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ALCOHOL-INDUCED INFLAMMASOME ACTIVATION IN THE INTESTINE, LIVER AND BRAIN

PhD thesis

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To my Dearest Family

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2. THE LIST OF ABBREVIATION

AIM2: absent in melanoma 2
ALD: alcoholic liver disease
AP-1: activator protein-1
ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP: adenosine triphosphate
BSA: bovine serum albumin
cDNA: complementary deoxyribonucleic acid
Ctr: control
DAMP: danger-associated molecular pattern
dI-dC: poly(deoxyinosinic-deoxycytidylic) acid
DSS: dextran sodium sulfate
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
EMSA: electrophoretic mobility shift assay
EtOH: Lieber-DeCarli ethanol diet
FBS: fetal bovine serum
GFAP: glial fibrillary acidic protein
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1: high-mobility group box-1
HRP: horseradish peroxidase
Iba1: ionized calcium-binding adaptor molecule-1
IBD: inflammatory bowel disease
IL: interleukin
IL-1R: IL-1 receptor
IL-1Ra: IL-1R antagonist
IP: immunoprecipitation
i.p.: intraperitoneal
KCl: potassium chloride
KO: knockout

LPS: lipopolysaccharide, endotoxin

LRR: leucine-rich repeat

MCP-1: monocyte chemotactic protein-1

miRNA: micro-RNA

mRNA: messenger RNA

MyD88: myeloid differentiation primary response gene (88)

Na3VO4: sodium orthovanadate

Na4P2O7: sodium pyrophosphate decahydrate

NACHT: NAIP (neuronal apoptosis inhibitor protein), C2TA (major histocompatibility complex class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)

NaF: sodium fluoride

NF-κB: nuclear factor-κB

NLRP1: NACHT, LRR and PYD domains-containing protein-1

NLRP3: NACHT, LRR and PYD domains-containing protein-3

NLRP6: nucleotide-binding oligomerization domain-like receptor-6

NLRC4: nucleotide-binding oligomerization-like receptor family caspase recruitment domain-containing protein 4

PAMP: pathogen associated molecular patterns

PBS: phosphate buffered saline

PMSF: phenylmethylsulfonyl fluoride

PSC: primary sclerosing cholangitis

PRR: pattern recognition receptor

PYD: pyrin domain

RAGE: receptor for advanced glycation end products

Reg3b: regenerating islet-derived protein III-β

RIPA: radioimmunoprecipitation assay

RNA: ribonucleic acid

rIL-1Ra: recombinant IL-1Ra

ROS: reactive oxygen species

SEM: standard error of the mean

TBS: tris buffered saline

TLR: Toll-like receptor

TNFα: tumor necrosis factor-α

UC: ulcerative colitis

v/v: volume per volume

WHO: World Health Organization

WT: wild type

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3. INTRODUCTION

3.1. Burden of alcohol-related diseases

Based on a WHO report from 2012, 5.9% of all global deaths (3.3 million) were attributable to alcohol consumption [1]. Globally, harmful use of alcohol has been ranked among the top five risk factors for diseases and death [2]. Alcohol consumption is associated with the risk of developing a range of health problems including neuropsychiatric conditions (i.e. alcohol dependence), gastrointestinal diseases (liver cirrhosis), malignancies (colorectal cancers), cardiovascular diseases (hemorrhagic stroke), pre-term birth complications, and injuries (such as road accidents) [3]. There is an estimate that alcohol use plays a causal role in almost 200 diseases [3]. Furthermore, regarding its vastness, international classification of disease (ICD-10) dedicated a code for mental and behavioral disorders due to alcohol. The amount of alcohol consumption, mainly containing ethanol, the drinking patterns and rarely the quality of alcohol are the major determinants of harmful outcome [4]. Worldwide, 13.5 gr/day pure alcohol (equal to 6.5 liters) is consumed by drinkers aged 15 and more of which 16% have pattern of heavy drinking episodes [4]. There is a large geographical variation in alcohol related morbidity, and Europe has the highest alcohol-attributable fraction rate [4]. Based on a 2010 report, Hungary was categorized among the countries with high consumption of alcohol per capita (≥12.5 liters) (Figure 1) [5].

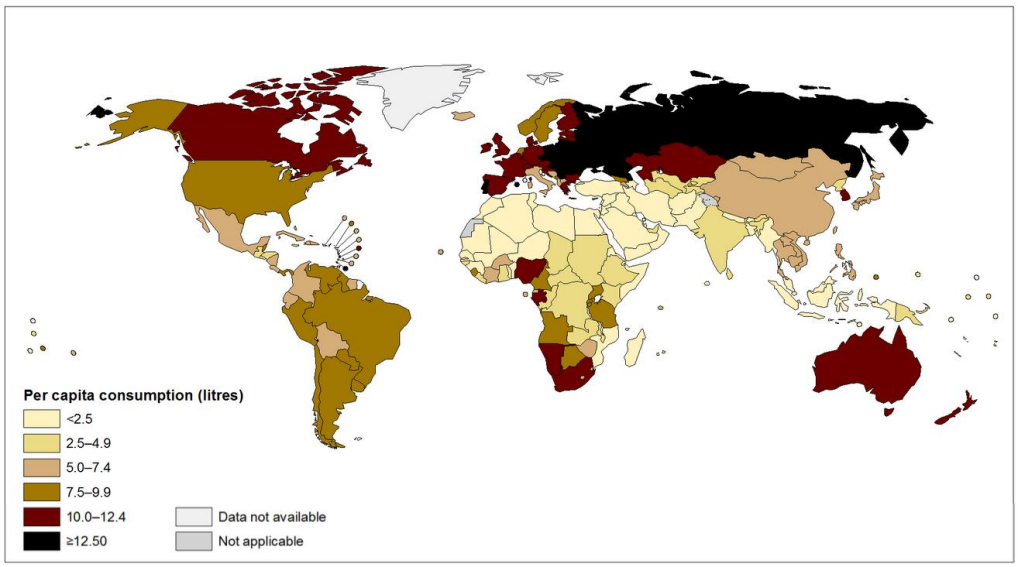

Figure 1. Total alcohol per capita (15+ years) consumption, in liters of pure alcohol, 2010 [5] (Reprinted with permission from publisher's URL: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_consumption_percapita_2010.png; date accessed 10-07-2015).

There is a gender difference in global death related to alcohol, as an epidemiologic study from Hungary in 2003 reported 27.5 and 23.2% casual, 7.7 and 31.3% mild, 2.5 and 17.7% severe alcohol consumption among female and male, respectively [6]. Overall based on a 2010 estimate, prevalence of alcohol use associated diseases and dependence in Hungary was 17.7% (31% for male, 6% for female) [7]. Increase in alcohol consumption per capita is strongly correlated with increase in alcohol-attributable deaths [8, 9]. In Hungary in 2010 alcohol-attributable death was 13.3% in spite of the decrease from 17.1% in 2005, it is still among the highest (Figure 2) [10].

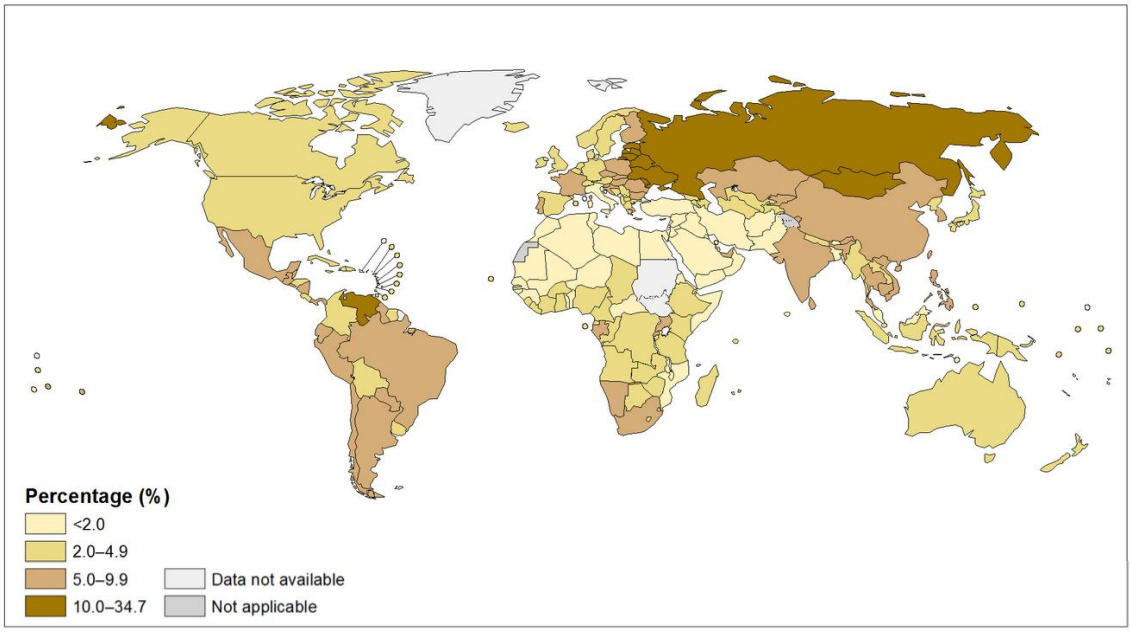

Figure 2. World: Proportion of all deaths attributed to alcohol (alcohol-attributable fractions, %; all ages), 2012 [10]. (Reprinted with permission from publisher's URL: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_deaths_2012.png; date accessed 10-07-2015).

The first line of treatment is prevention, cessation of alcohol use and proper nutrition. However, once alcoholic organ damage has emerged, even abstinence and our limited non-specific therapeutic options might be ineffective in preventing progression of the disease or death [11]. As a result of easy (even natural) availability, addictive effect and harmful consequences of alcohol our effort should focus on finding a safe, effective and economically beneficial treatment for alcohol related diseases. In order to achieve a refined treatment, understanding the mechanism, cellular and subcellular changes as a result of alcohol consumption is crucial.

3.2. Effects of alcohol

3.2.1. General

Long term use of alcohol generally affects major organ systems – digestive-, circulatory-, nervous-, endocrine-, immune system, etc. The effects include inflammatory changes and carcinogenesis. Furthermore alcohol intake affects the next generation as prevalence of fetal alcohol spectrum disorder as a result of parental alcohol intake is around 1% [12].

The impact of alcohol is not limited to the increased incidence of certain disease, it also affects the course and outcome of other diseases. The mechanism by which alcohol harms organs and tissues is not thoroughly understood, its range varies from direct irritation, conversion to other toxic agents such as acetaldehyde, induction of inflammatory by-products, acting as solvent for other toxic agents, slowing the detoxification of other processes, lowering level of nutrients (such as folate) to completely unknown pathways [13].

3.2.2 Intestine

During alcohol ingestion the oral mucosa, esophagus, stomach and small intestine are exposed to high concentrations of alcohol [14], which leads to the most severe direct and pure effect of alcohol seen among organs. Alcohol impairs the gastrointestinal barrier function by inducing inflammation, epithelial cell apoptosis and necrosis [14-18]. Furthermore, bacterial overgrowth, decreased antimicrobial peptide level and impaired epithelial tight junction is associated with chronic alcohol use in the proximal small intestine [19-21]. Affected intestinal barrier allows systemic translocation of pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) [22]. PAMPs and DAMPs activate pro-inflammatory pathways via pattern-recognition receptors (PRRs) [22]. The increased amount of bacterial cell wall, endotoxin / lipopolysaccharide (LPS), in the serum is suggestive of impaired gut barrier function, which is observed in animal models of alcoholism as well as patients with chronic alcohol use [23, 24].

Inflammation might occur within the intestinal wall following the loss of barrier integrity, or as a direct effect of alcohol, which could then contribute to further loss of

function. Duodenal inflammation is observed in alcoholic individuals, including increased presence of B lymphocytes [15, 25].

Nuclear factor-κB (NF-κB) activation and as a consequence tumor necrosis alpha (TNFα) production has been suggested to be part of the pathogenesis of intestinal inflammatory processes [26, 27]. Supporting this theory, dysbiosis-induced intestinal inflammation causes loss of barrier function via TNFα receptor which then leads to alcoholic liver disease in mice [28].

Inflammatory cytokine, interleukin-1β (IL-1β), produced by macrophages, contributes to immune tolerance of the intestine via regulating expansion of regulatory T cells through macrophages and dendritic cells [29]. Additionally, NF-κB pathway mediated IL-1β release is associated with increased permeability in Caco2 cell line [30]. Inflammasome activation occurs in dextran sodium sulfate (DSS) induced colitis model in mice and is observed to contribute to the healing process of the intestinal barrier via the maturation of IL-18 which is similarly produced to IL-1β [31].

So far there is no clear data on inflammasome activation, IL-1β or TNFα production or their possible role in alcohol induced inflammatory changes in the small intestine.

3.2.3. Liver

Alcohol and alcohol-induced P/DAMPs have easy access to hepatocytes, stellate and Kupffer cells in the liver because the sinusoids do not form a strong barrier. The major metabolism of alcohol occurs in the liver, therefore the cellular effects of harmful derivates, including acetaldehyde is mainly expected in the liver of alcoholics [32].

Consequences follow as chronic alcohol intake frequently induces steatohepatitis, cirrhosis and can result in hepatic cancer. The most common feature, steatohepatitis consists of fatty transformation and inflammatory changes [33]. Both Kupffer cells and hepatocytes are involved in the process of alcohol-induced inflammation where excess pro-inflammatory cytokine (TNFα and IL-1β) production is observed [34]. Chronic alcohol use induces hepatocyte apoptosis and necrosis [35], which can facilitate the inflammatory changes. Furthermore, long-term alcohol exposure-induced inflammation is associated with fibrotic changes of the liver mediated by stellate cells [34]. Fibrosis and chronic inflammation is associated with increased risk of hepatocellular carcinoma formation [36]. Cellular changes, inflammasome activation, pro-inflammatory cytokine

secretion, reactive oxygen species (ROS) production, apoptosis, changes in micro-RNA (miRNA) expression are all part of the underlying mechanism [34, 37, 38].

3.2.4. Brain

Alcohol can easily pass through the blood-brain barrier, including the endothelial sheet as well as the astrocyte wrap due to its small size and lipophilic property [39]. Neural cell apoptosis, defective neuroregeneration, microglia- and astrocyte activation are some of the cellular hallmarks of alcohol induced changes in the brain [40-44]. Primarily alcohol alters behavior via changes in neurotransmission, further alcoholism leads to dementia, ataxia via neurodegeneration and possibly neuroinflammation, which are mediated by innate immune responses [45, 46]. Long-term alcohol intake results in neuroinflammation and neurodegeneration both in human and animals [43, 44, 47, 48]**.** Chronic alcohol ingestion increases expression of monocyte chemotactic protein-1 (MCP-1), TNFα, and caspase-3 in the brain [43, 44, 47]. Inflammatory cytokines affect neurons and are associated with disease symptoms [49]. Toll-like receptor-4 (TLR4) signaling pathway plays an important role in the alcohol-induced production of inflammatory cytokines in different regions of the brain [46]. Cellular changes, pro-inflammatory cytokine secretion, ROS production, apoptosis, changes in miRNA expression are all part of the underlying mechanism of alcohol induced neuroinflammation and -degeneration.

Cerebellum although affected by alcohol consumption [50], there is no clear data on inflammasome activation, IL-1β or TNFα production or their possible role in alcohol induced inflammatory changes in this brain region.

3.3. Intestine-liver-brain axis

3.3.1. General considerations

The portal circulation and the biliary system makes the interaction between intestine and the liver bidirectionally strong. Circulation makes an easy channel to communicate between organs. Peripheral nervous system allows the brain to receive and act fast using neural reflexes.

The significance of gut-liver interaction is suggested by the evidences that hepatobiliary involvement in inflammatory bowel disease (IBD) is high; 70-80% of primary

sclerosing cholangitis (PSC) patients have ulcerative colitis (UC) [51]; alcoholic liver disease (ALD) patients have leaky gut and endotoxemia [52]; the altered intestinal microbiome is associated with various liver diseases [53].

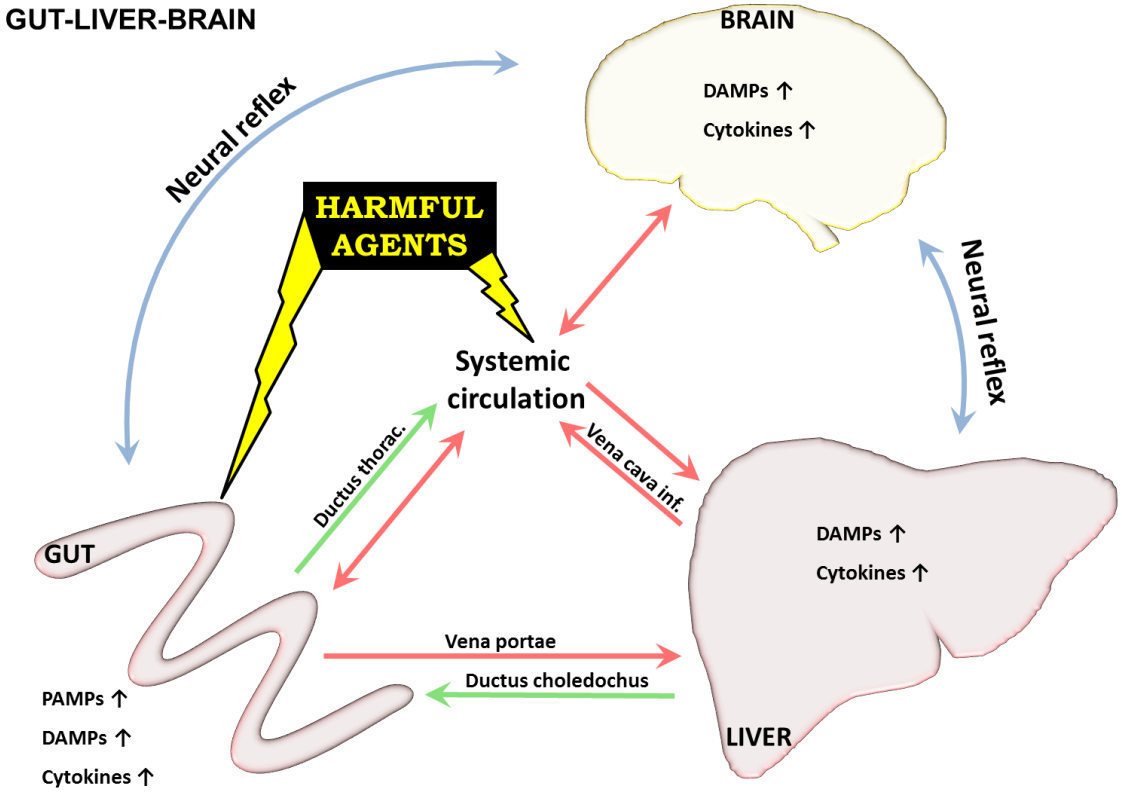

Figure 3. Intestine-liver-brain axis. Most harmful agents enter via the GI system, skin or lung and are distributed through systemic and/or lymph circulation inducing inflammation in different organs including the entering site. The harmful agents can go through first-pass metabolism in the liver and in original or metabolite form can be excreted to the bile entering the intestinal system. Similarly, but to a lesser extent kidney/lung/skin/GI tract, etc. have excretory ability. Neural reflexes quickly deliver signals to the brain and back to the organs, fast responding to the problem. DAMPs and cytokines induced in the inflammatory process can enhance inflammation at the site of origin or can use similar pathways to PAMPs and reach other organs to affect.

The involvement of brain in disease condition results in altered behavior, fatigue, anorexia, loss of appetite, occasionally fever which are caused by the circulating PAMPs and DAMPs including cytokines like TNFα and IL-1β of the affected organ, whereas others are results of direct neuronal interactions [54, 55]. Motor and secretory function of intestine or neutrophil accumulation of the liver can all be affected [54-57]. Consecutively the overall performance of the individual changes [54-57]. Emerging evidences suggest that intestinal-brain interactions play crucial role in the pathogenesis of IBS and IBD [58]. Chronic intestinal inflammation in mice reduces hippocampal neurogenesis [40]. LPS has an indirect role in neuroinflammation, as neuroimmune reflexes can sense and respond to peripheral injuries [49]. Moreover vagotomy

diminishes IL-1β response in the brain, but not on the periphery after intraperitoneal injection with LPS [59]. Inflammatory cytokine TNFα is rapidly induced and remains elevated for a prolonged period of time in the brain after peripheral LPS challenge [60]. Serum radiolabeled TNFα is being transported through the blood-brain barrier via endothelial receptors in a saturable manner [61]. Furthermore, LPS-induced intracerebral IL-6 signaling inhibits neurogenesis in the hippocampus [41].

This finely tuned live system, the intestine-liver-brain axis has a lot of possible connection routes (Figure 3).

3.3.2. Alcohol effects

Alcoholic individuals have altered and increased bacterial load in their intestine [21]. Bacterial endotoxin LPS seeping through alcohol-disrupted barrier of the intestine is increased in the sera of alcoholics [14-18, 23, 62, 63]. LPS contributes to alcoholic liver disease and therefore DAMP secretion from liver [62]. Reversing dysbiosis by certain antibiotics, probiotics or long-chain fatty acids can restore intestinal barrier function and decrease the subsequent liver damage [28, 64-66]. Intestinal mucus is increased in rats after alcohol feeding and mucin-2 deficiency can ameliorate alcoholic liver disease in mice [67, 68], which further underlines the importance of every aspect of the intestinal barrier.

The pro-inflamatory cytokines, PAMPs and DAMPs originating from the intestine enhance the inflammatory changes in other organs, including liver and brain via direct and indirect effects. Liver is a source of excessive inflammatory cytokine production in alcoholics which are released to the serum, as a response to alcohol and its toxic metabolites, as well as PAMPs and DAMPs originating from the intestine [69]. Both primary and secondary bile acids are increased in alcoholics and are currently suggested to play a role in the pathogenesis of colonic inflammation [70].

Brain damage is more common among alcoholics with cirrhosis and liver-failure than without liver disease [71]. High ammonia level is widely known for its neurotoxic effect, and can activate peripheral benzodiazepine receptors, contributing to astrocyte swelling and subsequent coma [72]. As a reverse interaction, intracerebral IL-1β injection results in hepatic recruitment of neutrophils [57]. It is noteworthy that alcohol

induces cortisol secretion modifying the immune system as part of the neuroimmune pathology [56].

The signaling and regulatory pathways might be similar to regulate gene expression in different organs, as there are similarities in gene expression profiles induced by alcohol in astrocytes and hepatocytes [34, 73, 74].

3.4. Inflammasome

Innate immune signaling pathways are hallmarks of alcohol-induced organ damage affecting intestine, liver, cardiovascular system, and brain [43, 69, 75]. Inflammation and these altered innate immune responses play pivotal roles in the pathogenesis of infectious and degenerative disorders [76, 77]. Part of these pro-inflammmatory changes, inflammatory cytokines and mediators including IL-1β, TNFα, high-mobility group protein-B1 (HMGB1) affect neurons and are associated with disease symptoms in animal models [49].

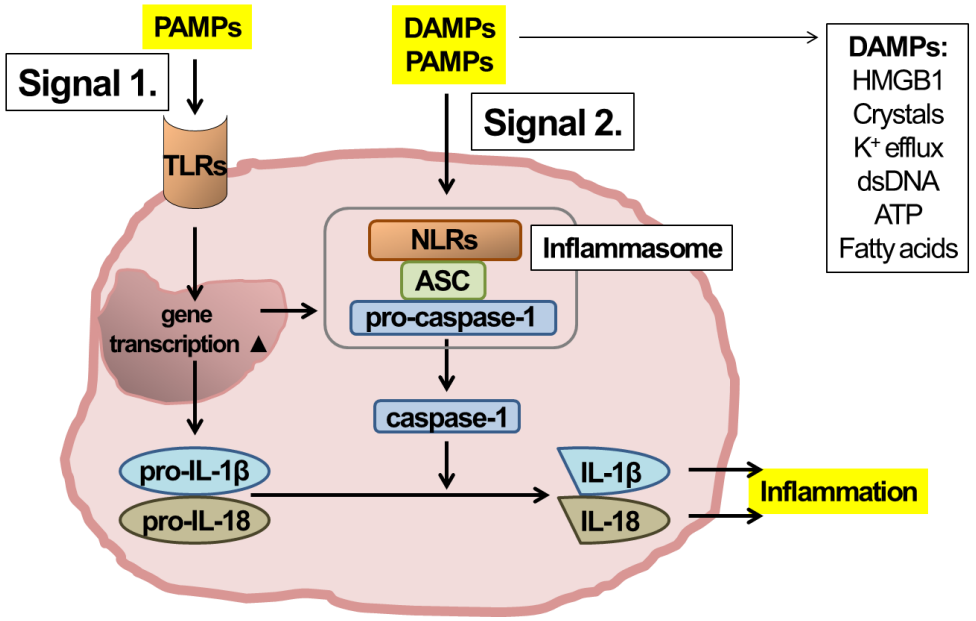

Figure 4. Inflammasome signaling [template [78] with some alterations].

Increased level of the inflammatory cytokine, IL-1β is found in the sera of alcoholics [79] and in brains and livers of rodents after alcohol intake [43, 80]. The inactive form of IL-1β, pro-IL-1β level is increased in response to PRR including TLR activation upon endogenous or exogenous danger signals [81]. Following activation of the receptors, inflammasome processes pro-IL-1β to mature, active IL-1β via caspase-1 activation [81]. Inflammasomes are multi-protein complexes mostly containing NOD-

like receptors (NLRs: NLRP1, NLRP3, NLRC4); an adapter molecule, ASC; and effector pro-caspase-1 [81]. At first, a priming signal, usually an inflammatory stimuli upregulate the expression of these inflammasome components and target proteins, pro-caspase-1 and pro-IL-1β [82]. A second signal is generally required for inflammasome activation [82]. Upon inflammasome activation, pro-caspase-1 is converted to caspase-1 (a proteolytic, effector enzyme) which then cleaves pro-IL-1β to IL-1β, the bioactive, secretable form (Figure 4) [81].

IL-1β exerts its biologic function via the type I IL-1 receptor (IL-1R) and amplifies inflammation through both autocrine and paracrine manner [83]. The endogenous inhibitor of IL-1R, IL-1R antagonist (IL-1Ra) occupies the IL-1R without transducing activation unlike activators IL-1α and IL-1β (Figure 5) [84].

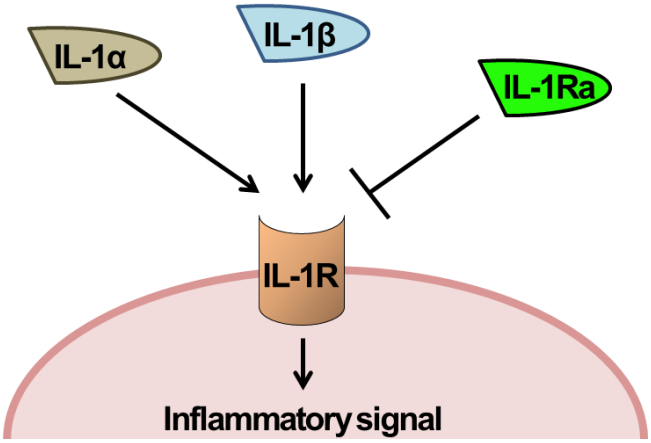

Figure 5. Interleukin-1-receptor signaling.

3.4.1. Signal 1.

PRR activation is needed for inflammatory gene induction [81]. PRR, TLR4 has a pivotal role in the pathogenesis of alcohol-induced liver damage and neuroinflammation [46, 62]. TLR activation via DAMPs and PAMPs leads to NF-κB activation and subsequently increased cytokine production [85].

Transcription factor NF-κB activation is involved in the pathogenesis of alcohol-induced hepatitis and neuroinflammation [45, 86]. NF-κB is known to induce the transcription of pro-inflammatory cytokines and chemokines, like TNFα, IL-1β and MCP1 [85, 87], all of which are increased in alcohol-induced hepatitis and neuroinflammation [34, 45, 46]. NF-κB activation and TNFα production have been suggested to play role in the pathogenesis of intestinal inflammatory processes [26, 27].

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3.4.2. Signal 2.

Posttranslational cleavage of pro-IL-1β to mature IL-1β is required for its functional activity and is executed by the inflammasome through caspase-1 activation [81]. PAMPs or DAMPs in sterile inflammation as a second signal can induce inflammasome activation [22]. DAMPs are molecules or cells that, upon cellular danger/damage, are actively excreted or passively released [88]. A recently widely recognized DAMP, HMGB1 is a nuclear protein that upon cytoplasmic translocation after phosphorylation or acetylation represents a danger signal and activates TLRs and the inflammasome (Figure 6) [89, 90].

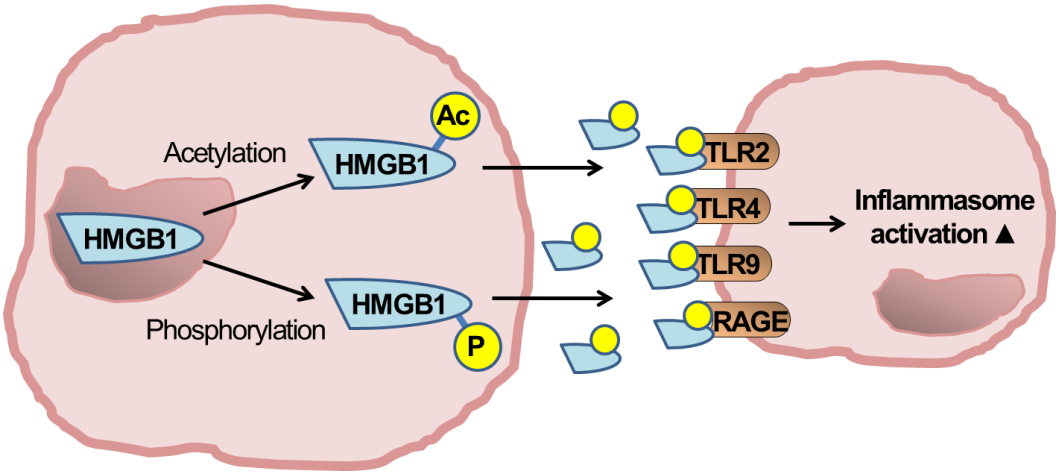

Figure 6. HMGB1 activation HMGB1 is passively released from damaged cells or as seen here can actively be excreted from cells after phosphorylation or acetylation.

Inflammasome activation and IL-1β signaling play a role in various liver diseases including acetaminophen injury and non-alcoholic steatohepatitis [91, 92]. Furthermore, inflammasome activation contributes to the pathogenesis of various neurologic disorders including traumatic and thromboembolic brain injury and Alzheimer's disease [93-96].

The significance of IL-1 signaling and inflammasome activation has not been evaluated in alcoholic intestinal, liver and brain injury which became the target of our research team, the main focus of this thesis is the effect of alcohol on the proximal small intestine and the cerebellum.

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4. OBJECTIVES

1. To evaluate the inflammasome activation in the proximal intestine of an animal model of alcoholism.
2. To evaluate the inflammasome activation in the cerebellum of an animal model of alcoholism.

5. METHODS

5.1. Animals

5.1.1. Ethical approval: These studies were conducted according to the regulations of the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School (Worcester, MA).

5.1.2. Genotypes: Six to eight weeks old female C57/BL6J wild type (WT); TLR4 knock-out (KO); apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) KO; NACHT, LRR and PYD domains-containing protein 3 (NLRP3) KO mice (backcrossed on a C57/BL6J background) were employed.

5.1.3. Diet: The animals on chronic ethanol-regime received 5% (v/v) ethanol (36% ethanol-derived calories) containing Lieber-DeCarli diet (EtOH) or pair-fed diet (Ctr) with an equal amount of calories where the alcohol-derived calories were substituted with dextran-maltose (Bio-Serv, Frenchtown, NJ, USA) for 5 weeks. The daily consumption of the diet was the same in the WT and KO mouse strains, approximately 10ml/animal. The animals had access to water ad libitum and the consumption was comparable among the different diet groups.

The animals on acute ethanol-regime received 5g/kg 50% (v/v) ethanol diluted in water or an equal amount of water via oral gavage for three consecutive days (otherwise indicated) using a stainless steel 22-gauge feeding tube.

5.1.4. Treatment: Where indicated, WT mice received daily intraperitoneal injection (i.p.) of 25 mg/kg recombinant IL-1Ra (anakinra; Amgen) or equal amount of saline throughout the whole experiment.

Some eight weeks old female C57/BL6J mice were anesthetized with Isoflurane, inhalation, to effect and received 30µl intracranial (posterior fontanelle) injection with recombinant mouse IL-1β (100ng/mouse) (R&D Systems, Inc., Minneapolis, MN) or equal amount of saline solution, these animals were sacrificed six hours after the procedure.

5.1.5. Sample collection and storage: Across the study animals were sacrificed in the morning following cheek bleeding. In the acute model this was 6 or 12 hours after the last gavage (otherwise indicated) and in the chronic alcohol model at 12 hours after the start of the last feeding. The sample collection after chronic feeding in the morning was approximately 3-5 hours following the actual end of the final feeding. Animals were sacrificed by cervical dislocation.

Serum was isolated from blood by centrifugation. The proximal third of the small-intestine was immediately isolated and kept in 2% fetal bovine serum (FBS) containing PBS on ice for further processing. Proximal intestines were immediately flushed with cold PBS, dissected longitudinally, washed further in PBS, until intestinal content was removed. Peyer patches were dissected and discarded. The 1st and the 3rd-6th cm of the proximal intestine were snap frozen in liquid nitrogen for protein evaluation. The 2nd cm was incubated in RNAlater (Qiagen, Hilden, Germany) at 4°C overnight for RNA extraction. Cerebella and cerebra were immediately isolated and were snap frozen or stored in RNAlater. All samples were stored at -80°C.

5.2. Laboratory methods

5.2.1. Alcohol concentration: Blood alcohol content in serum was measured using alcohol analyzer (Analox Instruments, Lunenburg, MA, USA).

5.2.2. Quantitative polymerase chain reaction (qPCR): RNA was extracted from the 2nd cm of proximal small-intestine, cerebellum or cerebrum using RNeasy kit (Qiagen, Maryland, USA). Optical density (260/280 and 260/230 ratios) was measured to check RNA quality. cDNA was transcribed from 1ug of total RNA by using Reverse Transcription System (Promega Corp., Madison, Wisconsin, USA) in a final volume of 30ul. Sybr-Green-based real-time quantitative PCR was performed using the iCycler (Bio-Rad Laboratories Inc., Hercules, California, USA). Each 25ul reaction contained 200nM of primer mix (1/1, v/v) and 2.5ul of diluted (1/4, v/v) cDNA. Primers used for the experiments are listed in Table 1. The PCR contained a denaturation step for 3 min at 95°C and 45 cycles of 30s at 95°C, 45s at gene-specific annealing temperature and 30s at 72°C for primer extension. MicroAmp optical 96-well reaction plate with optical tape was used for all amplifications and detections. The increase in fluorescence by

double-stranded DNA-binding Sybr-Green was detected each cycle, indicating PCR product accumulation. A dissociation melting curve was performed between 55 and 95°C. Comparative threshold cycle (ΔΔCt) method was used to calculate expressions relative to WT control groups. Ct for the target amplicon and the internal control, 18S gene, were determined. All results were normalized to their own internal control 18S to remove the differences in the amounts of nucleic acid added due to any deficiency of the reverse transcriptase PCR step (ΔCt), the values were then normalized to the average of the actual reference control sample group (ΔΔCt). The final results were expressed as fold changes ($2^{-\Delta\Delta Ct}$) between the sample and the controls corrected with 18S.

Table 1. Real-Time PCR Primers – forward and reverse sequence of the primers used in real-time PCR.

TARGET GENE	FORWARD PRIMER (5'>3')	REVERSE PRIMER (5'>3')
18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
ASC	GAA GCT GCT GAC AGT GCA AC	GCC ACA GCT CCA GAC TCT TC
HMGB1	CGC GGA GGA AAA TCA ACT AA	TCA TAA CGA GCC TTG TCA GC
IL-1R	GCC CAC CTA GAA GGG CGG GA	CCA GCG ACA GCA GAG GCA CC
IL-1Ra	GAG ACA GCC TGC CTG CCT GGG GGA	TCA CTG GCA TGG CCA CCT GC
NLRP1	TGG CAC ATC CTA GGG AAA TC	TCC TCA CGT GAC AGC AGA AC
NLRP3	AGC CTT CCA GGA TCC TCT TC	CTT GGG CAG CAG TTT CTT TC
NLRP6	CAGAAGGGCAAGCAAAAGAC	ACATTCAGCAACACGCTCAG
Pro-caspase-1	AGA TGG CAC ATT TCC AGG AC	GAT CCT CCA GCA GCA ACT TC
Pro-IL-1β	TCT TTG AAG TTG ACG GAC CC	TGA GTG ATA CTG CCT GCC TG
RAGE	GAA GGC TCT GTG GGT GAG TC	CCG CTT CCT CTG ACT GAT TC
Reg3b	TAC TGC CTT AGA CCG TGC TTT CTG	GAC ATA GGG CAA CTT CAC CTC ACA
TLR2	ACA ATA GAG GGA GAC GCC TTT	AGT GTC TGG TAA GGA TTT CCC AT
TLR4	GCC TTT CAG GGA ATT AAG CTC C	AGA TCA ACC GAT GGA CGT GTA A
TLR9	TGA AGT CTG TAC CCC GTT TCT	GTG GAC GAA GTC GGA GTT GT
TNFα	CAC CAC CAT CAA GGA CTC AA	AGG CAA CCT GAC CAC TCT CC

5.2.3. Electrophoretic mobility shift assay (EMSA): End labeling of double-stranded NF-κB oligonucleotide, 5'AGTTGAGGGGACTTTCGC3' was accomplished by treatment with T4 polynucleotide kinase in the presence of γ32P-ATP (PerkinElmer, Waltham, MA), followed by purification on a polyacrylamide copolymer column (Bio-Rad). Proximal intestinal or cerebellar whole cell lysates (5μg) were incubated with 1μl labeled oligonucleotide (50,000 cpm), 4μl dI-dC (Affymetrix Inc., Santa Clara, CA) and 5X gel buffer (containing 20mM HEPES pH7.9 (Sigma, St. Louis, MO), 50mM KCl (Sigma, St. Louis, MO), 0.1mM EDTA (Boston BioProducts Inc., Ashland, MA), 1mM DTT (Sigma, St. Louis, MO), 5% glycerol (Fisher Scientific, Fair Lawn, NJ), 200 μg/ml

BSA in sterile water). A 20 µl final volume was reached by adding nuclease-free water. For cold competition reaction a 20-fold excess of specific unlabeled double-stranded probe was added to the reaction mixture 20 minutes prior to adding the labeled oligonucleotide. Samples were incubated at room temperature for 20 minutes. Reactions were run on a 4% polyacrylamide gel. Gels were then dried and exposed to an X-ray film at -80°C for 6 hours or overnight where appropriate. Kodak X-OMAT 2000A Processor was used for film development in the darkroom. The films were scanned and densitometry was performed on the images using Multi Gauge Ver.3.2 image software (Fujifilm Corp., USA).

5.2.4. Enzyme-linked immunosorbent assay (ELISA): Tissue lysates were prepared from proximal intestines, cerebella and cerebra in RIPA buffer containing protease and phosphatase inhibitors (1mM PMSF, 1mM NaF, 2mM Na3VO4, 20mM Na4P2O7, protease and phosphatase inhibitor tablet). First, the tissue was homogenized with stainless steel bead (Qiagen, Maryland, USA) in TissueLyser II (Qiagen, Maryland, USA) then clarified by centrifugation. The tissue lysate supernatant was stored at -80°C. Protein level was measured by ELISA reader using Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories Inc., Hercules, CA). TNFα (BD Biosciences, San Diego, CA) and IL-1β (R&D Systems, Inc., Minneapolis, MN) were measured in whole tissue lysates using specific anti-mouse ELISAs.

5.2.5. Enzyme-activity assay: Caspase-1 colorimetric assay was used to determine the enzymatic activity (R&D Systems, Inc., Minneapolis, MN) from cerebellar and cerebral tissue lysates. It has been shown earlier that the results of caspase-1 activity assay correspond to the Western blot results of cleaved capase-1 p10 protein in liver [34], we further evaluated the method for brain tissue using Western blots (Figure 15E-F).

5.2.6. Endotoxin: Endotoxin levels were evaluated in serum and cerebellar lysates with Limulus Amebocyte Lysate assay (Lonza Group Ltd, Basel, Switzerland).

5.2.7. Western blot: Equal amounts of protein from tissue lysates were run on 15% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane overnight

then blocked for two hours in blocking buffer-1 or 2. Primary antibodies against mouse Reg3b (R&D Systems Inc., Minnesota, USA), IL-1β (R&D Systems, Inc., Minneapolis, MN), caspase-1 p10 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), HMGB1 (Abcam, Cambridge, MA), and beta-actin (Abcam, Cambridge, MA) were used overnight at 4˚C at different dilution rates varying from 1:100 to 1:30,000 in blocking buffer-1 or 2, followed by three washing steps. For detection, appropriate donkey anti-sheep, goat anti-rat, anti-rabbit or anti-mouse secondary horseradish peroxidase–labeled secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used for one hour at a dilution rate of 1:5000 in blocking buffer-1 or 2. The immunoreactive bands of interest were detected by chemiluminescence using Pierce ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) and LAS-4000IR Ver.2.02 (Fujifilm Corp., USA) for image acquisition. The results were quantified by densitometry analysis using Multi Gauge Ver.3.2 image software (Fujifilm Corp., USA). Blocking buffer-1: 0.1% TWEEN-20 TBS 5% milk. Blocking buffer-2: 0.1% TWEEN-20 TBS 5% BSA.

5.2.8. Immunoprecipitation: Equal amounts of proteins from cerebellar tissue lysates were pre-cleaned with anti-rabbit Ig IP beads (eBioscience, Inc., San Diego, CA). Beads were removed by centrifuge and supernatants were incubated and rotated overnight at 4˚C with 5 μg anti-HMGB1 or anti-acetyl lysine Ab (Abcam, Cambridge, MA) or normal rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as a negative control. Samples were partially rotated for one hour with anti-rabbit Ig IP beads at 4˚C. The formed immune complexes were collected by centrifugation, washed three times and boiled with 2X Laemmli's sodium dodecyl sulfate-sample buffer (Boston BioProducts, Ashland, MA) to dissociate from the beads. The beads were then removed by centrifugation. Protein was separated from the supernatants by polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane and blocked for two hours in blocking buffer-2 or TBS with 3% BSA, 0.1% gelatin and 0.2% TWEEN-20. The membrane was incubated overnight at 4˚C with 3 μg/ml anti-phosphoserine or 1 μg/ml anti-HMGB1 Ab (Abcam, Cambridge, MA) in blocking buffer-2 or 3. The membrane was washed three times and incubated for an hour with HRP-conjugated secondary anti-rabbit IgG Ab (eBioscience, Inc., San Diego, CA) at 1:5000 dilution in

blocking buffer-2 or 3. The immunoreactive bands were detected by chemiluminescence using Pierce ECL Western blotting substrate and LAS-4000IR. The results were quantified by densitometry using Multi Gauge Ver.3.2 image software. The loading control was detected on a separate gel by monoclonal mouse anti-beta-actin. Blocking buffer-3: 0.2% TWEEN-20 TBS containing 0.5% BSA.

5.3. Statistical analysis:

Since data was not normally distributed, statistical analysis was performed using Kruskal-Wallis nonparametric test. Data are shown as average ± standard error of the mean (SEM). Differences were considered statistically significant at P value ≤ 0.05.

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6. RESULTS

6.1. Proximal small intestine

6.1.1. Serum alcohol content in alcohol-fed mice

Mice receiving either one-time alcohol gavage (Figure 7A) or 5 weeks of alcohol feeding (Figure 7B) have significantly higher blood alcohol content than their appropriate controls.

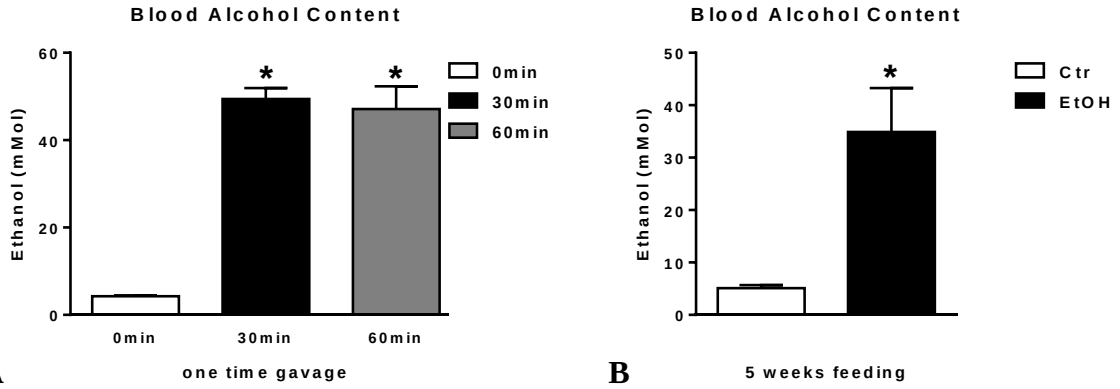

Figure 7. Serum alcohol content in alcohol-fed mice. Wild type (WT; n=6 or 7) mice were fed with ethanol (EtOH) or isocaloric (Ctr) diet for 5 weeks, or had a single oral gavage of 50% 5g/kg ethanol (WT; n=6) where blood was collected 30 or 60min after. Blood alcohol content in serum was measured using alcohol analyzer (A-B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate isocaloric or 0min controls, respectively, by Kruskal-Wallis non-parametric test).

6.1.2. Serum-endotoxin level in alcohol-fed mice

Mice receiving either three days alcohol gavage (Figure 8A) or 5 weeks of alcohol feeding (Figure 8B) have significantly higher serum-endotoxin levels than their appropriate controls. Twelve hours after alcohol gavage, no difference of serum-endotoxin level is found between alcohol gavage and control groups (Figure 8A).

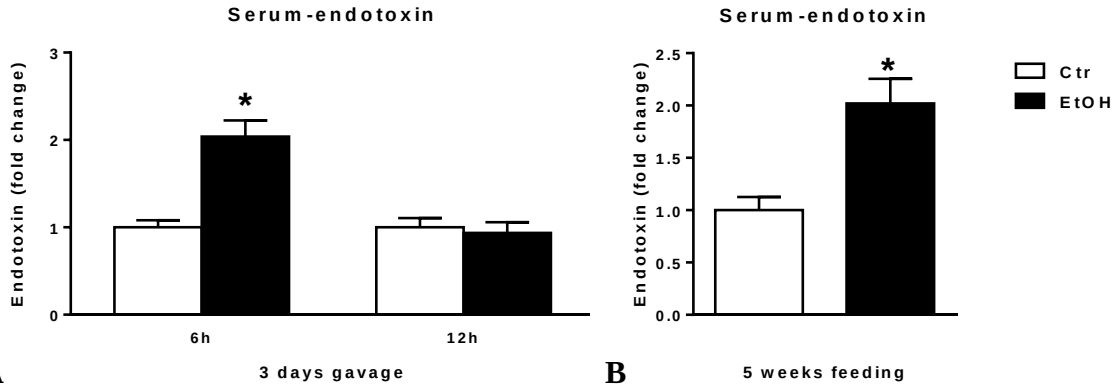

Figure 8. Serum-endotoxin level in alcohol-fed mice. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=6) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Serum-endotoxin measurement was executed using Limulus Amebocyte Lysate-assay (A-B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

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6.1.3. Intestinal antimicrobial protein expression in alcohol-fed mice

Both mRNA (Figure 9A) as well as protein level (Figure 9C, E) of Reg3b is significantly higher in the small intestine of mice receiving alcohol gavage than their controls.

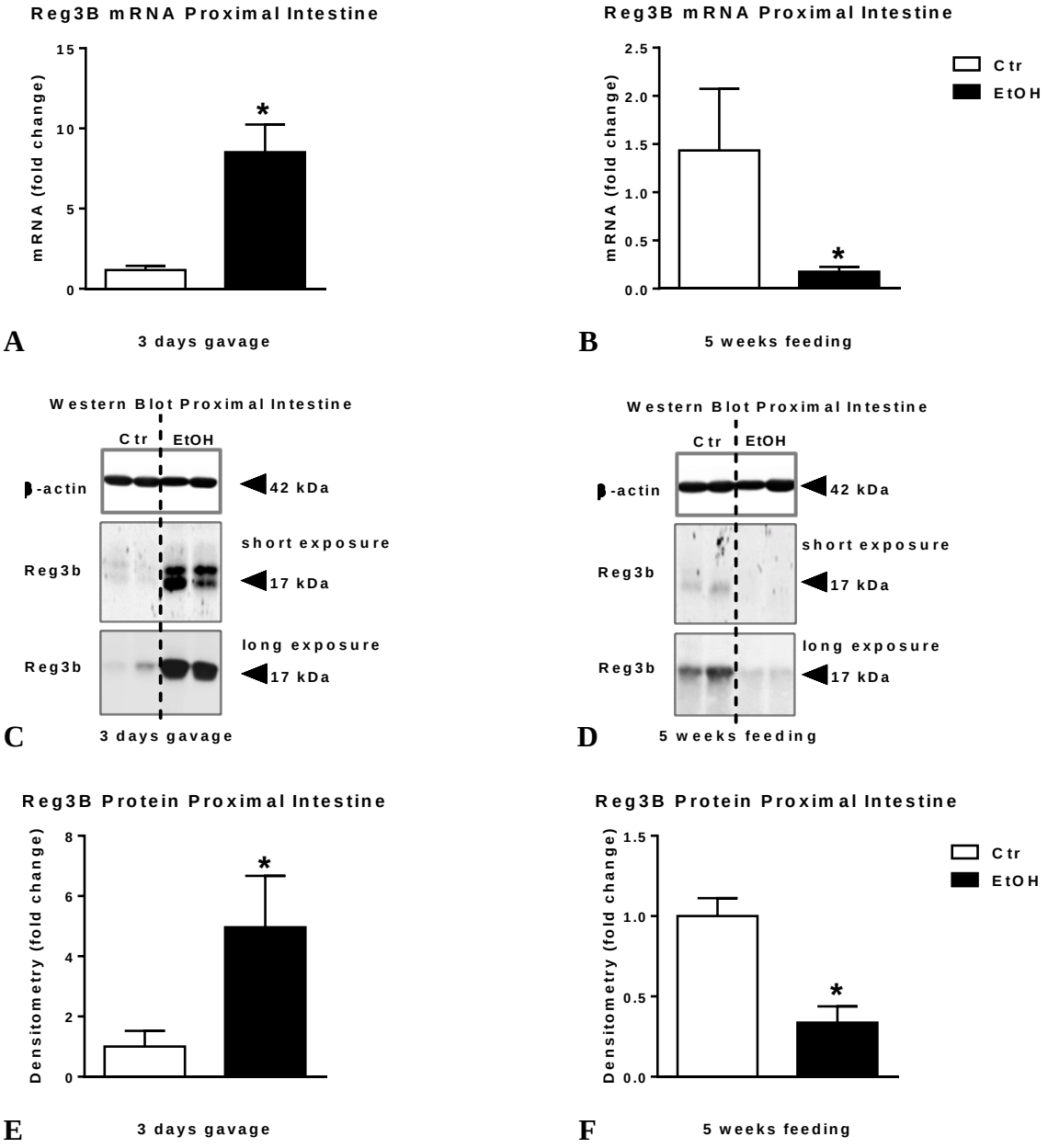

Figure 9. Intestinal antimicrobial protein expression in alcohol-fed mice. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=6) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Regenerating islet-derived protein III-beta (Reg3b) mRNA was assessed by qRT-PCR in proximal small intestine using 18S internal control (A-B). Reg3b protein in whole SB lysates was assessed by Western blot using β-actin loading control (C-D), and further quantified by densitometry (E-F). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

In contrast, the expression of Reg3b in the small intestine of chronic alcohol-fed mice is significantly lower both at mRNA (Figure 9B) as well as protein level compared to controls (Figure 9D, F). (Average; SEM of density values of saline vs. alcohol gavage 248; 98 vs. 1226; 426 and PF vs. EtOH-feeding [longer exposure] 265; 20 vs. 107; 31, respectively).

6.1.4. Intestinal NF-κB activation in alcohol-fed mice

Both alcohol gavage and chronic alcohol feeding results in significantly higher NF-κB activation and DNA binding in proximal small intestine of mice compared to controls (Figure 10A-D). The magnitude of increase is significantly greater in mice with chronic compared to acute binge alcohol administration.

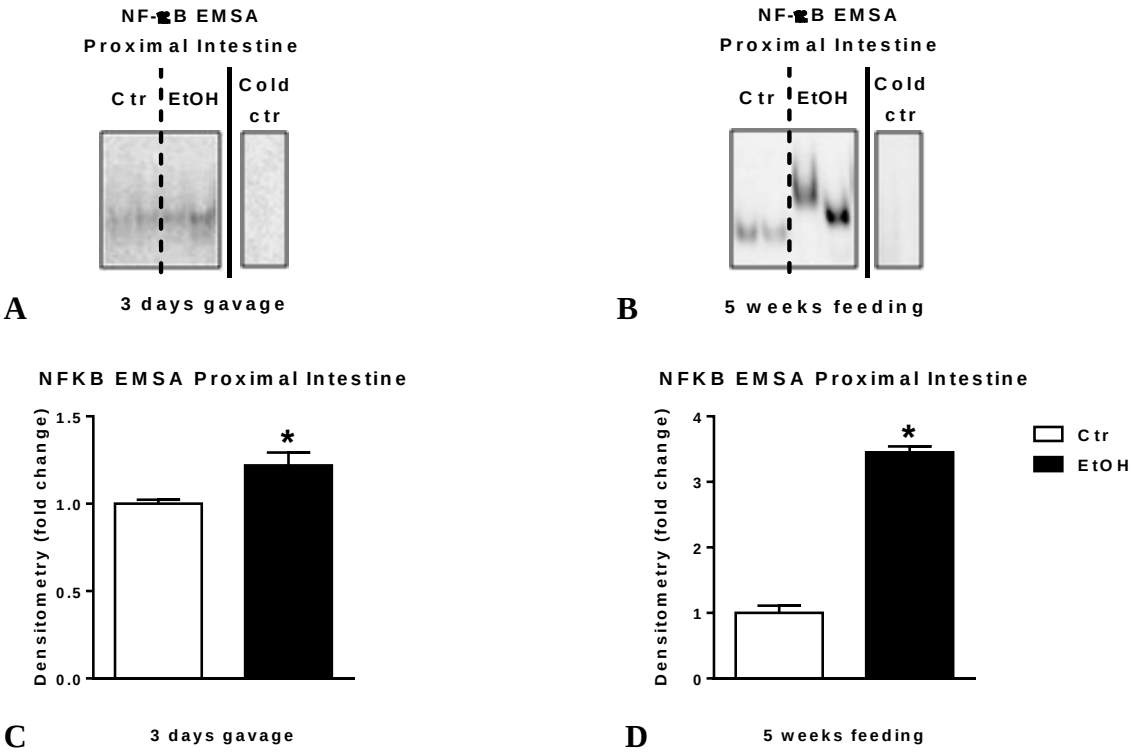

Figure 10. Intestinal NF-κB activation in alcohol-fed mice. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=6) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Nuclear factor-κB (NF-κB) activation of whole proximal small intestinal lysates was assessed by EMSA (A-B), loading equal amounts of protein, using EtOH-fed intestinal sample for cold competition control (cold ctr), and further quantified by densitometry (C-D). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.1.5. Intestinal TNFα level in alcohol-fed mice

TNFα mRNA levels are significantly higher in the proximal small intestine of mice after both acute binge and chronic alcohol feeding compared to controls (Figure 11A-B).

However, murine proximal small intestinal TNFα protein levels are significantly higher after chronic alcohol feeding (Figure 11D), no difference of TNFα protein level is found between alcohol gavage and control groups (Figure 11C).

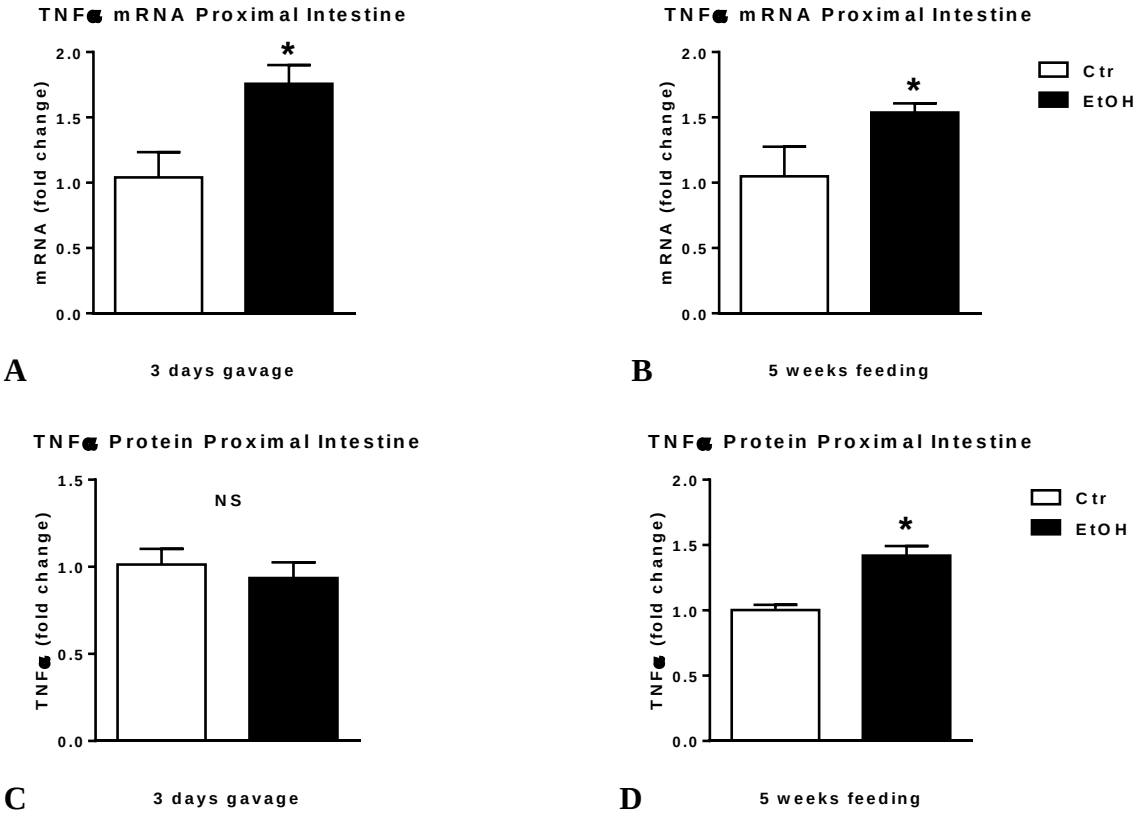

Figure 11. Intestinal TNFα level in alcohol-fed mice. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=6) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Tumor necrosis factor-α (TNFα) mRNA was assessed by qRT-PCR in proximal small intestine using 18S internal control (A-B). TNFα protein in whole proximal small intestinal lysates was assessed by specific ELISA (C-D). Bars represent mean±SEM (*: P value<0.05; NS: not significant; relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.1.6. Intestinal IL-1β level in alcohol-fed mice

No difference of murine proximal intestinal pro-IL-1β mRNA (Figure 12A-B) or IL-1β protein (Figure 12C-D) level is found between either alcohol gavage or chronic alcohol-feeding and appropriate control groups [unpublished data].

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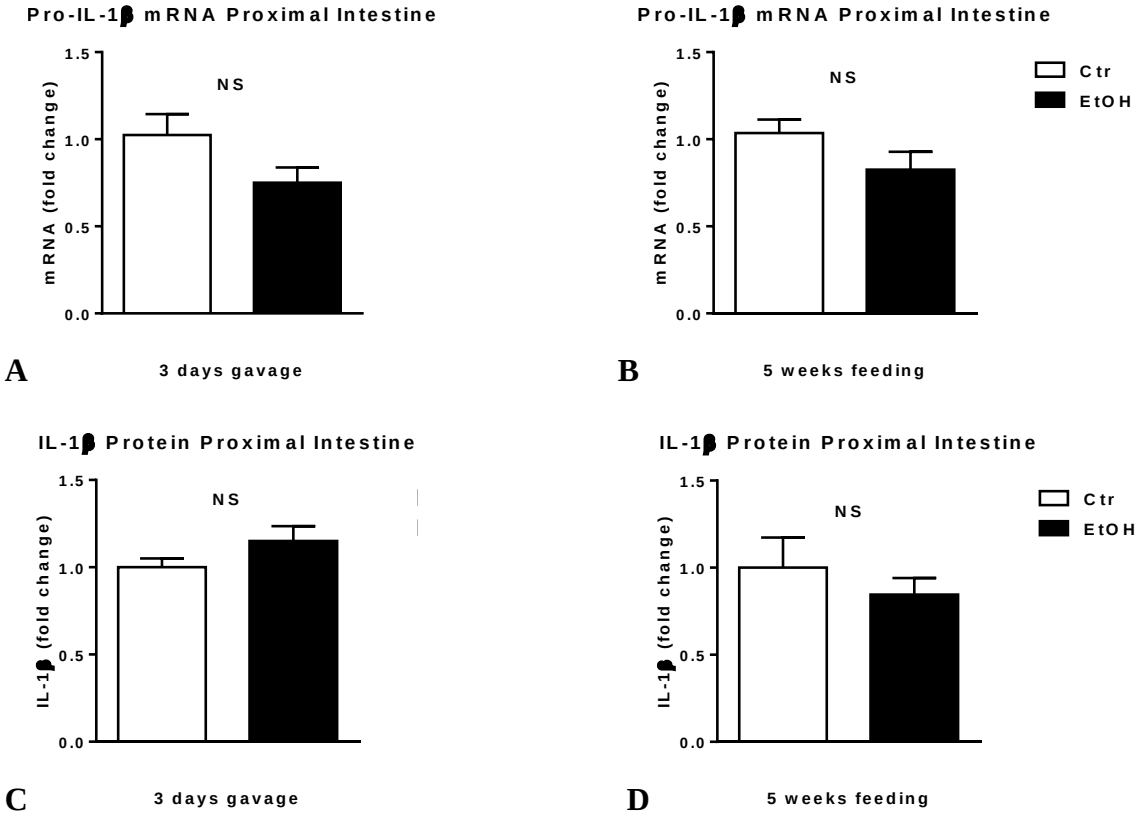

Figure 11. Intestinal IL-1β level in alcohol-fed mice [unpublished data]. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=6) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Pro-interleukin-β (pro-IL-1β) mRNA was assessed by qRT-PCR in proximal small intestine using 18S internal control (A-B). IL-1β protein in whole proximal small intestinal lysates was assessed by specific ELISA (C-D). Bars represent mean±SEM (NS: not significant relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.1.7. Intestinal inflammasome expression in alcohol-fed mice

No difference of murine proximal small intestinal NLRP3, NLRP6, ASC and pro-caspase-1 mRNA is found between either alcohol gavage (Figure 12A) or chronic alcohol-feeding (Figure 12B) and appropriate control groups [unpublished data].

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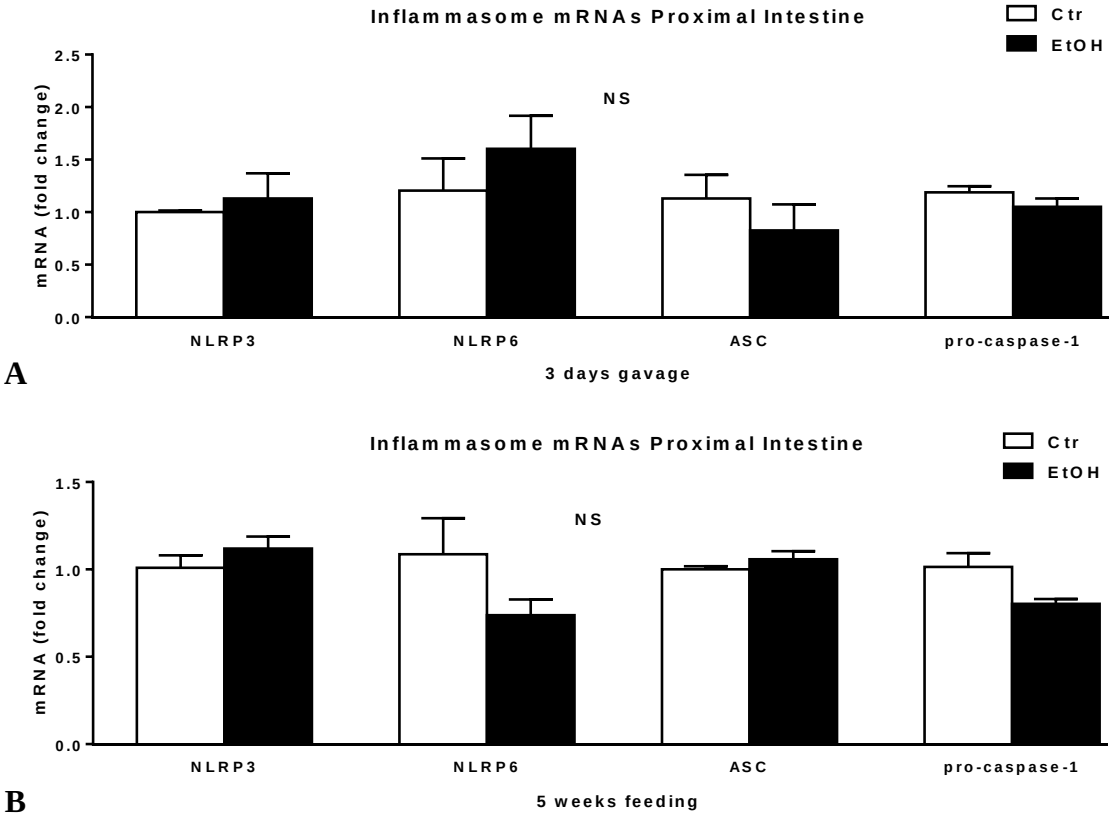

Figure 12. Intestinal inflammasome expression in alcohol-fed mice [unpublished data]. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=6) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Inflammasome sensors (NLRP3 and NLRP6), inflammasome adaptor (ASC) and inflammasome effector (pro-caspase-1) mRNA was assessed by qRT-PCR in proximal small intestine using 18S internal control (A-B). Bars represent mean±SEM (NS: not significant relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2. Cerebellum

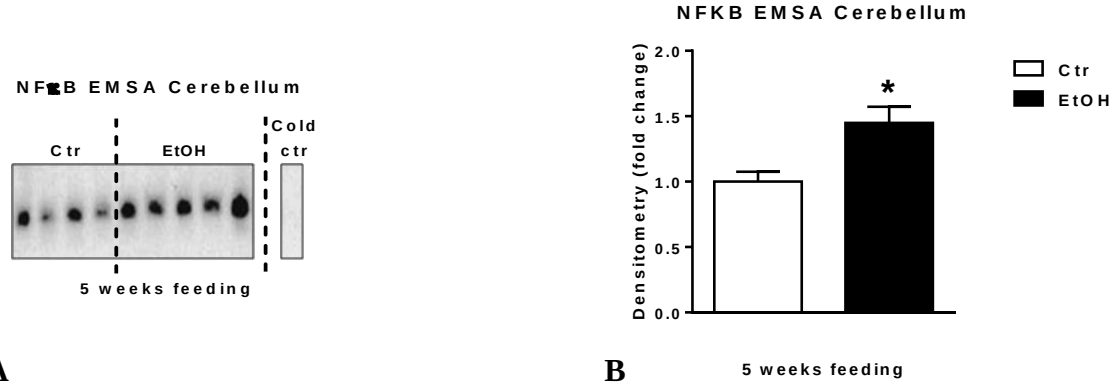

Figure 13. Cerebellar NF-κB activation in chronic alcohol-fed mice. Wild type mice were gavaged daily 50% 5g/kg ethanol (EtOH; n=6) or equal amount of saline (Ctr; n=5) for three consecutive days or were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Nuclear factor- κB (NF-κB) activation of whole cerebellar lysates was assessed by EMSA (A), loading equal amounts of protein, using EtOH-fed cerebellar sample for cold competition control (cold ctr), and further quantified by densitometry (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.1. Cerebellar NF-κB activation in chronic alcohol-fed mice

Chronic alcohol feeding results in significantly higher NF-κB activation in cerebella of mice compared to controls (Figure 13A-B).

6.2.2. Cerebellar TNFα level in chronic alcohol-fed mice

Both TNFα mRNA (Figure 14A) and protein (Figure 14B) levels are significantly higher in the cerebella of chronic alcohol-fed than control mice.

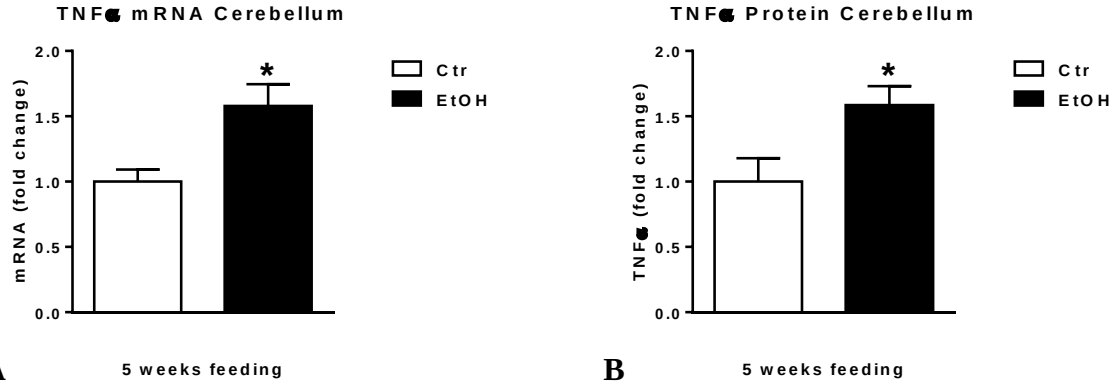

Figure 14. Cerebellar TNFα level in chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. Tumor necrosis factor-α (TNFα) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control (A). TNFα protein in whole cerebellar lysates was assessed by specific ELISA (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.3. Cerebellar IL-1β level in chronic alcohol-fed mice

Pro-IL-1β mRNA (Figure 15A) as well as protein (Figure 15C) levels are significantly higher in the cerebella of mice after chronic alcohol feeding compared to controls. Similar to cerebellar IL-1β protein levels detected by specific ELISA (Figure 15C), Western blot result shows significantly higher mature IL-1β protein levels in the cerebellum of chronic alcohol-fed compared to control mice (Figure 15E-F). Both pro-IL-1β mRNA (Figure 15B) and IL-1β protein (Figure 15D) are significantly higher in the cerebral cortex of chronic alcohol-fed compared to control mice. The magnitude of IL-1β protein increase does not differ between murine cerebellar and cerebral cortex upon chronic alcohol feeding (Figure 15G).

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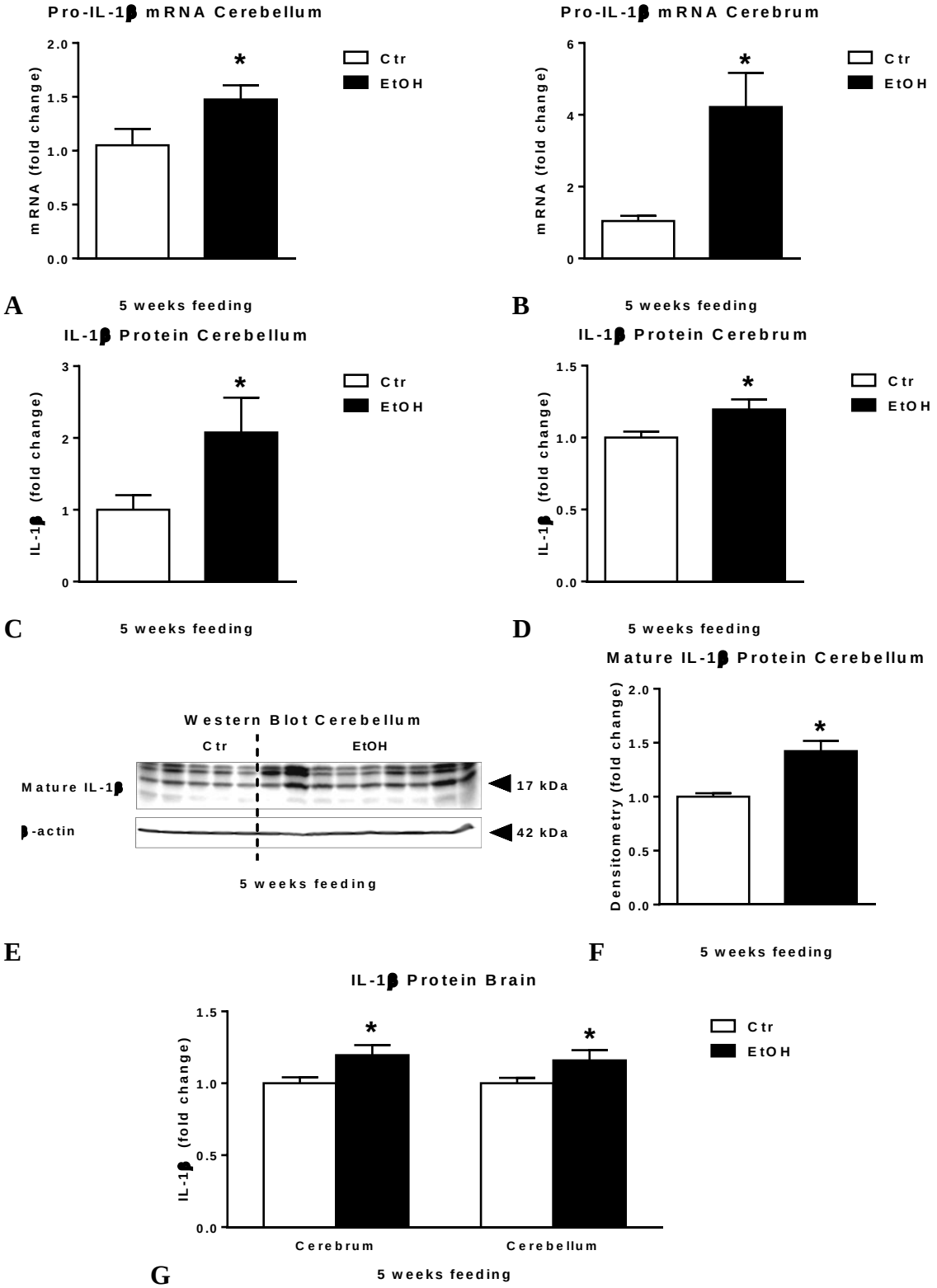

Figure 15. Cerebellar IL-1β level in chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. Pro-interleukin-1β (pro-IL-1β) mRNA was assessed by qRT-PCR in cerebellum (A) or cerebrum (B) using 18S internal control. IL-1β protein in whole cerebellar (C) or cerebral (D) lysates was assessed by specific ELISA (G). Mature IL-1β in whole cerebellar lysates was assessed by Western blot using β-actin loading control (E), and further quantified by densitometry (F). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

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6.2.4. Cerebellar inflammasome expression in chronic alcohol-fed mice

Murine cerebellar NLRP1, NLRP3, ASC and pro-caspase-1 mRNAs are significantly higher after chronic alcohol-feeding compared to controls (Figure 16A). Similarly the levels of cerebral NLRP1, NLRP3, ASC and pro-caspase-1 mRNAs are significantly higher in chronic alcohol-fed compared to control mice (Figure 16B).

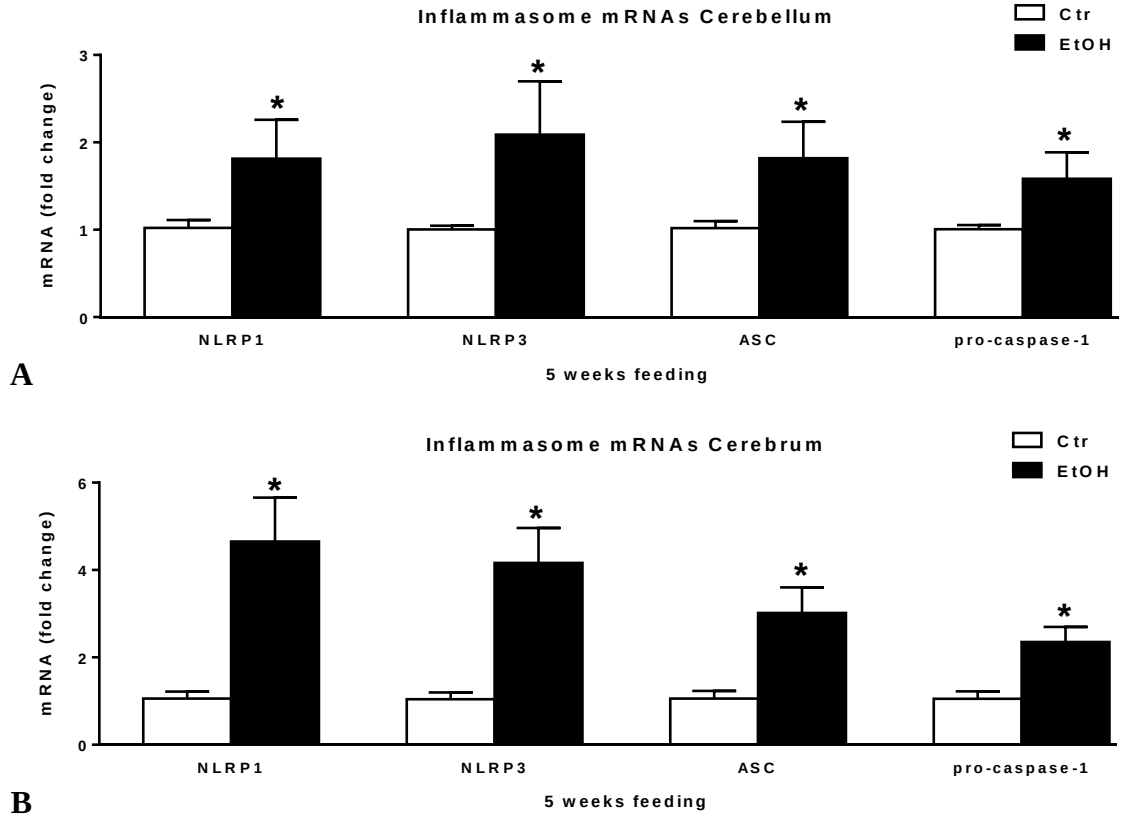

Figure 16. Cerebellar inflammasome expression in chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. Inflammasome sensors (NLRP1 and NLRP3), inflammasome adaptor (ASC) and inflammasome effector (pro-caspase-1) mRNA was assessed by qRT-PCR in cerebellum (A) or cerebrum (B) using 18S internal control. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.5. Cerebellar inflammasome activation in chronic alcohol-fed mice

Caspase-1 activity is significantly higher in murine cerebellum (Figure 17A) and cerebrum (Figure 17B) after chronic alcohol feeding compared to controls. Pro-caspase-1 and caspase-1 p10 are significantly higher in murine cerebellum after chronic alcohol feeding compared to controls (Figure 17C-D).

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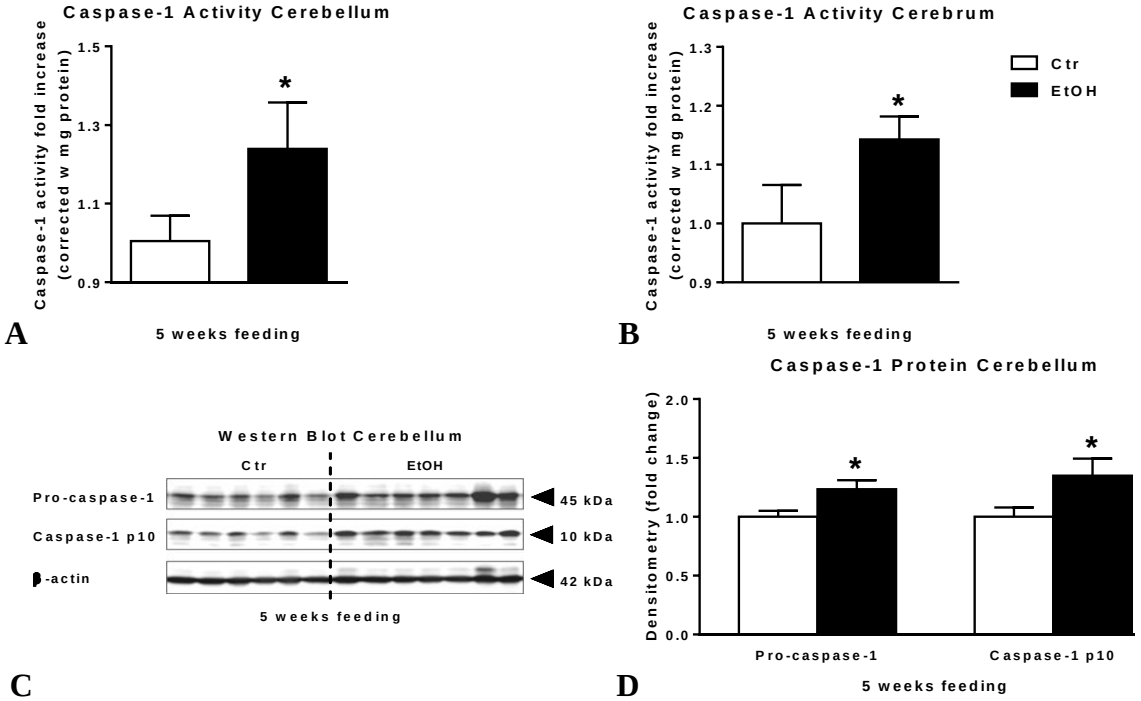

Figure 17. Cerebellar inflammasome activation in chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. Inflammasome activity was measured by caspase-1 colorimetric assay from whole cerebellar (A) or cerebral (B) lysates. Pro-caspase-1 and caspase-1 p10 levels were visualized on Western blot using β-actin loading control (C), and further quantified by densitometry (D). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.6. Serum alcohol content in chronic alcohol-fed mice with different genotypes

Mice receiving 5 weeks of alcohol feeding have significantly higher blood alcohol content than their appropriate controls regardless of their genotype (WT; TLR4-KO; NLRP3-KO; ASC-KO) (Figure 18). No statistically significant difference of blood alcohol content of mice is found among different genotypes (WT vs TLR4-KO; WT vs NLRP3-KO and ASC-KO) after 5 weeks of alcohol feeding (Figure 18).

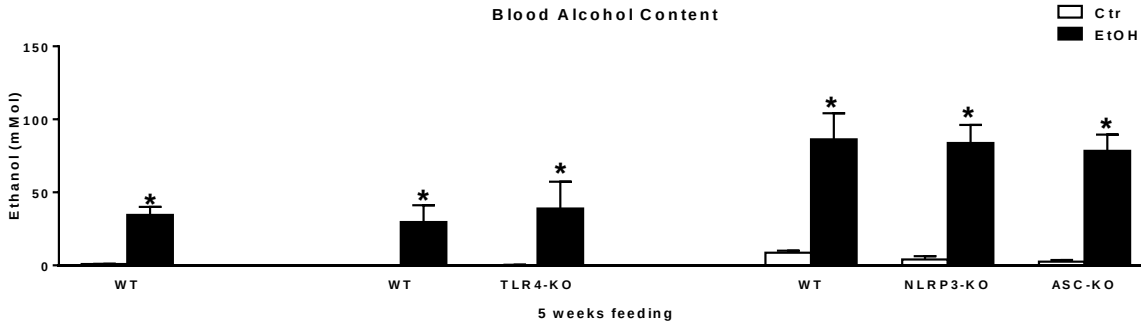

Figure 18. Serum alcohol content in chronic alcohol-fed mice with different genotypes. Wild type (WT; n=7-14 or 6-8), TLR4-KO (n=13 or 8), NLRP3-KO (n=10 or 8) and ASC-KO (n=12 or 8) mice were fed with ethanol (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. Blood alcohol content in serum was measured using alcohol analyzer. Bars represent mean±SEM (*: P value<0.05 relative to appropriate isocaloric controls by Kruskal-Wallis non-parametric test).

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6.2.7. Cerebellar IL-1β level in chronic alcohol-fed NLRP3-KO and ASC-KO mice

Cerebellar IL-1β protein level is significantly higher after chronic alcohol feeding in wild type (WT) mice whereas no significant difference of cerebellar IL-1β protein level is found between alcohol-fed NLRP3-KO or ASC-KO and their appropriate control mice (Figure 19A-B). Cerebellar mature IL-1β protein level is significantly lower in chronic alcohol-fed NLRP3-KO compared to WT mice (Figure 19A-B).

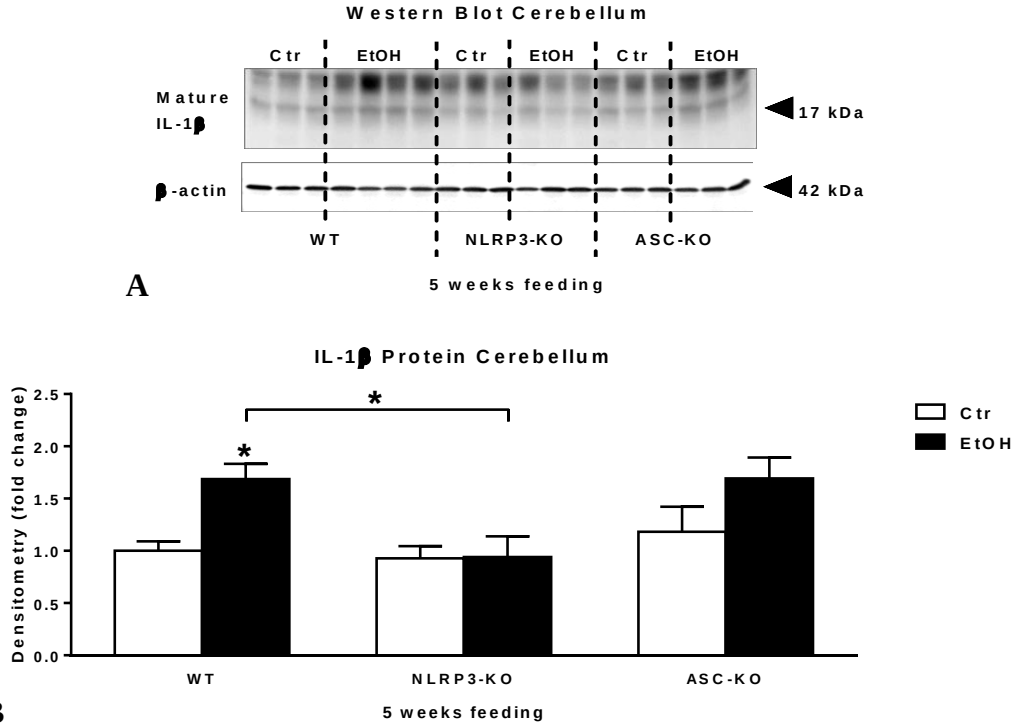

Figure 19. Cerebellar IL-1β level in chronic alcohol-fed NLRP3-KO and ASC-KO mice. Wild type (WT; n=14 or 7), NLRP3-KO (n=10 or 8), and ASC-KO (n=12 or 9) mice were fed with 5% ethanol containing (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. Mature IL-1β in whole cerebellar lysates was assessed by Western blot using β-actin loading control (A), and further quantified by densitometry (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr or WT-EtOH controls by Kruskal-Wallis non-parametric test).

6.2.8. Cerebellar inflammasome activation in chronic alcohol-fed NLRP3-KO and ASC-KO mice

Caspase-1 activity is significantly higher in WT murine cerebellum after chronic alcohol feeding compared to controls (Figure 17B, 20). No difference of cerebellar caspase-1 activity is found between chronic alcohol-fed NLRP3-KO or ASC-KO and their appropriate control mice (Figure 20).

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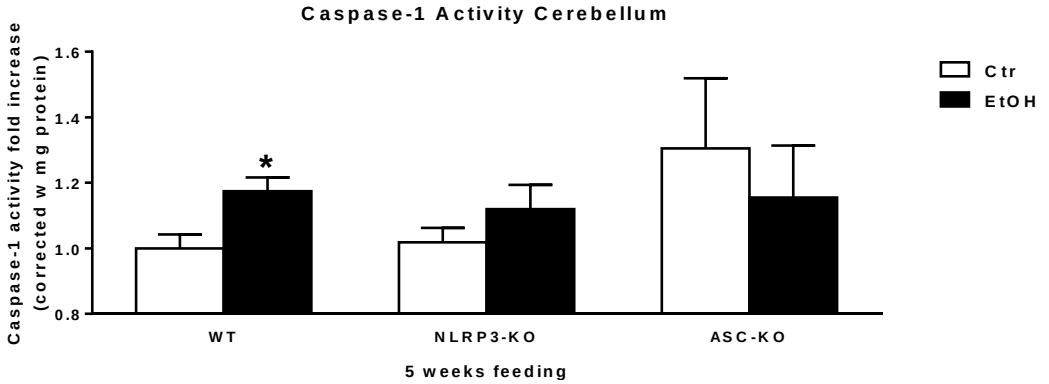

Figure 20. Cerebellar inflammasome activation in chronic alcohol-fed NLRP3-KO and ASC-KO mice. Wild type (WT; n=14 or 7), NLRP3-KO (n=10 or 8), and ASC-KO (n=12 or 9) mice were fed with 5% ethanol containing (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. Inflammasome activity was measured by caspase-1 colorimetric assay from whole cerebellar lysates. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.9. Cerebellar receptor expression in chronic alcohol-fed mice

Murine cerebellar Toll-like receptors, TLR2, TLR4, TLR9 and receptor for advanced glycation end products (RAGE) mRNAs are significantly higher after chronic alcohol-feeding compared to controls (Figure 21A).

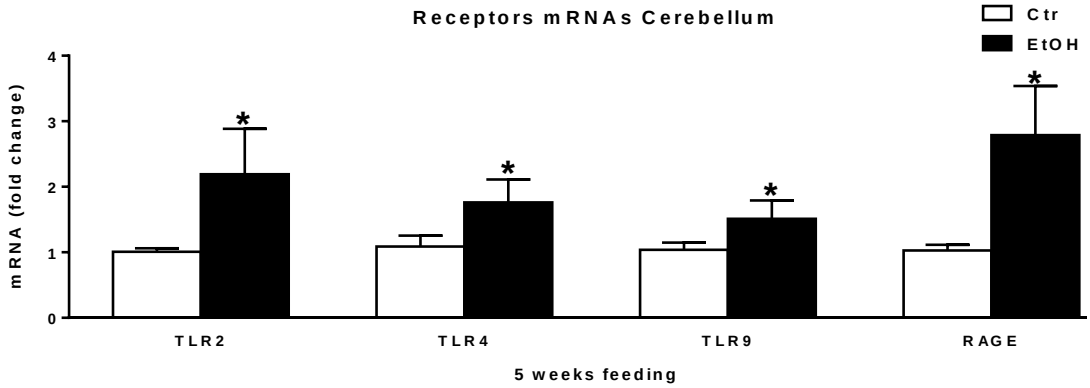

Figure 21. Cerebellar receptor expression in chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. Toll-like receptors (TLR2, TLR4, TLR9) and receptor for advanced glycation end products (RAGE) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.10. Cerebellar TNFα level in chronic alcohol-fed TLR4-KO mice

Murine cerebellar TNFα protein levels are significantly higher after chronic alcohol feeding (Figure 14B, 22). No significant difference of cerebellar TNFα protein level is found between chronic alcohol-fed TLR4-KO and isocaloric control mice (Figure 22). Furthermore, cerebellar TNFα protein level is significantly lower in chronic alcohol-fed TLR4-KO compared to WT mice (Figure 22).

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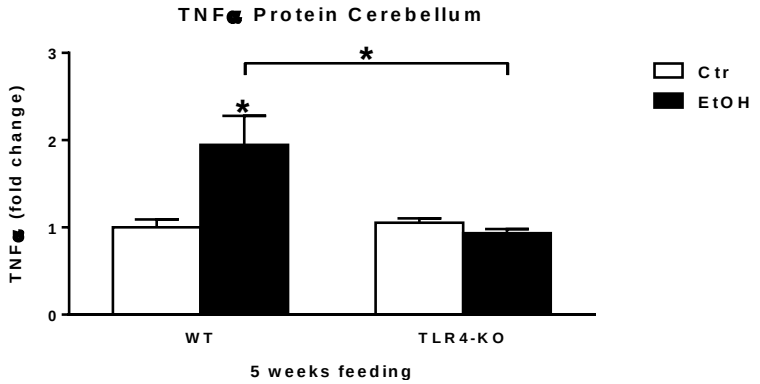

Figure 22. Cerebellar TNFα level in chronic alcohol-fed TLR4-KO mice. Wild type (WT; n=7 or 8) and Toll-like receptor-4 knockout (TLR4-KO; n=13 or 8) mice were fed with 5% ethanol containing (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. TNFα protein in whole cerebellar lysates was assessed by specific ELISA. Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr or WT-EtOH controls by Kruskal-Wallis non-parametric test).

6.2.11. Cerebellar IL-1β level in chronic alcohol-fed TLR4-KO mice

Pro-IL-1β mRNA level is significantly higher in the cerebella of chronic alcohol-fed compared to control mice (Figure 15A, 23A). Similarly murine cerebellar IL-1β protein levels are significantly higher after chronic alcohol feeding (Figure 15C, 23B). The levels of cerebellar pro-IL-1β mRNA (Figure 23A) and IL-1β protein (Figure 23B) are significantly higher in chronic alcohol-fed TLR4-KO compared to isocaloric control mice. The levels of cerebellar pro-IL-1β mRNA (Figure 23A) and IL-1β protein (Figure 23B) are significantly lower in either isocaloric or chronic alcohol-fed TLR4-KO compared to WT mice.

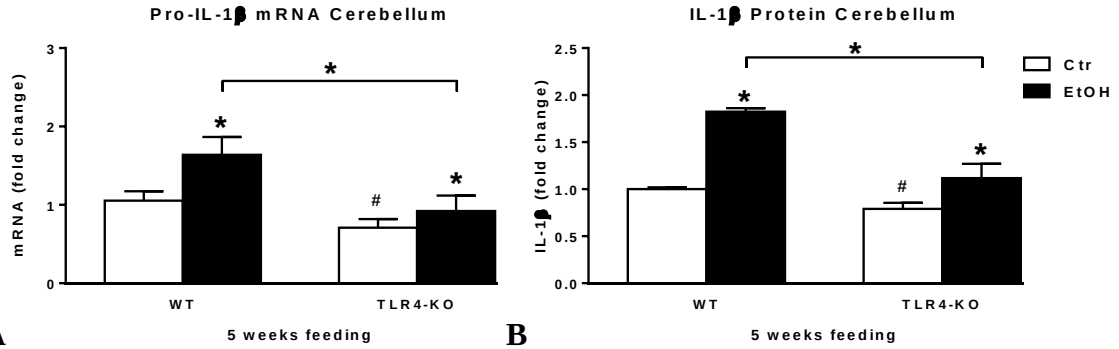

Figure 23. Cerebellar IL-1β level in chronic alcohol-fed TLR4-KO mice. Wild type (WT; n=7 or 8) and Toll-like receptor-4 knockout (TLR4-KO; n=13 or 8) mice were fed with 5% ethanol containing (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. Pro-interleukin-1β (pro-IL-1β) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control (A). IL-1β protein in whole cerebellar lysates was assessed by specific ELISA (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr or WT-EtOH controls; #: P value<0.05 relative to appropriate WT-Ctr controls by Kruskal-Wallis non-parametric test).

6.2.12. Cerebellar inflammasome expression in chronic alcohol-fed TLR4-KO mice

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Murine cerebellar TLR4, NLRP1, NLRP3, ASC and pro-caspase-1 mRNAs are significantly higher in chronic alcohol-fed compared to control mice (Figure 16A, 21A, 24A-E).

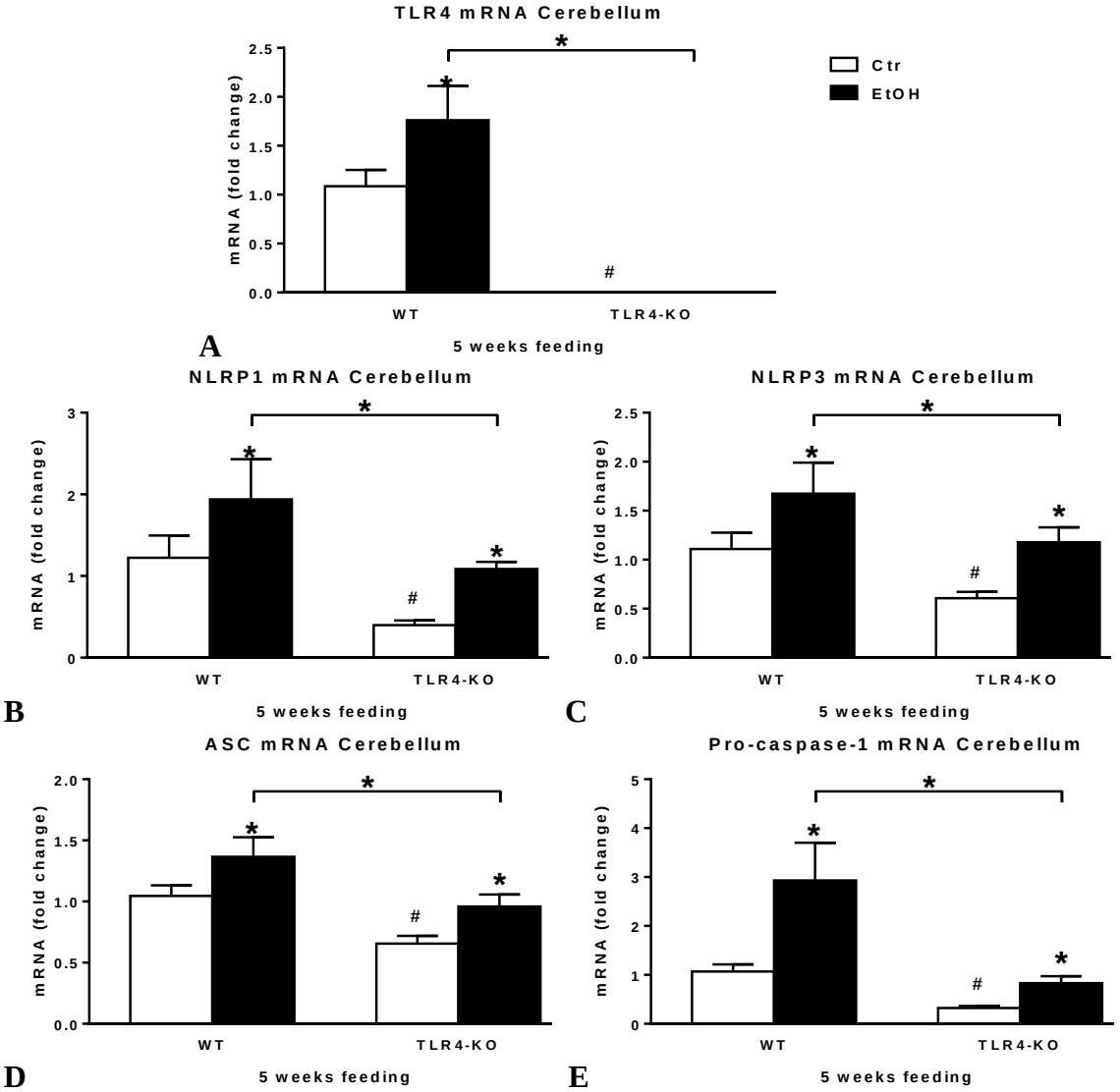

Figure 24. Cerebellar inflammasome expression in chronic alcohol-fed TLR4-KO mice. Wild type (WT; n=7 or 8) and Toll-like receptor-4 knockout (TLR4-KO; n=13 or 8) mice were fed with 5% ethanol containing (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. TLR4 (A), inflammasome sensors NLRP1 (B) and NLRP3 (C), inflammasome adaptor ASC (D) and inflammasome effector pro-caspase-1 (E) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control. Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr or WT-EtOH controls; #: P value<0.05 relative to appropriate WT-Ctr controls by Kruskal-Wallis non-parametric test).

Cerebellar TLR4 is not detectable in TLR4-KO mice and therefore is significantly lower than WT controls receiving either chronic alcohol or isocaloric diet (Figure 24A). The level of cerebellar NLRP1 (Figure 24B), NLRP3 (Figure 24C), ASC (Figure 24D) and pro-caspase-1 (Figure 24E) mRNA is significantly higher in chronic alcohol-fed WT or

TLR4-KO compared to isocaloric control mice. The level of cerebellar NLRP1 (Figure 24B), NLRP3 (Figure 24C), ASC (Figure 24D) and pro-caspase-1 (Figure 24E) mRNA is significantly lower in either isocaloric or chronic alcohol-fed TLR4-KO compared to WT mice.

6.2.13. Cerebellar inflammasome activation in chronic alcohol-fed TLR4-KO mice

Caspase-1 activity is significantly higher in WT murine cerebellum after chronic alcohol feeding compared to controls (Figure 17B, 20, 25). Similarly caspase-1 activity is significantly higher in TLR4-KO murine cerebellum after chronic alcohol feeding compared to controls (Figure 25). No significant difference of cerebellar caspase-1 activity is found between chronic alcohol-fed WT and TLR4-KO mice (Figure 25).

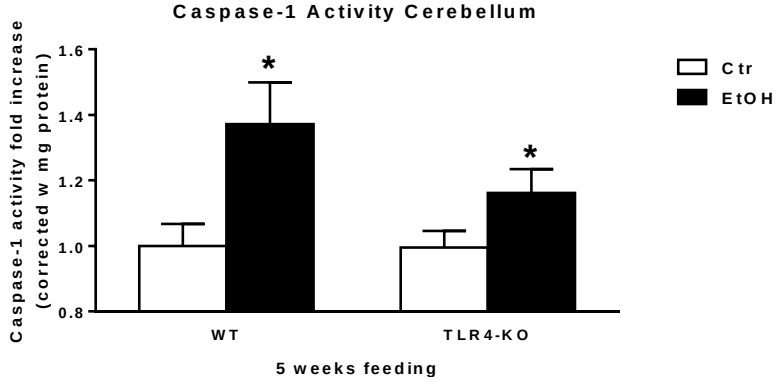

Figure 25. Cerebellar inflammasome activation in chronic alcohol-fed TLR4-KO mice. Wild type (WT; n=7 or 8) and Toll-like receptor-4 knockout (TLR4-KO; n=13 or 8) mice were fed with 5% ethanol containing (EtOH) or isocaloric (Ctr) diet for 5 weeks, respectively. Inflammasome activity was measured by caspase-1 colorimetric assay from whole cerebellar lysates. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

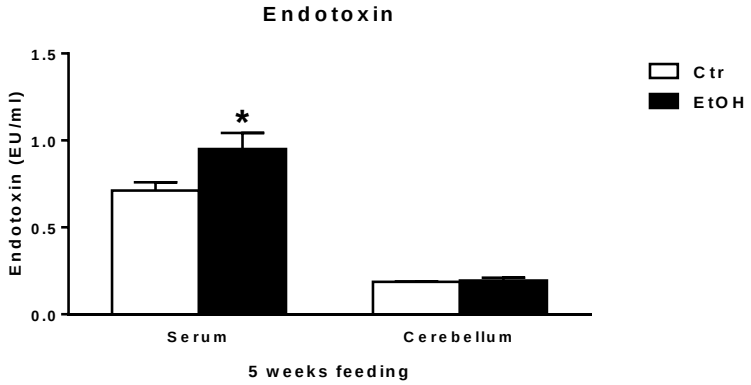

Figure 26. Endotoxin level in the cerebellum of chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=8) or isocaloric (Ctr; n=7) diet for 5 weeks. Endotoxin measurement was executed in serum and whole cerebellar lysates using Limulus Amebocyte Lysate-assay. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.14. Endotoxin level in the cerebellum of chronic alcohol-fed mice

Despite the higher serum-endotoxin levels in alcohol-fed mice (Figure 7B, 26), no difference of cerebellar endotoxin level is found between chronic alcohol-fed and control mice (Figure 26).

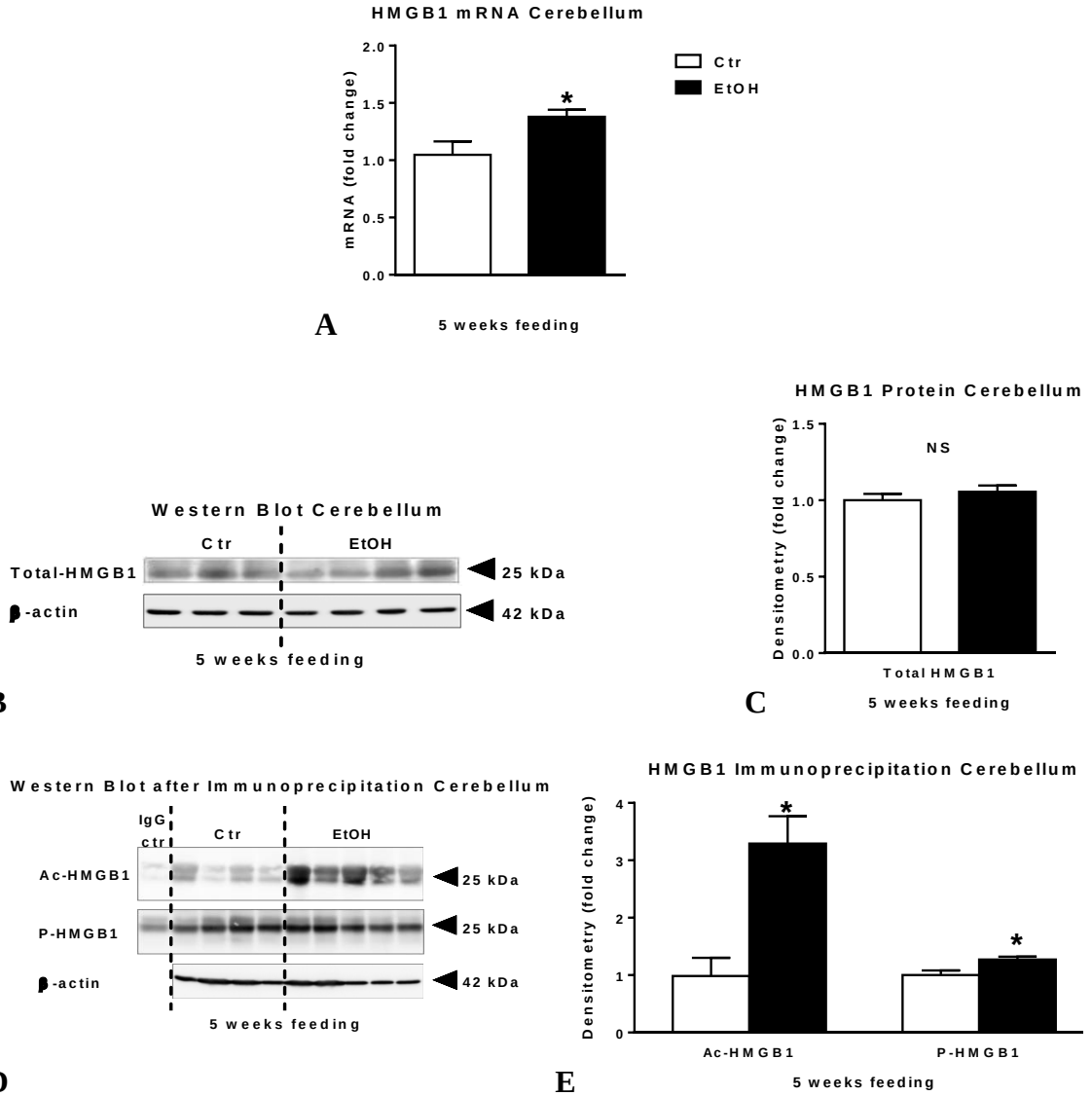

Figure 27. Cerebellar HMGB1 level and activation of chronic alcohol-fed mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. HMGB1 mRNA was assessed by qRT-PCR in cerebellum using 18S internal control (A). Total HMGB1 of whole cerebellar lysates was analyzed by Western blot using β-actin loading control (B) and quantified further by densitometry (C). Acetyl- (Ac-) and phospho- (P-) HMGB1 of whole cerebellar lysates were analyzed by IP using β-actin Western blot for loading control on a separate gel (D) and assessed further by densitometry (E). Cerebellar lysate of one representative alcohol-fed mouse was applied for IgG control (IgG ctr), using the same amount of protein. Bars represent mean±SEM (*: P value<0.05; NS: not significant; relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.15. Cerebellar HMGB1 level and activation of chronic alcohol-fed mice

Cerebellar HMGB1 mRNA is significantly higher in chronic alcohol-fed compared to control mice (Figure 27A). No significant difference of cerebellar total HMGB1 protein is found between chronic alcohol-fed and control mice (Figure 27B-C). Using immunoprecipitation, both acetylated- and phosphorylated-HMGB1 levels are significantly higher in the cerebellum of chronic alcohol-fed compared to control mice (Figure 27D-E).

6.2.16. Interleukin-1 receptor and interleukin-1 receptor antagonist in the cerebellum of chronic alcohol-fed mice

No difference of cerebellar interleukin-1 receptor (IL-1R) is found between chronic alcohol-fed and control mice (Figure 28) [unpublished data]. Mice receiving 5 weeks of alcohol feeding have significantly higher endogenous IL-1R antagonist levels in the cerebellum compared to controls (Figure 28).

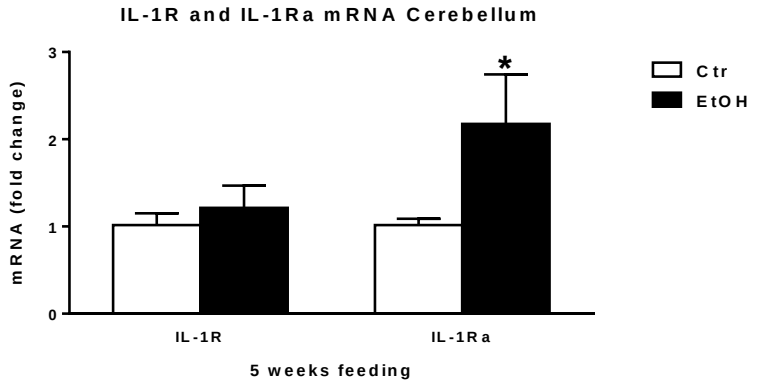

Figure 28. Interleukin-1 receptor and interleukin-1 receptor antagonist in the cerebellum of chronic alcohol-fed mice [partially unpublished data]. Wild type mice were fed with 5% ethanol containing (EtOH; n=6) or isocaloric (Ctr; n=8) diet for 5 weeks. Interleukin-1 receptor (IL-1R) and IL-1R antagonist (IL-1Ra) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.17. Cerebellar TNFα level in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice

Both cerebellar TNFα mRNA (Figure 14A, 29A) and protein (Figure 14B, 22, 29B) levels are significantly higher in chronic alcohol-fed compared to control mice. No significant difference of cerebellar TNFα mRNA is found between chronic alcohol-fed and isocaloric control mice after recombinant interleukin-1 receptor antagonist (rIL-1Ra) treatment (Figure 29A). In contrast cerebellar TNFα protein is significantly higher in chronic alcohol-fed rIL-1Ra compared to appropriate isocaloric control mice (Figure

29B). Both cerebellar TNFα mRNA (Figure 29A) and protein (Figure 29B) levels are significantly lower in rIL-1Ra compared to saline treated mice after chronic alcohol feeding.

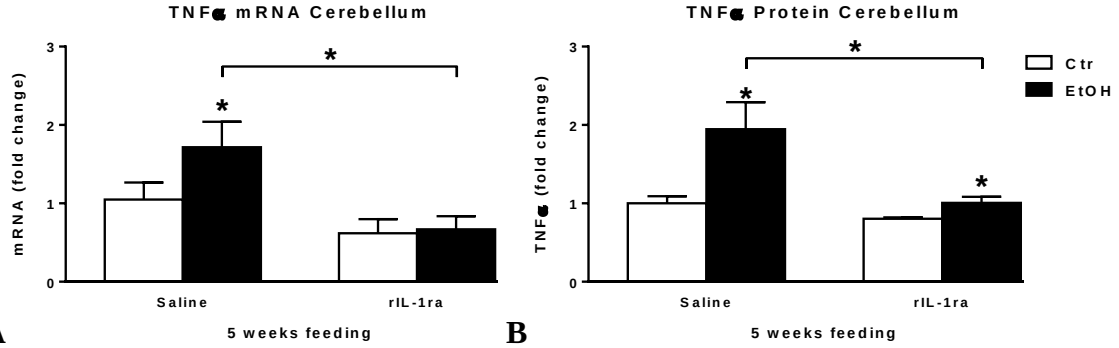

Figure 29. Cerebellar TNFα level in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=9 or 7) or isocaloric (Ctr; n=5 or 8) diet and received daily intra peritoneal recombinant interleukin-1 receptor antagonist (rIL-1Ra; anakinra: 25 mg/kg) or an equal amount of saline injections for 5 weeks, respectively. Tumor necrosis factor-α (TNFα) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control (A). TNFα protein in whole cerebellar lysates was assessed by specific ELISA (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr or saline-EtOH controls by Kruskal-Wallis non-parametric test).

6.2.18. Cerebellar IL-1β level in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice

Both cerebellar pro-IL-1β mRNA (Figure 15A, 23A, 30A) and IL-1β protein (Figure 15C, 23B, 30B) levels are significantly higher after chronic alcohol feeding compared to control mice.

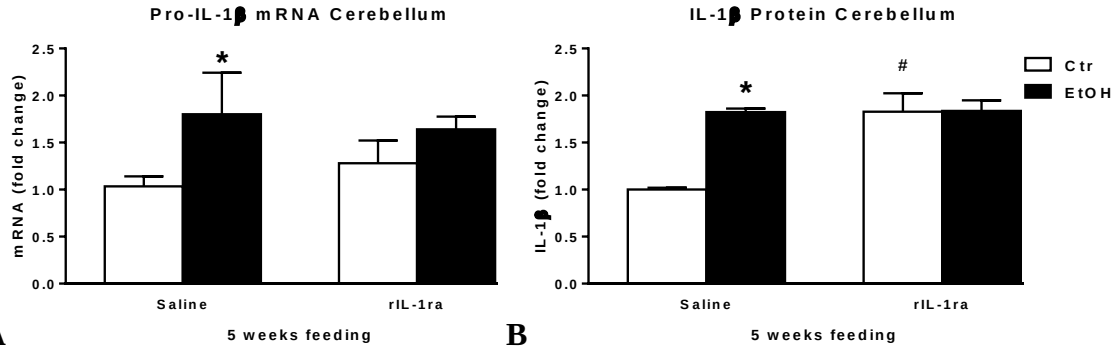

Figure 30. Cerebellar IL-1β level in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=9 or 7) or isocaloric (Ctr; n=5 or 8) diet and received daily intra peritoneal recombinant interleukin-1 receptor antagonist (rIL-1Ra; anakinra: 25 mg/kg) or an equal amount of saline injections for 5 weeks, respectively. Pro-interleukin-1β (pro-IL-1β) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control (A). IL-1β protein in whole cerebellar lysates was assessed by specific ELISA (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr controls; #: P value<0.05 relative to appropriate saline-Ctr controls by Kruskal-Wallis non-parametric test).

No significant difference of either cerebellar pro-IL-1β mRNA (Figure 30A) or IL-1β protein (Figure 30B) is found between chronic alcohol-fed recombinant interleukin-1 receptor antagonist (rIL-1Ra) and appropriate isocaloric control mice. The background of cerebellar IL-1β protein level is significantly higher in rIL-1Ra compared to saline treated mice receiving isocaloric diet (Figure 30B).

6.2.19. Cerebellar receptor and inflammasome expression in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice

No significant difference of cerebellar receptor (TLR2, TLR4, TLR9), inflammasome (NLRP1, NLRP3, ASC, pro-caspase-1), IL-1R or endogenous IL-1Ra mRNA is found between chronic alcohol-fed rIL-1Ra and isocaloric control mice (Figure 31) [unpublished data].

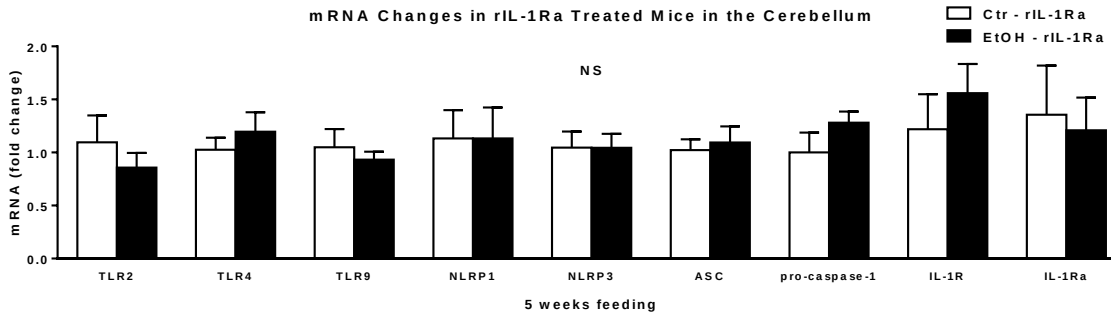

Figure 31. Cerebellar receptor and inflammasome expression in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice [unpublished data]. Wild type mice were fed with 5% ethanol containing (EtOH; n=9 or 7) or isocaloric (Ctr; n=5 or 8) diet and received daily intra peritoneal recombinant interleukin-1 receptor antagonist (rIL-1Ra; anakinra: 25 mg/kg) or an equal amount of saline injections for 5 weeks, respectively. Toll-like receptors (TLR2, TLR4, TLR9), inflammasome sensors (NLRP1 and NLRP3), inflammasome adaptor (ASC), inflammasome effector (pro-caspase-1), interleukin-1 receptor (IL-1R) and IL-1R antagonist (IL-1Ra) mRNA was assessed by qRT-PCR in cerebellum using 18S internal control. Bars represent mean±SEM (NS: not significant relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.20. Cerebellar inflammasome activation in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice

Caspase-1 activity is significantly higher in WT murine cerebellum after chronic alcohol feeding compared to controls (Figure 17B, 20, 25, 32). Similarly caspase-1 activity is significantly higher in chronic alcohol-fed rIL-1Ra treated compared to appropriate isocaloric control mice (Figure 32). Cerebellar caspase-1 activity is significantly lower in rIL-1Ra treated compared to saline treated mice after chronic alcohol feeding (Figure 32).

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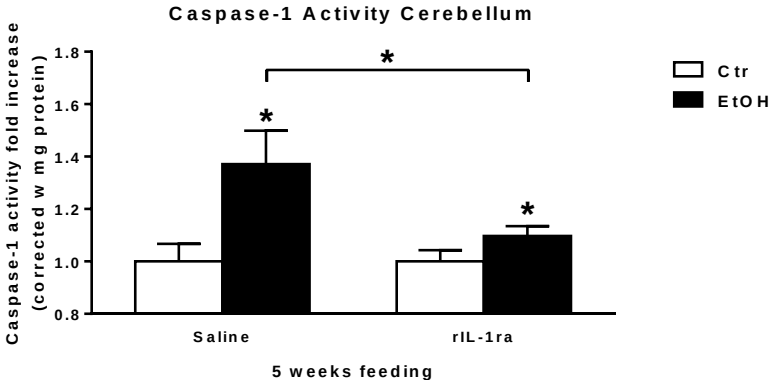

Figure 32. Cerebellar inflammasome activation in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=9 or 7) or isocaloric (Ctr; n=5 or 8) diet and received daily intra peritoneal recombinant interleukin-1 receptor antagonist (rIL-1Ra; anakinra: 25 mg/kg) or an equal amount of saline injections for 5 weeks, respectively. Inflammasome activity was measured by caspase-1 colorimetric assay from whole cerebellar lysates. Bars represent mean±SEM (*: P value<0.05 relative to appropriate Ctr or saline-EtOH controls by Kruskal-Wallis non-parametric test).

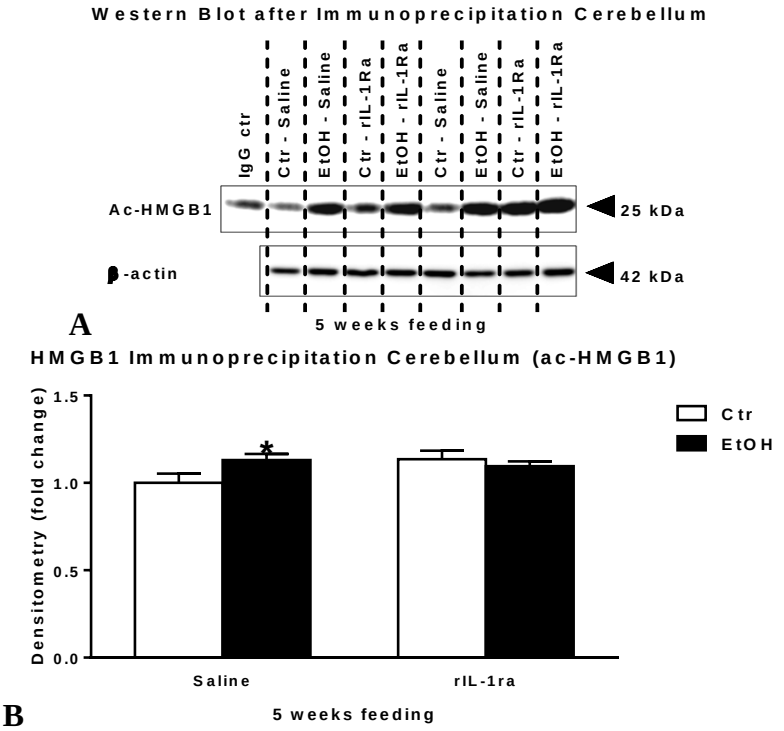

Figure 33. Cerebellar HMGB1 activation in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice. Wild type mice were fed with 5% ethanol containing (EtOH; n=9 or 7) or isocaloric (Ctr; n=5 or 8) diet and received daily intra peritoneal recombinant interleukin-1 receptor antagonist (rIL-1Ra; anakinra: 25 mg/kg) or an equal amount of saline injections for 5 weeks, respectively. Acetyl- (Ac-) HMGB1 of whole cerebellar lysates were analyzed by IP using β-actin Western blot for loading control on a separate gel (A) and assessed further by densitometry (B). Cerebellar lysate of one representative alcohol-fed mouse was applied for IgG control (IgG ctr), using the same amount of protein. Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

6.2.21. Cerebellar HMGB1 activation in chronic alcohol-fed recombinant interleukin-1 receptor antagonist treated mice

Using immunoprecipitation, acetylated-HMGB1 level is significantly higher in the cerebellum of chronic alcohol-fed compared to control mice (Figure 27D-E, 33A-B). However no significant difference of cerebellar acetylated-HMGB1 is found between chronic alcohol-fed rIL-1Ra treated and appropriate isocaloric control mice (Figure 33A-B).

6.2.22. Cerebellar TNFα and pro-IL-1β levels in response to intracranial recombinant IL-1β injection in mice

Cerebellar TNFα and pro-IL-1β mRNA (Figure 34A) as well as TNFα protein (Figure 34B) are significantly higher in mice receiving intracranial recombinant IL-1β injection compared to saline treated controls.

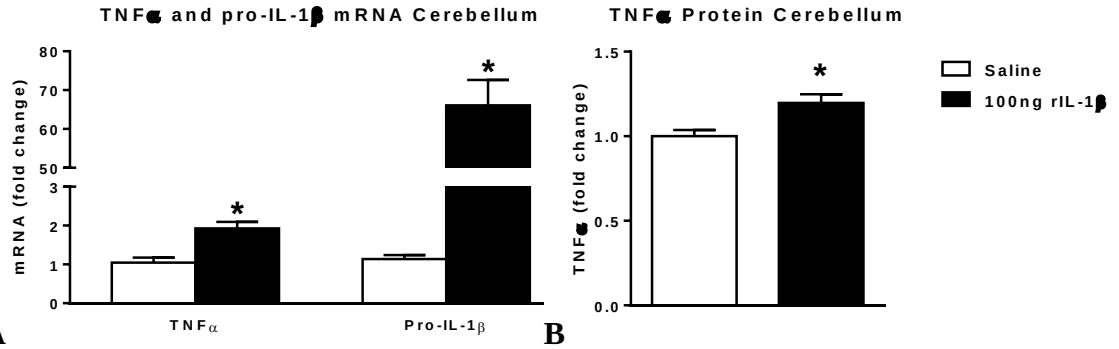

Figure 34. Cerebellar TNFα and pro-IL-1β levels in response to intracranial recombinant IL-1β injection in mice. Wild type mice received 100 ng (30 µl) intracranial mouse recombinant interleukin-1β (rIL-1β) (n=7) or an equal amount of saline solution (n=8). Tumor necrosis factor-α (TNFα) and pro-IL-1β mRNA was assessed by qRT-PCR in cerebellum using 18S internal control (A). TNFα protein in whole cerebellar lysates was assessed by specific ELISA (B). Bars represent mean±SEM (*: P value<0.05 relative to appropriate controls by Kruskal-Wallis non-parametric test).

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7. DISCUSSION

7.1. General considerations

These studies were executed in animal models, main conclusions should be cautiously drawn on generalizability in human. Human studies are necessary to confirm our findings. As a general role process of preparing knock-out animals might affect the outcome as theoretically removal of a gene can alter other mechanisms. Therefore other forms of pharmaceutical silencing should confirm our findings. The homogeneity of our animals (including age, gender, etc.) and the laboratory conditions (including air-, light conditions, diet, etc.) removed all known confounding variations. As cases and controls were treated equally, any possible factor should affect both groups in a similar direction. In this study our target was to evaluate the intestinal mucosa, therefore we discarded immune cell clusters within the Peyer patches which are involved in the pathogenesis of inflammation. Though it is a standard type of method for mucosal research. Using particular parts of the body like the cerebellum and proximal small intestine allows us to narrow down our conclusion toward specific sites.

7.2. Proximal small intestine

Mice receiving either one-time alcohol gavage or 5 weeks of alcohol feeding have significantly higher blood alcohol content than their appropriate controls.

Alcohol use, both acute binge and chronic alcohol intake, has numerous negative health effects on different organs including the intestine [14, 97]. The first line of attack of alcohol in the body is its entrance route, mainly the gastrointestinal system [14]. As a result the integrity of the mucosal barrier is lost and bacterial cell wall particles seep through into the circulation leading to elevated endotoxin levels in the sera of alcoholic patients [52]. Our observation confirmed previous reports of increased endotoxin levels in chronic alcohol feeding [98] and demonstrated that increase in serum endotoxin after acute alcohol binge was transient.

In this study we focused on the proximal small intestine which is in the first line of contact with alcohol and has a different pattern of gene activation than other parts. Antibacterial proteins are important elements of the mucosal innate immune system and are reportedly decreased in chronic alcoholics [19]. Similarly, in our model chronic

alcohol feeding reduces the antibacterial protein, Reg3b, level in the proximal small intestine. Interestingly, acute alcohol binge increases Reg3b level while increased serum endotoxin is still implying the loss of barrier function. We speculate that increased Reg3b may represent a compensatory mechanism to repair or maintain gut barrier after an acute alcohol binge. While this mechanism is likely to be exhausted after repeated and sustained alcohol exposure in a chronic model, resulting in decreased Reg3b levels affirmed by others' and our findings. These observations suggest that both acute binge and chronic alcohol intake disturbs the gut mucosal barrier function.

It is notable that the control group of the chronic feeding has higher amount of intestinal Reg3b protein (longer exposure) than acute gavage controls. The increased baseline Reg3b production in chronic feeding might be attributable to the high fat content of the applied diet, as in high-fat diet model the transcription of Reg3b of the neighbor mesenteric adipose tissue is induced [99]. However we cannot rule out the possibility of post-transcriptional or post-translational modifications of Reg3b in our models.

Once there is a mucosal barrier breach inflammatory changes start developing at the site. Based on previous studies NF-κB activation and pro-inflammatory cytokine, TNFα release play an important role in regulating intestinal epithelial function in inflammatory bowel disease [26, 27]. TNFα secreting cells are increased in the inflamed mucosa in IBD [100]. Upregulation of TNFα involves activation of transcription factors, such as NF-κB [101] or activator protein-1 (AP-1) [102]. NF-κB is a major regulator in proinflammatory pathways and can upregulate multiple proinflammatory cytokines and chemokines, including TNFα and pro-IL-1β [85, 87]. We established that TNFα protein increased only in chronic alcohol feeding in the proximal small intestine. Both acute and chronic alcohol consumption increase TNFα mRNA and yet acute binge only minimally increases NF-κB. NF-κB is not the sole mediator of TNFα induction, therefore in our acute binge model there might be other transcription factors involved in the upregulation of TNFα mRNA, such as AP-1 [102].

Despite the fact that acute alcohol increases TNFα mRNA, TNFα protein levels remained unchanged while in chronic alcohol feeding TNFα protein levels were elevated. This phenomenon could be in part due to post-transcriptional modification by miR-155, which is induced in chronic alcohol feeding in contrast to the lack of induction of miR-155 in the acute binge drinking model. MiRNA-155 is a positive

regulator of TNFα by increasing its mRNA stability in macrophages [103]. Further work on other possible post-transcriptional or post-translational modifications are necessary.

The data suggest that chronic alcohol leads to a robust inflammatory response in proximal small intestine characterized by increased TNFα, NF-κB activation and reduced expression of the antimicrobial protein, Reg3b. Our results indicate that in contrast to chronic alcohol feeding, acute alcohol binge results in a transient increase in serum endotoxin and a further increase in Reg3b leading to the speculation that rapid restoration of the gut barrier may occur after barrier disruption caused by acute alcohol binge, whereas chronic alcohol-fed mice might lose this reserve capacity during time.

NF-κB pathway also mediates IL-1β increase and augmented IL-1β is associated with enhanced permeability in Caco2 cell line [30]. Furthermore, IL-1β gene polymorphisms are associated with the course and severity of IBD [104]. Colitis driven by specific microbiota is associated with induced monocytic IL-1β release [105]. Recent data involving small number of patients is inconclusive on whether IL-1β signaling blockage by IL-1Ra treatment is beneficial or not in IBD [106, 107]. Based on alcohol induced intestinal NF-κB activity and increased endotoxin level, we evaluated IL-1β production in the chronic alcohol-fed mouse model where the inflammatory changes seemed to be more robust than in the acute binge model. Even though intestinal NF-κB activity is increased in both alcohol gavage and chronic alcohol feeding model there is no difference of pro-IL-1β mRNA or IL-1β protein between either alcohol gavage or chronic alcohol-feeding and appropriate controls. Similarly there is no difference of mRNA expression of inflammasome components between either alcohol gavage or chronic alcohol-feeding and appropriate controls. These data suggest no inflammasome involvement in the proximal intestine of alcohol-fed mice. Interestingly, a recent report shows increased murine proximal intestinal IL-1β mRNA level one hour after intraperitoneal administration of 20% 5.5 or 8.25g/kg ethanol [108]. The differences between the methodologies certainly could account for the discrepancies in the findings, as our three times 50% 5g/kg oral alcohol administration was checked at 6h after the last gavage. Additionally in our study the Peyer patches, a collection of immune cells, were discarded, which might be responsible for inflammasome activation. More detailed

studies are warranted to understand the relation between inflammatory response of proximal intestine and the harmful effects of alcohol.

7.3. Liver

Alcohol, DAMPs and PAMPs originating from the intestine can affect other organs, including the site of first pass metabolism, the liver. Amplification of TNFα production by chronic alcohol in Kupffer cells involves alcohol-induced induction of NF-κB activation [103]. Our results showed that TLR4 signaling and activation of inflammasome complex followed by IL-1β production are involved in the pathogenesis of alcoholic liver disease [34, 62]. Furthermore, caspase-1, ASC and IL-1R deficiency protected chronic alcohol-fed mice against from developing alcoholic hepatitis and decreased the level of steatosis and liver damage [34].

However, it has been showed that NLRP3 deficient alcohol-fed B6 mice were protected against alcohol-induced liver injury [109]. The possible explanations for the differences might be due to discrepancies in methodology, the increased levels of IL-18 and the possibility of increased leakage of LPS from altered microbiota [109, 110]. Similarly, ASC deficient mice are prone to develop liver injury in a non-alcoholic fatty liver disease / steatohepatitis model [110]. Further detailed studies are warranted to understand the role of ASC/NLRP3 on microbiota and inflammation in the context of alcohol.

Results from our lab showed that IL-1R blockage with rIL-1Ra treatment prevents the development and progression of alcoholic liver disease [34]. Currently there is an ongoing clinical trial evaluating the efficacy of rIL-1Ra in severe acute alcoholic hepatitis [111].

7.4. Cerebellum

Alcohol, PAMPs and DAMPs originating from intestine and liver via circulation and/or neural feedback mechanisms can affect the brain.

Chronic ethanol feeding results in neuroinflammatory changes in cortical, hippocampal and cerebellar brain regions [45, 46]. Our study focused mainly on changes in the cerebellum after chronic alcohol intake. The immunological aspect of ethanol induced cerebellar damage has not yet been extensively studied and the high cellular density and

easy accessibility of cerebellum provides a preferable candidate for research. Alcohol intake impairs integration of sensory perception, coordination and motor control, which are functions of the cerebellum [112, 113]. In long term, alcohol consumption can lead to cerebellar atrophy which is in part due to malnutrition. Increasing evidences suggest that long-term neurodegenerative changes in the cerebellum of alcoholics are not solely due to lack of dietary factors and that immunological pathways might be involved [50].

The neuroinflammatory changes include induction of pro-inflammatory cytokines and chemokines, DAMPs, NF-κB, inflammasome and iNOS activation, NADPH-oxidase and ROS mediated pathways [45, 46]. To evaluate the mechanism by which cerebellum can be affected in our model of chronic alcohol feeding, we first evaluated NF-κB activation. Consistent with previous studies, there is an increase in NF-κB DNA binding in the cerebellum of chronic alcohol-fed WT mice compared to controls.

While IL-1β protein production is largely dependent on caspase-1 activation, pro-IL-1β mRNA induction is NF-κB mediated [114]. We aimed to evaluate whether activated NF-κB can upregulate TNFα and pro-IL-1β in our animal model as a part of the inflammatory changes. Similar to the effects of long-term ethanol feeding in a rat model, our results indicate that chronic alcohol-fed mice have increased cerebellar protein levels of inflammatory mediators including TNFα and IL-1β [46]. Short-term ethanol administration in mice also results in increased brain TNFα levels, while IL-1β is only increased after additional intra-peritoneal LPS injection [43]. We observed that IL-1β induction by alcohol was identical in the cerebral cortex and the cerebellum of alcohol-fed mice suggesting that investigation of the cerebellum is one of the representative sites of alcohol-induced neuroinflammation.

The rise in cerebellar IL-1β and TNFα could have serum origin, as chronic alcohol feeding increases serum cytokine levels and there are saturable transporter systems at the BBB [34, 115]. However in our study we observed an increase in NF-κB and inflammasome activation which is suggestive of a local cytokine production by cerebellar cells.

Our data revealed IL-1β increment in the brain after chronic alcohol administration, therefore we evaluated the role of inflammasome activation as a contributor to IL-1β production. We showed that the mRNA expression of several inflammasome components including receptors, (NLRP1, NLRP3), adaptor (ASC) and effector (pro-

caspase-1) are significantly increased in the cerebellum and cerebrum after chronic alcohol administration in mice. Caspase-1 is the effector enzyme of the inflammasome complex and it exerts its proteolytic effect on pro-IL-1β when the 45-kDa pro-caspase-1 protein is cleaved into its active forms, caspase-1 p20- and p10-kDa subunits [81]. In our model caspase-1 activity is increased in the cerebella and cerebra of chronic alcohol-fed compared to pair-fed mice and increased levels of the active, cleaved caspase-1 p10 on Western blots support this finding (Figure 17).

Several studies have shown the importance of the inflammasome in neuroinflammation. NLRP3 activation is involved in inflammation and tissue damage in response to amyloid-β in Alzheimer's [93]. Attenuation of ASC or the NLRP1 receptor improves traumatic brain injury [96, 116]. Caspase-1 is involved in the pathogenesis of both neurodegenerative and neuroinfectious diseases [94, 95, 117]. Molecules such as NLRC4 [81, 118], absent in melanoma 2 (AIM2) receptors [119] and the pannexin-1 channel [120] facilitate intracellular DAMP transport, stimulate the NLRP3 inflammasome, and can be activated in the neural system [81, 118].

To further investigate the mechanistic role of inflammasome activation in alcohol-induced IL-1β formation we evaluated neuroinflammation in mice deficient in the inflammasome sensor, NLRP3, or the adaptor molecule, ASC. In our animal model, NLRP3 and ASC are both necessary for inflammasome activation and IL-1β production as both NLRP3-KO and ASC-KO mice were protected from caspase-1 activation and increased IL-1β production. This was in contrast with the significant increase in cerebellar caspase-1 activity and mature IL-1β level in alcohol-fed WT mice. Our data suggest that the NLRP3 inflammasome complex is active and necessary in the cerebella of mice with chronic ethanol-feeding. This effect should not be the result of less alcohol intake in KO mice as animals were fed equally and serum alcohol levels were comparable between the genotypes.

As two separate signals are required for NLRP3 inflammasome activation: priming via TLRs to upregulate the expression of inflammasome components and a second signal to activate the inflammasome complex to cleave caspase-1 [82], we first evaluated the expression of some candidate receptors. Chronic alcohol feeding increases cerebellar mRNA of TLR2, TLR4, TLR9 and RAGE, as expected. NLRP3 inflammasome can be induced by either MyD88 or TRIF signaling pathways that are downstream of TLRs

[82]. Previous studies indicate that TLR4 ligands promote TNFα and MCP-1 production which contribute to microglia accumulation, astrogliosis and inflammatory cytokine production in alcoholic neuroinflammation [42, 46]. We investigated the role of TLR4 in inflammasome activation in the brain. In contrast to WT mice, alcohol-fed TLR4-KO mice have no increase in TNFα protein levels in the cerebellum compared to controls.

Previous studies showed that the cerebral cortex of TLR4-KO rats is protected from alcohol-induced IL-1β induction [46], a central mediator of neuroinflammation [121]. In our model there is an overall significantly attenuated expression of the inflammasome components (NLRP1, NLRP3, ASC) and effector molecule (pro-caspase-1) and decrease in pro-IL-1β mRNA and IL-1β protein levels in alcohol-fed TLR4-KO compared to WT mice, however the levels remain induced upon alcohol challenge. Alcohol-induced caspase-1 activity is comparable between TLR4-KO and WT mice. These data suggest that TLR4 only partially contributes to the expression, likely to the priming of inflammasome induction in the brain after alcohol feeding and that activation of the inflammasome is independent of TLR4.

PAMPs, including LPS, activate the TLR4 signaling pathway [122]. LPS mediates alcoholic liver disease and is increased in the serum of chronic alcohol-fed mice [62] as well as in patients with alcoholic cirrhosis [63]. LPS could increase IL-1β directly via the TLR4-mediated pathway in the brain. Therefore we investigated the possible direct role of LPS in alcohol-induced inflammasome activation in the brain. Despite increased endotoxin levels in the serum, we found no detectable LPS in the brain of alcohol-fed mice. These data suggest that TLR4-mediated pathways are responsible for alcohol-induced TNFα increment in the cerebellum even in the absence of LPS in the brain. Moreover, increased IL-1β in the cerebellum after alcohol-feeding was not fully TLR4- or directly LPS-mediated. Previous studies are controversial on the effect of alcohol on the integrity of the blood-brain-barrier. Some have found no increase in permeability [123-125], while others suggest impairment [126, 127]. Reports also indicate an indirect role for LPS in neuroinflammation [49]. Neuroimmune reflexes have been shown to sense and respond to peripheral injuries [49]. Vagotomy in animals intraperitoneally injected with LPS diminishes the IL-1β response in the brain, but not on the periphery or the pituitary gland [59]. While we cannot rule out indirect effects of LPS via extra-

cerebral targets, our data suggest that LPS is not directly involved in neuroinflammation induced by alcohol.

These observations led us to explore other danger molecules that could induce inflammation in our model. HMGB1, a DAMP, was shown to mediate ischemic brain damage [128] and nasal introduction of small interfering-RNA targeting HMGB1 in a post-ischemic brain injury model resulted in significant neuroprotection [129]. Interestingly, HMGB1 antibodies or small interfering RNA targeting HMGB1 were used effectively in arthritis, sepsis, cancer or post-ischemic brain injury models, all of which are associated with induction of the inflamamsome-IL-1β cascade [130]. Furthermore, recent reports suggest that HMGB1 can contribute to the activation of the inflammasome complex to cleave pro-IL-1β into its mature (17kD), biologically active form [90, 131]. NLRP3 and ASC, independent of caspase-1, are necessary for HMGB1 release [130] and IL-1β can induce HMGB1-release from monocytes and macrophages [132, 133]. Posttranslational modification by acetylation and phosphorylation is important for translocation of nuclear HMGB1 to the cytoplasm and its subsequent release [134] as a result acetylated or phosphorylated HMGB1 is recognized as a danger signal [135, 136]. Although HMGB1 itself does not exert proinflammatory effects, upon phosphorylation, HMGB1 can enhance inflammation by binding to other cytokines and initiating or promoting signaling through TLR2, TLR4, TLR9, RAGE or IL-1R [89, 135-137]. Similar to alcoholic steatohepatitis [138], HMGB1 receptor mRNA expression (TLR2, TLR4, TLR9 and RAGE) as well as phosphorylated and acetylated HMGB1 levels are induced by alcohol in murine cerebellum. These data suggest that HMGB1 is a possible DAMP that could induce signaling through TLRs to augment the inflammasome response and IL-1β production in alcoholic neuroinflammation. HMGB1 also exerts neuronal apoptosis via caspase-3 and induces iNOS, TNFα, COX2 and IFNγ expression in primary microglia [139]. Increase in caspase-3 activity, iNOS, COX2 and IFNγ are observed in alcohol feeding [46]. Thus, it is feasible that in addition to activation of the inflammasome-caspase-1 complex, increased phosphorylated- and acetylated-HMGB1 could also contribute to caspase-3 activation in the brain of chronic alcoholic mice.

IL-1β exerts its biologic function via the IL-1 receptor (IL-1R) and amplifies inflammation [83]. Although IL-1R mRNA remains unchanged after chronic alcohol

feeding, its activator IL-1β and natural endogenous inhibitor IL-1Ra are both increased. The inhibitor of IL-1R, IL-1Ra occupies the IL-1R without transducing activation unlike the activators, IL-1α and IL-1β [84]. The cerebellar alcohol-induced increase of IL-1Ra suggests the possible activation of a negative feedback loop in order to establish a new steady state.

Recombinant IL-1Ra, anakinra, is a disease-modifying anti-inflammatory medication for active rheumatoid arthritis and has been suggested for treating a variety of diseases with excessive IL-1β production, including recurring-fever syndromes, gout or diabetes mellitus [140]. To evaluate the involvement of IL-1/IL-1R signaling in alcohol-induced neuroinflammation, we treated mice with daily doses of 25mg/kg recombinant IL-1Ra, anakinra, during alcohol feeding [34]. In short-term ethanol administration in mice, IL-1Ra pretreatment efficiently reduced the sedation and motor impairment recovery time [141] supporting the observation that IL-1 signaling is involved in alcohol-induced neuroinflammatory changes. Moreover our in vivo experiments suggest that rIL-1Ra interrupts the circuit of neuroinflammation and prevents the excessive activation of the inflammasome complex and neuroinflammation in the cerebellum of chronic alcohol-fed mice. Recombinant IL-1Ra treatment prevented the alcohol-induced mRNA expression of receptors (TLR2, TLR4, TLR9), inflammasome components (NLRP1, NLRP3, ASC, procaspase-1), proinflammatory cytokines (TNF-α, pro-IL-1β) and IL-1Ra in the cerebellum. Furthermore, rIL-1ra administration significantly attenuated TNF-α protein levels in alcohol-fed mice, suggesting interruption of the positive-feedback loop in inflammatory cytokine production. Importantly, there was no increase in IL-1β protein levels, and caspase-1 activity was significantly attenuated in rIL-1ra treated alcohol-fed mice. There is increasing evidence for a feed-forward activation between acetylated HMGB1 and IL-1 as IL-1β can induce HMGB1 acetylation and therefore contributes to its release in monocytes [133]. Consistent with this notion, rIL-1Ra treatment prevented the increase in acetylated HMGB1 in alcohol-fed mice in our model.

To evaluate whether a positive pro-inflammatory feedback loop was induced by IL-1β in the cerebellum, some mice received intracranial injection of recombinant mouse IL-1β, which resulted in increased TNFα and IL-1β mRNA as well as TNFα protein levels in the cerebellum. Altogether these data suggest an interruption of the positive feedback

loop in inflammatory cytokine/DAMP production by rIL-1Ra. All of these findings might be also beneficial for the patients in the ongoing trial evaluating the efficacy of rIL-1Ra in severe acute alcoholic hepatitis [111].

7.5. Concluding remarks and human relevance

Structure (histology) of human and murine organs (small intestine, liver or cerebellum) are similar [142]. There are evolutionary preserved biological responses to harmful agents, as alcohol induced immune response identified in murine or human intestine, liver and brain are similar corroborated by research on cadavers, biopsy samples and cell lines [23, 24, 43-47, 143]. Alcohol induced gut permeability is increased, neuroinflammation is present in both species and increasing evidence suggest that these pathways are leastwise overlapping [23, 24, 43-47, 143]. Alcohol impairs hippocampal neurogenesis and cerebral innate immune response has been linked to the addictive effect of alcohol [47, 144, 145]. The presented data raise new questions, including where exactly the organ damage cascade begin; what the first organ is to be affected by alcohol in inducing inflammation; can this inflammatory cascade be stopped and where; is there an organ-organ crosstalk which is suggested by the findings of the intracerebral injection of IL-1β; these questions warrant further research. All of the research effort is in order to find feasible intervention for the inflammatory pathways of alcohol-induced end organ damage.

7.6. Future perspectives

- The change from augmented to suppressed antimicrobial protein levels in the proximal intestine in acute vs chronic models is not fully understood. Further mechanical studies are warranted.
- Due to alcohol-induced colonic mucosal and microbiota changes large intestinal inflammasome activation might be informative. In addition to IL-1β, studying other members of the same signaling pathway might shed light to the underlying mechanism.
- The association between gender and the inflammasome activation is not known, and there is no evidence of difference. However, it might be of value to do

studies using both male and female mice to recognize possible gender differences.

- Both astrocytes and microglia has been shown to be involved in the inflammasome activation in the cerebral cortical regions, however no data has emerged on the cellular distribution of IL-1β induction and its main targets in the cerebellum. Therefore it would be of value to further evaluate this in animal models and cell cultures.
- As inflammasome is activated in chronic alcohol model in the cerebellum, the timeline of its occurrence might be of interest, therefore acute gavage models could be of benefit.
- To address whether HMGB1 is indeed a major harmful agent in the alcohol induced neuroinflammatory process, HMGB-1 silencing might be informative in in vivo and in vitro models.
- HMGB1 pathway has been considered as a candidate for treatment for alcohol induced inflammatory changes. It is noteworthy to test siHMGB1 whether it is able to halt or reverse the inflammatory processes.

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8. CONCLUSIONS

Based on our animal model we could conclude the following:

- Both acute and chronic alcohol intake impair intestinal barrier function; the increased serum endotoxin after acute alcohol binge is transient.
- Increased serum endotoxin after chronic alcohol administration is associated with reduced Reg3b whereas acute alcohol administration induces Reg3b production.
- Chronic alcohol intake induces inflammation in the gut-liver-brain axis, including NF-κB activation and TNFα production.
- Chronic alcohol administration up-regulates and activates the NLRP3/ASC inflammasome leading to caspase-1 activation and IL-1β production in the liver and cerebellum in contrast to the proximal small intestine.
- Chronic alcohol intake increases the levels of phosphorylated and acetylated forms of HMGB1 in the cerebellum.
- Disruption of IL-1/IL-1R signaling by rIL-1Ra prevents alcohol-induced inflammasome activation, neuroinflammation and steatohepatitis by inhibiting inflammasome activation, IL-1β and TNFα production.
- IL-1β amplifies neuroinflammation and steatohepatitis.

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9. SUMMARY

9.1. English summary

Introduction: Alcohol-induced neuroinflammation and steatohepatitis is mediated by proinflammatory cytokines, including IL-1β. IL-1β production requires caspase-1 activation by inflammasomes-multiprotein complexes that are assembled in response to danger signals. **Hypothesis:** We hypothesized that alcohol-induced inflammasome activation contributes to increased IL-1β in the gut-liver-brain axis. **Methods:** WT and TLR4-, NLRP3-, and ASC-deficient (KO) mice received an ethanol-containing or isocaloric control diet for 5 weeks, and some received the rIL-1Ra, anakinra, or saline treatment. Inflammasome activation, proinflammatory cytokines, endotoxin, and HMGB1 were measured in murine proximal intestine and cerebellum. **Results:** Expression of inflammasome components (NLRP1, NLRP3, ASC) and proinflammatory cytokines (TNF-α, MCP-1) was increased in brains and partially in intestines of alcohol-fed compared to control mice. Increased caspase-1 activity and IL-1β protein in ethanol-fed mice indicated inflammasome activation in the brain, in contrast to the intestine. TLR4 deficiency protected from TNF-α, MCP-1, and attenuated alcohol-induced IL-1β increases in the brain. The TLR4 ligand, LPS, was increased in the serum, but not in the cerebellum of alcohol-fed mice. We found up-regulation of acetylated and phosphorylated HMGB1 and increased expression of the HMGB1 receptors (TLR2, TLR4, TLR9, RAGE) in alcohol-fed mice in the brain. NLRP3- or ASC-deficient mice were protected from caspase-1 activation and alcohol-induced IL-1β increase in the brain. Furthermore, in vivo treatment with rIL-1Ra prevented alcohol-induced inflammasome activation and IL-1β, TNF-α induction in the brain, and prevented acetylated HMGB1 increase in the cerebellum. **Conclusion:** In conclusion, alcohol induces inflammation in the gut-liver-brain axis. Alcohol up-regulates and activates the NLRP3/ASC inflammasome, leading to caspase-1 activation and IL-1β increase in the cerebellum and liver in contrast to the intestine. IL-1β amplifies neuroinflammation, and steatohepatitis. Disruption of IL-1/IL-1R signaling prevents alcohol-induced inflammasome activation, neuroinflammation and steatohepatitis. Increased levels of acetylated and phosphorylated HMGB1 may contribute to alcoholic neuroinflammation.

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9.2. Magyar összefoglalás

Bevezetés: Az alkohol okozta neuroinflammációt és steatohepatitist proinflammatorikus citokinek mediálják, mint pl. az IL-1β. Az IL-1β képzéséhez az inflammaszóma multiprotein-komplex általi kaszpáz-1 aktivációra van szükség. Az inflammaszóma összeszerelése vészjelekre adott válasz. **Feltevés:** Az alkohol okozta inflammaszóma aktiváció hozzájárul a magasabb IL-1β-hoz a bél-máj-agy tengelyen. **Módszerek:** vad típusú, TLR4-, NLRP3-, és ASC-hiányos (KO) egerek alkohol tartalmú vagy isokalorikus kontroll diétaát kaptak 5 héten át. Néhány állat rIL-1Ra, anakinra, vagy fiziológiás sóoldatot kapott. Inflammaszóma aktiváció, proinflammatorikus citokinek, endotoxin és HMGB1 mérése történt egér proximalis vékonybélben és cerebellumban. **Eredmények:** Inflammaszóma komponensek (NLRP1, NLRP3, ASC) és proinflammatorikus citokinek (TNF-α, MCP-1) expressziója megnövekedett az agyban és részben a bélben alkohol diétában részsülő egereknél összehasonlítva a kontrollal. A megnövekedett kaszpáz-1 aktivitás és IL-1β fehérje mennyisége alkohol diétában részesült egerekben az inflammaszóma aktivációjára utalt az agyban, ellentétben a béllel. A TLR4 hiánya meggátolta a TNF-α és MCP-1, valamint csökkentette az alkohol okozta IL-1β növekedést az agyban. A TLR4 ligand, LPS, magasabb volt az alkoholos egerek szérumában, de nem a cerebellumában. Alkohol diétán tartott egerek agyában magasabb az acetilált és foszforilált HMGB1 szintje, valamint az HMGB1 receptorok expressziója (TLR2, TLR4, TLR9, RAGE). Az NLRP3- vagy ASC-hiánya meggátolta a kaszpáz-1 aktivációt és az alkohol okozta IL-1β emelkedést az agyban. In vivo rIL-1Ra kezelés meggátolta az alkohol okozta inflammaszóma aktivációt, valamint az IL-1β és a TNF-α emelkedését az agyban, továbbá elmaradt az acetilált HMGB1 emelkedés a cerebellumban. **Következtetések:** Az alkohol gyulladást okoz a bél-máj-agy tengelyen. Az alkohol növeli és aktiválja az NLRP3/ASC inflammaszómát, mely kaszpáz-1 aktiváción keresztül IL-1β növekedéshez vezet a cerebellumban és a májban, ellentétben a béllel. Az IL-1β felerősíti a neuroinflammációt és steatohepatitist. Az IL-1/IL-1R jelátviteli útvonal gátlása megelőzi az alkohol okozta inflammaszóma aktivációt, a neuroinflammációt és steatohepatitist. A megnövekedett mennyiségű acetil és foszforilált HMGB1 hozzájárulhat az alkohol okozta neuroinflammációhoz.

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11. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

11.1. Publications related to the theme of the Ph.D. thesis:

1. Petrasek J, Bala S, Csak T, Lippai D, Kodys K, Menashy V, Barrieau M, Min SY, Kurt-Jones EA, Szabo G. (2012) IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest, 122: 3476-3489.
2. Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, Szabo G. (2013) Alcohol-induced IL-1beta in the brain is mediated by NLRP3/ASC inflammasome activation that amplifies neuroinflammation. J Leukoc Biol, 94: 171-182.
3. Lippai D, Bala S, Csak T, Kurt-Jones EA, Szabo G. (2013) Chronic alcohol-induced microRNA-155 contributes to neuroinflammation in a TLR4-dependent manner in mice. PLoS One, 8: e70945.
4. Lippai D, Bala S, Catalano D, Kodys K, Szabo G. (2014) Micro-RNA-155 deficiency prevents alcohol-induced serum endotoxin increase and small bowel inflammation in mice. Alcohol Clin Exp Res, 38: 2217-2224.

11.2. Other publications and abstracts related to the theme of the Ph.D. thesis:

11.2.1. Oral presentations:

1. Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, Szabo G. (2013) Alcohol-induced IL-1β Production is Mediated by NLRP3/ASC Inflammasome Activation in the Brain. Symposium: Neuroimmune Activation, Microglia and Alcohol Addiction. The 36th annual RSA scientific meeting, Orlando, FL, US.
2. Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, Szabo G. (2012) Chronic Alcohol-Induced IL-1β is mediated by the NALP3/ASC Inflammasome Activation in a TLR4-Independent Manner in the Brain and Prevented by IL-1 Receptor Antagonist Treatment. The 45th annual meeting of the Society for Leukocyte Biology, Maui, HI, US.

11.2.2. Posters:

1. Lippai D, Bala S, Donna C, Kodys K, Szabo G (2014) Alcohol-induced Small Bowel Inflammation is Prevented by Micro-RNA-155 Deficiency. 9th Meeting on Alcoholic liver and pancreatic disease symposium, Szeged, HU.
2. Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, Szabo G. (2012) Chronic Alcohol-Induced IL-1β is mediated by the NALP3/ASC Inflammasome Activation in a TLR4-Independent Manner in the Brain and Prevented by IL-1 Receptor Antagonist Treatment. Clinical and Translational Science Research Retreat, UMASS, Worcester MA, US.
3. Petrasek J, Bala S, Lippai D, Kodys K, Menashy V, Barrieau M, Kurt-Jones EA, Szabo G. (2012) Caspase-1-dependent, IL-1β-mediated alcoholic steatohepatitis is ameliorated by IL-1 receptor antagonist treatment in mice. Clinical and Translational Science Research Retreat, UMASS, Worcester MA, US.
4. Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, Szabo G. (2012) Chronic Alcohol-Induced IL-1β is mediated by the NALP3/ASC Inflammasome Activation in a TLR4-Independent Manner in the Brain and Prevented by IL-1 Receptor Antagonist Treatment. The 45th annual meeting of the Society for Leukocyte Biology, Maui, HI, US.
5. Petrasek J, Bala S, Lippai D, Kodys K, Kurt-Jones EA, Szabo G. (2012) Inflammasome Complex Components, ASC and Caspase-1, Mediate Alcoholic Steatohepatitis via Interleukin-1 in Mice. The 45th annual meeting of the Society for Leukocyte Biology, Maui, HI, US.
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11.3. Publications not related to the theme of the Ph.D. thesis:

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