. Importantly, conflictive data have highlighted that alcohol use was not associated with the increased incidence of infectious post-injury complications or mortality, or even alcohol increased the risk for development of in-hospital complications [42, 43]. The authors correlate their findings to possible anti-inflammatory characteristic of alcohol, since in numerous studies, alcohol was reported to significantly impact the immune response by suppressing the pro-inflammatory response. Our data confirm the anti-inflammatory influence of an alcohol use. Acute exposure to alcohol significantly diminished both, IL-1 β induced IL-6 release and PMN adhesion to lung epithelial A549 cells. Regarding the specificity of the applied inflammatory stimulus, previously, we have demonstrated alcohols' anti-inflammatory effects in A549 cells, which were stimulated with LPS from *E. coli* [34]. Although, we did not evaluate the underlying mechanisms in that preliminary study, we have observed significant anti-inflammatory potential of EtOH, when applied in the same dose and equal incubation period, which were used in the underlying study. In the referred study, LPS stimulation resulted in increased release of IL-8 and TGF- β as well as enhanced neutrophil adhesion to treated A549 cells. EtOH has lowered IL-8 and neutrophil adhesion. Furthermore, after incubation of phorbol-12-myristate-13-acetate (PMA)-stimulated A549 cells, EtOH (170 mM) significantly reduced IL-6 release compared to untreated PMA-stimulated cells (data not shown). Therefore, EtOH

exerts significant and relevant anti-inflammatory potential. With regard to other cell types, IL-8-reducing influence of EtOH in IL-1 β -stimulated human Chang liver (CL) cells and in another pulmonary cell line, A427, after their stimulation with IL-1 β has been observed as well (data not shown). Taken together, an acute exposure of various cellular entities to EtOH upon their inflammatory stimulation *in vitro* results in anti-inflammatory effects. Goral et al. reported decreased IL-6 synthesis in macrophages of mice, who received a single dose alcohol beforehand [44]. Exposing monocytes to acute alcohol (25-100 mM) impaired TNF- α , IL-6 and IL-1 β production but elevated IL-10 levels *in vitro* [17, 45]. Additionally, clinical studies revealed acute alcohol use to decrease post-traumatic cytokine levels in trauma patients [8, 16].

Further analyses of the mechanistical pathways are in line with the data from others, that alcohols' mode of action has been linked to the deactivation of the transcription factor NF- κ B, which is triggering the systemic and local propagation of inflammation [19, 27]. In addition, we could demonstrate that IL-1 β increased both, p50 as well as p52 activation in human lung epithelial cells. Consecutive exposure of the stimulated cells to alcohol reduced p50 activation, while p52 activation remained unaltered. Specific inhibition of either the canonical (p50) or the non-canonical (p52) NF- κ B signaling uncovered that alcohol reduces specifically the canonical NF- κ B signaling in this model. The general impact on the NF- κ B suppression is supported by other studies. For example, alcohol was demonstrated to prevent LPS-induced nuclear translocation of p65 and p50 in human monocytes [46]. Also, acute exposure to alcohol for one hour resulted in decreased phosphorylation of p65 in human blood monocytes [47]. Regarding the specific NF- κ B signaling, Mandrekar et al. exposed human blood monocytes to 25 mM EtOH for one hour, as comparable to our setting, and found significantly elevated nuclear p50-levels matching decreased cytoplasmic p50-levels [19]. Mandrekar et al. have reported before, that *in vitro* acute EtOH treatment of monocytes in the absence of bacterial stimulus increased NF- κ B binding [19]. Moreover, in the presence of a bacterial stimulus, such as LPS, acute EtOH exposure of monocytes decreased NF- κ B DNA binding, which was responsible for transactivation of target genes [46]. Summarized, our data indicate at possibly similar mechanisms in lung epithelial cells. While p50 activation was increased by acute exposure of those cells to ethanol in the absence of an inflammatory stimulus, in the presence of IL-1 β , ethanol decreased specifically p50 activation. Taken together, acute exposure to EtOH may differentially affect the NF- κ B regulatory subunits, depending on the inflammatory state of the cell. The underlying mechanisms are yet to be investigated. Mandrekar's group assumed that alcohol rather leads to the formation of p50/p50 homodimers. Since the p50/p50 homodimer has been described to exert inhibiting effects on NF- κ B regulated gene expression, this might be in line with alcohol's assumed immune-suppressive properties. However, both mechanisms, p50/p50 upregulation on the one hand, and p65/p50 downregulation on the other hand, might be involved in alcohol's inhibiting effect on NF- κ B controlled gene expression. Balancing between these mechanisms might be dose-dependent. While Mandrekar used 25 mM, we use alcohol in a dose of 170 mM, which is a significantly higher dose. Dose dependent effects of alcohol have been reported previously by others and our own group [35, 48]. In an *in vitro* study involving A549 cells, our group found alcohol to dose-dependently (85 mM and 170 mM) affect IL-8 release after IL-6 stimulation [35].

Regarding a possible clinical approach, NF- κ B as well as its inhibition have been under investigation in several inflammatory diseases. Torices et al. found a TLR10 missense (I437T) to be associated with a more severe course of inflammatory disease in patients with rheumatoid arthritis (RA) [49]. This effect is so far interesting, since this TLR10 variant lacked the inhibitory capacity of NF- κ B signalling, as shown by increased transcriptional activity of NF- κ B compared to wild-type [49]. In macrophages, which play an important role in the RA pathogenesis, NF- κ B subunits p50 and p65 were present in the majority of cells in patients suffering from RA, supporting the concept of NF- κ B promoting rheumatoid synovialitis *via* macrophage-derived cytokines [50, 51]. Here, IL-1 β application induced a translocation of the p65, p50, and c-rel NF- κ B subunits into the nucleus [52]. Similarly, in patients suffering from

chronic kidney disease, increased muscle inflammation has been associated with increased NF- κ B expression [53]. Summarized, these findings clearly indicate at the therapeutical relevance of both, the pro-inflammatory IL-1 β as well as NF- κ B inhibition in inflammatory conditions. Patients undergoing trauma exert a pro-inflammatory response, which has been linked to detrimental outcome [13]. With regard to alcohol, several clinical studies report beneficial effects of an acute alcohol use prior traumatic brain injury (TBI). Even more, lower mortality rates in TBI patients with low and moderate blood alcohol concentrations (BAC) were reported [54, 55]. Interestingly, positive BAC has been associated with lower leukocyte counts as well as systemic IL-6 levels in severe TBI or in major trauma patients [8, 16]. However, contradictory data showing deteriorating effects of an alcohol intoxication also on patients' outcome have been reported as well [43, 56, 57]. Nonetheless, the therapeutic use of alcohol is surely to be assessed critically because of its well-described side effects, notably regarding the central nervous system, e.g. neuronal loss [58]. Yet, the side effects have been usually described for a prolonged exposure to high dose of alcohol, neglecting that a low dose in an acute setting of acute inflammation may provide anti-inflammatory benefits. To avoid the discussion on alcohol application, due to known pathological mechanisms, new targets, as described in this study as well, may be therapeutically used. As one of those, ethyl pyruvate (EP) has shown a comparable mode of action as alcohol, and is currently being investigated *in vitro* as well as *in vivo* in models of acute inflammation [35, 36].

Further studies are warranted involving longer exposure times to alcohol in order to uncover the effects of its prolonged use. Additionally, only one dose of alcohol was used here. Since dose-dependent effects of alcohol have been described above, different doses should be implemented in further studies.

Conclusion

Summing up, we have demonstrated a decrease of pro-inflammatory IL-6 release from human lung epithelial A549 cells upon their stimulation with IL-1 β and following acute exposure to alcohol. Furthermore, stimulating cells with sera from trauma patients increased PMN adhesion to epithelial cells, and the consecutive treatment with alcohol markedly decreased this effect. With regard to the specific mechanism, IL-1 β induced a strong activation of both, canonical (p50) and non-canonical (p65) pathway. Nonetheless, due to diminishing p50, representing the canonical, but not altering p52 activation, representing the non-canonical NF- κ B signaling, alcohol's mode of action for its anti-inflammatory effects is apparently mediated *via* decreasing the canonical NF- κ B signaling pathway in our model.

Abbreviations

BAC (blood alcohol concentration); Ca (calcium); CO₂ (carbon dioxide); DFG (Deutsche Forschungsgemeinschaft, German Research Foundation); DNA (Deoxyribonucleic acid); ELISA (enzyme-linked immunosorbent assay); EtOH (ethanol); FCS (fetal calf serum); g (earth's gravitational acceleration); HV (healthy volunteers); IKK-NBD (I κ B protein kinase-NEMO binding domain); IL (Interleukin); l (liter); mg (milligram, magnesium); mm (millimeter); mM (millimolar); min (minute); ml (milliliter); NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells); p (p-value); PMN (polymorphonuclear leukocytes); pg (picogram); RNA (Ribonucleic acid); s (second); sem (standard error of the mean); si (small interfering); SIRS (systemic inflammatory response syndrome); STROBE (strengthening the reporting of observational studies in epidemiology); TBI (traumatic brain injury); TNF (tumor necrosis factor); TP (trauma patients); U (unit); *vs* (*versus*); °C (Celsius); μ (micro); μ l (microliter); % (percent); ‰ (per mille).

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Disclosure Statement

The authors have nothing to declare. The authors have no conflicts of interest.

References

- Alberdi F, Garcia I, Atutxa L, Zabarte M, Trauma, Neurointensive Care Work Group of the S: Epidemiology of severe trauma. *Med Intensiva* 2014;38:580-588.
- Bardenheuer M, Obertacke U, Waydhas C, Nast-Kolb D: [Epidemiology of the severely injured patient. A prospective assessment of preclinical and clinical management. AG Polytrauma of DGU]. *Unfallchirurg* 2000;103:355-363.
- Lefering R, Paffrath T: [Reality of care based on the data from the Trauma Registry of the German Society of Trauma Surgery]. *Unfallchirurg* 2012;115:30-32.
- Pickett W, Schmid H, Boyce WF, Simpson K, Scheidt PC, Mazur J, Molcho M, King MA, Godeau E, Overpeck M, Aszmann A, Szabo M, Harel Y: Multiple risk behavior and injury: an international analysis of young people. *Arch Pediatr Adolesc Med* 2002;156:786-793.
- Field CA, Claassen CA, O'Keefe G: Association of alcohol use and other high-risk behaviors among trauma patients. *J Trauma* 2001;50:13-19.
- Rivara FP, Jurkovich GJ, Gurney JG, Seguin D, Fligner CL, Ries R, Raisys VA, Copass M: The magnitude of acute and chronic alcohol abuse in trauma patients. *Arch Surg* 1993;128:907-912; discussion 912-903.
- Jurkovich GJ, Rivara FP, Gurney JG, Fligner C, Ries R, Mueller BA, Copass M: The effect of acute alcohol intoxication and chronic alcohol abuse on outcome from trauma. *JAMA* 1993;270:51-56.
- Relja B, Menke J, Wagner N, Auner B, Voth M, Nau C, Marzi I: Effects of positive blood alcohol concentration on outcome and systemic interleukin-6 in major trauma patients. *Injury* 2016;47:640-645.
- Hadjibashi AA, Berry C, Ley EJ, Bukur M, Mirocha J, Stolpner D, Salim A: Alcohol is associated with a lower pneumonia rate after traumatic brain injury. *J Surg Res* 2012;173:212-215.
- Lustenberger T, Inaba K, Barmparas G, Talving P, Plurad D, Lam L, Konstantinidis A, Demetriades D: Ethanol intoxication is associated with a lower incidence of admission coagulopathy in severe traumatic brain injury patients. *J Neurotrauma* 2011;28:1699-1706.
- Relja B, Hohn C, Bormann F, Seyboth K, Henrich D, Marzi I, Lehnert M: Acute alcohol intoxication reduces mortality, inflammatory responses and hepatic injury after haemorrhage and resuscitation *in vivo*. *Br J Pharmacol* 2012;165:1188-1199.
- Lenz A, Franklin GA, Cheadle WG: Systemic inflammation after trauma. *Injury* 2007;38:1336-1345.
- Wutzler S, Lustenberger T, Relja B, Lehnert M, Marzi I: [Pathophysiology of multiple trauma : intensive care medicine and timing of treatment]. *Chirurg* 2013;84:753-758.
- Gebhard F, Pfetsch H, Steinbach G, Strecker W, Kinzl L, Bruckner UB: Is interleukin 6 an early marker of injury severity following major trauma in humans? *Arch Surg* 2000;135:291-295.
- Nelson S, Bagby GJ, Bainton BG, Summer WR: The effects of acute and chronic alcoholism on tumor necrosis factor and the inflammatory response. *J Infect Dis* 1989;160:422-429.
- Wagner N, Akbarpour A, Mors K, Voth M, Stormann P, Auner B, Lehnert M, Marzi I, Relja B: Alcohol Intoxication Reduces Systemic Interleukin-6 Levels and Leukocyte Counts After Severe TBI Compared With Not Intoxicated TBI Patients. *Shock* 2016;46:261-269.
- Szabo G, Mandrekar P, Girouard L, Catalano D: Regulation of human monocyte functions by acute ethanol treatment: decreased tumor necrosis factor- α , interleukin-1 β and elevated interleukin-10, and transforming growth factor- β production. *Alcohol Clin Exp Res* 1996;20:900-907.
- Boe DM, Nelson S, Zhang P, Bagby GJ: Acute ethanol intoxication suppresses lung chemokine production following infection with *Streptococcus pneumoniae*. *J Infect Dis* 2001;184:1134-1142.

- 19 Mandrekar P, Catalano D, Szabo G: Alcohol-induced regulation of nuclear regulatory factor-kappa beta in human monocytes. *Alcohol Clin Exp Res* 1997;21:988-994.
- 20 Singh AK, Jiang Y: Differential activation of NF kappa B/RelA-p50 and NF kappa B/p50-p50 in control and alcohol-drinking rats subjected to carrageenin-induced pleurisy. *Mediators Inflamm* 2004;13:255-262.
- 21 Gilmore TD: Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 2006;25:6680-6684.
- 22 Tak PP, Firestein GS: NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 2001;107:7-11.
- 23 Varfolomeev E, Vucic D: Intracellular regulation of TNF activity in health and disease. *Cytokine* 2016
- 24 Brasier AR: The NF-kappaB regulatory network. *Cardiovasc Toxicol* 2006;6:111-130.
- 25 Hoesel B, Schmid JA: The complexity of NF-kappaB signaling in inflammation and cancer. *Mol Cancer* 2013;12:86.
- 26 Sun SC: Non-canonical NF-kappaB signaling pathway. *Cell Res* 2011;21:71-85.
- 27 Mandrekar P, Catalano D, White B, Szabo G: Moderate alcohol intake in humans attenuates monocyte inflammatory responses: inhibition of nuclear regulatory factor kappa B and induction of interleukin 10. *Alcohol Clin Exp Res* 2006;30:135-139.
- 28 Driessler F, Venstrom K, Sabat R, Asadullah K, Schottelius AJ: Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. *Clin Exp Immunol* 2004;135:64-73.
- 29 Elsharkawy AM, Oakley F, Lin F, Packham G, Mann DA, Mann J: The NF-kappaB p50:p50:HDAC-1 repressor complex orchestrates transcriptional inhibition of multiple pro-inflammatory genes. *J Hepatol* 2010;53:519-527.
- 30 Robin MA, Demeilliers C, Sutton A, Paradis V, Maisonneuve C, Dubois S, Poirel O, Letteron P, Pessayre D, Fromenty B: Alcohol increases tumor necrosis factor alpha and decreases nuclear factor-kappaB to activate hepatic apoptosis in genetically obese mice. *Hepatology* 2005;42:1280-1290.
- 31 Maraslioglu M, Oppermann E, Blattner C, Weber R, Henrich D, Jobin C, Schleucher E, Marzi I, Lehnert M: Chronic ethanol feeding modulates inflammatory mediators, activation of nuclear factor-kappaB, and responsiveness to endotoxin in murine Kupffer cells and circulating leukocytes. *Mediators Inflamm* 2014;2014:808695.
- 32 Wang F, Yang JL, Yu KK, Xu M, Xu YZ, Chen L, Lu YM, Fang HS, Wang XY, Hu ZQ, Li FF, Kan L, Luo J, Wang SY: Activation of the NF-kappaB pathway as a mechanism of alcohol enhanced progression and metastasis of human hepatocellular carcinoma. *Mol Cancer* 2015;14:10.
- 33 von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP, Initiative S: The Strengthening of Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. *Int J Surg* 2014;12:1495-1499.
- 34 Relja B, Omid N, Konradowitz K, Jurida K, Oppermann E, Stormann P, Werner I, Juengel E, Seebach C, Marzi I: Decreased inflammatory responses of human lung epithelial cells after ethanol exposure are mimicked by ethyl pyruvate. *Mediators Inflamm* 2014;2014:781519.
- 35 Relja B, Omid N, Schaible A, Perl M, Meier S, Oppermann E, Lehnert M, Marzi I: Pre- or post-treatment with ethanol and ethyl pyruvate results in distinct anti-inflammatory responses of human lung epithelial cells triggered by interleukin-6. *Mol Med Rep* 2015;12:2991-2998.
- 36 Relja B, Omid N, Wagner N, Mors K, Werner I, Juengel E, Perl M, Marzi I: Ethanol, ethyl and sodium pyruvate decrease the inflammatory responses of human lung epithelial cells via Akt and NF-kappaB *in vitro* but have a low impact on hepatocellular cells. *Int J Mol Med* 2016;37:517-525.
- 37 Relja B, Horstmann JP, Konradowitz K, Jurida K, Schaible A, Neunaber C, Oppermann E, Marzi I: Nlrp1 inflammasome is downregulated in trauma patients. *J Mol Med (Berl)* 2015;93:1391-1400.
- 38 Hietbrink F, Koenderman L, Rijkers G, Leenen L: Trauma: the role of the innate immune system. *World J Emerg Surg* 2006;1:15.
- 39 Wu X, Schwacha MG, Dubick MA, Cap AP, Darlington DN: Trauma-Related Acute Lung Injury Develops Rapidly Irrespective of Resuscitation Strategy in the Rat. *Shock* 2016
- 40 Volpin G, Cohen M, Assaf M, Meir T, Katz R, Pollack S: Cytokine levels (IL-4, IL-6, IL-8 and TGFbeta) as potential biomarkers of systemic inflammatory response in trauma patients. *Int Orthop* 2014;38:1303-1309.
- 41 Mimasaka S, Funayama M, Hashiyada M, Nata M, Tsunenari S: Significance of levels of IL-6 and IL-8 after trauma: a study of 11 cytokines post-mortem using multiplex immunoassay. *Injury* 2007;38:1047-1051.
- 42 Beydoun H, Teel A, Crowder C, Khanal S, Lo BM: Past blood alcohol concentration and injury in trauma center: propensity scoring. *J Emerg Med* 2014;47:387-394.

- 43 Pandit V, Patel N, Rhee P, Kulvatunyou N, Aziz H, Green DJ, O'Keeffe T, Zangbar B, Tang A, Gries L, Friese RS, Joseph B: Effect of alcohol in traumatic brain injury: is it really protective? *J Surg Res* 2014;190:634-639.
- 44 Goral J, Kovacs EJ: *In vivo* ethanol exposure down-regulates TLR2-, TLR4-, and TLR9-mediated macrophage inflammatory response by limiting p38 and ERK1/2 activation. *J Immunol* 2005;174:456-463.
- 45 Mandrekar P, Catalano D, Girouard L, Szabo G: Human monocyte IL-10 production is increased by acute ethanol treatment. *Cytokine* 1996;8:567-577.
- 46 Mandrekar P, Catalano D, Szabo G: Inhibition of lipopolysaccharide-mediated NF κ B activation by ethanol in human monocytes. *Int Immunol* 1999;11:1781-1790.
- 47 Mandrekar P, Jeliaskova V, Catalano D, Szabo G: Acute alcohol exposure exerts anti-inflammatory effects by inhibiting IkappaB kinase activity and p65 phosphorylation in human monocytes. *J Immunol* 2007;178:7686-7693.
- 48 Goral J, Karavitis J, Kovacs EJ: Exposure-dependent effects of ethanol on the innate immune system. *Alcohol* 2008;42:237-247.
- 49 Torices S, Julia A, Munoz P, Varela I, Balsa A, Marsal S, Fernandez-Nebro A, Blanco F, Lopez-Hoyos M, Martinez-Taboada V, Fernandez-Luna JL: A functional variant of TLR10 modifies the activity of NF κ B and may help predict a worse prognosis in patients with rheumatoid arthritis. *Arthritis Res Ther* 2016;18:221.
- 50 Handel ML, McMorrow LB, Gravalles EM: Nuclear factor-kappa B in rheumatoid synovium. Localization of p50 and p65. *Arthritis Rheum* 1995;38:1762-1770.
- 51 Yanni G, Whelan A, Feighery C, Bresnihan B: Synovial tissue macrophages and joint erosion in rheumatoid arthritis. *Ann Rheum Dis* 1994;53:39-44.
- 52 Crofford LJ, Tan B, McCarthy CJ, Hla T: Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. *Arthritis Rheum* 1997;40:226-236.
- 53 Verzola D, Bonanni A, Sofia A, Montecucco F, D'Amato E, Cademartori V, Parodi EL, Viazzi F, Venturelli C, Brunori G, Garibotto G: Toll-like receptor 4 signalling mediates inflammation in skeletal muscle of patients with chronic kidney disease. *J Cachexia Sarcopenia Muscle* 2017;8:131-144.
- 54 Berry C, Salim A, Alban R, Mirocha J, Margulies DR, Ley EJ: Serum ethanol levels in patients with moderate to severe traumatic brain injury influence outcomes: a surprising finding. *Am Surg* 2010;76:1067-1070.
- 55 Tien HC, Tremblay LN, Rizoli SB, Gelberg J, Chughtai T, Tikuisis P, Shek P, Brenneman FD: Association between alcohol and mortality in patients with severe traumatic head injury. *Arch Surg* 2006;141:1185-1191; discussion 1192.
- 56 Wang H, Zhang Y, Bai R, Wang M, Du S: Baicalin Attenuates Alcoholic Liver Injury through Modulation of Hepatic Oxidative Stress, Inflammation and Sonic Hedgehog Pathway in Rats. *Cell Physiol Biochem* 2016;39:1129-1140.
- 57 Zhang J, Wu J, Zeng W, Yao K, Zu H, Zhao Y: Function of Thymosin Beta-4 in Ethanol-Induced Microglial Activation. *Cell Physiol Biochem* 2016;38:2230-2238.
- 58 Walker DW, Barnes DE, Zornetzer SF, Hunter BE, Kubanis P: Neuronal loss in hippocampus induced by prolonged ethanol consumption in rats. *Science* 1980;209:711-713.