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Article type : Original Article

Ethanol upregulates the P2X7 purinergic receptor in human macrophages

Running head: Ethanol upregulates P2X7R in human macrophages

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ABSTRACT

Alcohol consumption is considered to be the third leading cause of death in the United States. In addition to its direct toxicity, ethanol has two contrasting effects on the immune system: the nucleotide oligomerization domain-like receptor pyrin domain-containing-3 (NLRP3) inflammasome is inhibited by acute ethanol exposure but activated by chronic ethanol exposure. Purinergic receptors (especially the P2X7 receptor) are able to activate the NLRP3 inflammasome, and are involved in many ethanol-related diseases (such as gout, pulmonary fibrosis, alcoholic steatohepatitis and certain cancers). We hypothesized that ethanol regulates purinergic receptors and thus modulates the NLRP3 inflammasome's activity. In experiments with monocyte-derived macrophages, we found that interleukin (IL)-1β secretion was inhibited after 7h of exposure (but not 48 h of exposure) to ethanol. The disappearance of ethanol's inhibitory effect on IL-1β secretion after 48 h was not mediated by the upregulated production of IL-1β, IL-1α, IL-6 or the inflammasome components NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain, and caspase 1. P2X7R expression was upregulated by ethanol, whereas expression of the P2X4 and P2X1 receptors was not. Taken as a whole, our results suggest that ethanol induces NLRP3 inflammasome activation by upregulating the P2X7 receptor. This observation might have revealed a new mechanism for inflammation in ethanol-related diseases.

KEYWORDS: ethanol, NLRP3, P2X7, monosodium urate, inflammation, macrophage

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ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASC: apoptosis-associated speck like protein containing a CARD
ATP: adenosine triphosphate
BSA: bovine serum albumin
BzATP: 2',3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate
DAMPs: danger-associated molecular patterns
EtOH: ethanol
FCS: fetal calf serum
GABA: gamma-aminobutyric acid
LPS: lipopolysaccharide
MSU: monosodium urate
NMDA: N-methyl-D-aspartate
NLRP3: nucleotide oligomerization domain-like receptor pyrin domain containing 3
PAMPs: pathogen-associated molecular patterns
P2X1R: purinergic receptor P2X1
P2X4R: purinergic receptor P2X4
P2X7R: purinergic receptor P2X7
PBS: phosphate buffer saline
PRRs: pattern recognition receptors
ROS: reactive oxygen species
SEM: standard error of the mean

INTRODUCTION

According to the 2015 National Survey on Drug Use and Health (conducted by the US government's Substance Abuse and Mental Health Services Administration), 138.3 million of Americans aged 12 or older reported current use of alcohol. Nearly half of these current alcohol users (66.7 million people) reported binge alcohol use in the previous month, and 17.3 million reported heavy alcohol use in the previous month. Alcohol is considered to be among the most harmful drugs with regard to physical injury, social damage, and addiction (1). Furthermore, alcohol consumption is considered to be the third leading cause of death in the USA, mainly from cancer, liver cirrhosis, and injury. The pathophysiological effects of alcohol are now being better characterized. The production of ethanol-induced reactive oxygen species (ROS) is now known to increase membrane fluidity, and ROS are also involved in the induction of apoptosis and alcoholic liver diseases such as fibrosis and cirrhosis (2–4). Interestingly, ethanol has several contrasting effects on the immune system. Acute alcohol consumption leads to immunosuppression, whereas chronic exposure leads to inflammation (5,6). It was recently reported that the nucleotide oligomerization domain-like

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receptor pyrin domain-containing-3 (NLRP3) inflammasome was involved in this dual effect: acute ethanol exposure was found to inhibit the NLRP3 inflammasome pathway in leukocytes, whereas chronic exposure activated the pathway (7–9).

Inflammasomes are part of the pattern recognition receptor family of intracellular innate immune system sensors. The best known of these is the NLRP3 inflammasome, which is mainly expressed by myeloid cells like monocytes and macrophages. The NLRP3 inflammasome enables the production of IL-1β and IL-18 in response to pathogen-associated molecular patterns such as lipopolysaccharide (LPS). However, this first signal only enhances pro-IL-1β production, and is not enough to activate the release of active IL-1β from macrophages; a second signal (such as a danger-associated molecular pattern (DAMP) or pathogen-associated molecular pattern (PAMP) is required (10). This second step leads to the oligomerization of three main effectors: NLRP3, the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC, an adaptor protein), and procaspase 1. This assembly converts procaspase-1 into mature caspase 1, which can cleave pro-IL-1β and pro-IL-18 into their respective active forms. These pro-inflammatory cytokines activate the acute inflammation pathway (11). Over-activation of the inflammasome leads to pyroptosis, a type of programmed cell death that amplifies local inflammatory reactions (12). The NLRP3 inflammasome can be activated by many different DAMPs and PAMPs, such as ATP or his synthetic analog BzATP, nigericin, and crystals of silica, cholesterol or uric acid (13–15). These molecular patterns activate the inflammasome through several pathways: potassium efflux triggered by purinergic receptors, and lysosomal breakdown triggered by crystals, for example. Membrane pore formation, ROS production, and ASC phosphorylation are also involved in activation of the NLRP3 inflammasome (16,17). However, genetic mutations of NLRP3 protein lead to pathologies such as cryopyrin-associated periodic syndrome. Similarly, inflammasome overactivation is involved in several diseases, such as atherosclerosis, diabetes, or gout (7,18). In view of the NLRP3 inflammasome's involvement in inflammatory diseases, cognate inhibitors are being developed as drug candidates. Interestingly, treatment with these compounds *in vitro* and *in vivo* is associated with reductions in neuroinflammation and colonic inflammation (19,20). Furthermore, colchicine (an old compound used for the symptomatic treatment of various inflammatory diseases, including gout, Behçet syndrome, and familial Mediterranean fever) has been found to inhibit the NLRP3 inflammasome (21,22). This observation highlighted new potential indications in the inhibition of atherosclerosis-associated inflammation (23). Recently, molecular design

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studies has shown that benzene sulfonamide analogs can inhibit the NLRP3 inflammasome, and these compounds are also being developed as drug candidates (24). However, given that two steps are required to activate the NLRP3 inflammasome, studying the factors able to modulate the second step is a challenge in anti-inflammatory drug research. As mentioned above, the stimulation of purinergic receptors (especially the P2X7 receptor (P2X7R)) has been shown to activate the NLRP3 inflammasome (25).

Purinergic receptors are ubiquitous receptors involved in nociception and the inflammatory response. They are activated by extracellular nucleotides (26). Purinergic receptors are classified into two families: P1 receptors are selective for adenosine, and P2 receptors mainly bind ATP and ADP (27). The P2 receptors can be subclassified into P2Y G-protein-coupled receptors and P2X ligand-gated ion channels (28). Purinergic receptors are known to be involved in several disease processes. Recently, P2Y6R was linked to the development of pulmonary fibrosis (29), and P2X7R is thought to be involved in Parkinson's disease, pulmonary hypertension, gout, allergy, pulmonary fibrosis, and alcoholic and non-alcoholic steatohepatitis (30–35). Furthermore, high P2X7R expression levels may indicate a poor prognosis for patients with colorectal cancer (36). In the present paper, we used BzATP, a potent agonist of P2X7R, in order to activate NLRP3 inflammasome through purinergic receptor pathway. Given that ethanol is able to regulate purinergic receptor P2X4 and P2X7 activity in a microglia cell line (37), we hypothesized that this alcohol might promote inflammatory disease regulating purinergic receptors in monocyte-derived macrophage (MdMs).

Thus, the objective of the present study was to gain a better understanding of the mechanisms linking ethanol, the NLRP3 inflammasome and purinergic receptors in MdMs.

MATERIALS AND METHODS

Reagents

Phosphate-buffered saline (PBS), RPMI 1640 medium, penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Eugene, OR, USA). Fetal calf serum (FCS) was from Hyclone (Logan, UT, USA), bovine serum albumin was from Eurobio (Les Ulis, France), ultrapure E. coli 0111:B4 LPS was purchased from InvivoGen (Toulouse, France), and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-

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CSF) was from R&D Systems Europe (Lille, France). 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT), and 2',3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) were obtained from Sigma Aldrich (St. Louis, MO, USA). Specific antibodies against P2X7R and P2X4R were purchased from Alomone (Jerusalem, Israel).

Crystal preparation

Monosodium urate (MSU) crystals were prepared by recrystallization from uric acid (38). Briefly, the crystals were obtained by dissolving 1.68 mg of MSU in 500 mL of 0.01 M NaOH preheated to 70°C (pH 7.1-7.2). The solution was slowly and continuously agitated at room temperature, until crystals formed. The crystals were washed twice with 100% ethanol, dried, autoclaved, and weighed under sterile conditions. Crystals were resuspended in PBS by sonication and examined with phase microscopy prior to use in our experiments. The crystal size (greatest dimension) ranged from 2 µm to 20 µm.

Preparation and treatment of MdMs

Peripheral blood mononuclear cells were obtained from human buffy coat (French Blood Establishment, Rennes, France) by differential centrifugation on UNI-SEP® U-10 (Novamed, Jerusalem, Israel). The experiments were performed in compliance with the French legislation on blood donation and blood product use and safety. Monocytes from healthy donors were enriched using a human CD14 separation kit (Microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany), plated at a density of $0.5x10^6$ cells/well in 24 well-plates and cultured at 37°C with 5% humidified CO_2 in RPMI 1640 medium supplemented with 100 IU/mL penicillin - 100 µg/mL streptomycin, 2 mM L-glutamine, 1% sodium pyruvate, and 10% FCS. Macrophages were obtained after differentiation from monocytes by incubation with 50 ng/mL rhGM-CSF in RPMI 1640 medium. After 7 days, the supernatant was removed, and cells were exposed to various treatments. The MdMs were incubated overnight with 0.1 µg/mL ultrapure E. coli 0111:B4 LPS, incubated with ethanol for 1 h or for 42 h, and then incubated with ethanol and MSU or ethanol and BzATP for a further 6 h. At each change of treatment, the supernatant is removed in order to renew the ethanol-containing medium.

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Cell viability

Cytotoxicity was assessed using MTT colorimetric assays. Briefly, after medium removal, 500 µL of RPMI medium containing MTT (0.5 mg/ml) was added to each well, and the cells were incubated for 2 h at 37°C. The water-insoluble formazan was dissolved in 500 µL of DMSO, and absorbance was measured at 540 nm.

Cytokine production assays

The concentrations of IL-1β, IL-1α and IL-6 in the culture supernatant were measured using Duoset® ELISA kits (R&D Systems, Abingdon, United Kingdom), according to the manufacturer's instructions.

Real-time PCR (RT-qPCR) analysis

After lysis of the MdMs, total RNA was isolated using a Macherey-Nagel NucleoSpin® RNAII kit, according to the manufacturer's protocol. Total RNA (1 µg) was reverse-transcribed into first-strand cDNA using a High-Capacity cDNA Achieve Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. RNA quantity and purity were assessed with a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). qPCR was performed using the fluorescent dye SYBR Green method, with SYBR Green PCR Master Mix in 384-well plates and the StepOnePlus™ system (Applied Biosystems). Human GAPDH was used as the reference gene. Relative quantification values were expressed using the $2^{\Delta\Delta Ct}$ method as fold changes in the target gene, normalized to the reference gene, and related to the expression level in control experiments.

Flow cytometry detection of P2X4R and P2X7R proteins on MdMs

Cells were detached with trypsin, washed twice in PBS, and fixed for 10 min in 4% paraformaldehyde in PBS. After the paraformaldehyde had been discarded, the MdMs were resuspended in PBS supplemented with 2.5% nonimmune rabbit serum (in order to block nonspecific sites). Isotype control immunoglobulins and primary antibodies against P2X4R and P2X7R were diluted 1/100, 1/100 and 1/75 respectively, and then incubated with the

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MdMs for 1 h. After washing with PBS, the cells were incubated for 1 h with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1/100. After a final wash with PBS, the cells were analyzed by flow cytometry (using a BD LSRFortessa™ X-20 cell analyzer and BD FACSDiva™ software). The MdMs were first gated according to their forward- and side-scatter profiles, and then defined as $P2X4R^+$ and/or $P2X7R^+$. FlowLogic software (version 7.2.1, Inivai Technologies, Mentone, Australia) was used to analyze the flow cytometry data.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Intergroup differences as a function of the treatment were probed in a one-way analysis of variance (ANOVA), with a Bonferroni *post hoc* test for group comparisons. The significance of intergroup differences in the PCR analysis was determined using a Mann-Whitney test. All analyses were performed using Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). All tests were two-sided, and the threshold for statistical significance was set to $p<0.05$.

RESULTS

Acute exposure to ethanol dose-dependently inhibits IL-1β hypersecretion by MdMs

Since acute ethanol exposure has been shown to inhibit the NLRP3 inflammasome in macrophages (7,8), we looked at whether this alcohol was able to dose-dependently inhibit interleukin production in response to BzATP (Figure 1) or MSU (Figure 2). BzATP and MSU were used in order to stimulate NLRP3 inflammasome through P2X7R pathway. We found that ethanol inhibited IL-1β production at a concentration of 30 mM or more when the MdMs had been previously stimulated with BzATP and at a concentration of 100 mM or more when the cells had been stimulated with MSU (Figure 1A; 2A). Interestingly, IL-6 and IL-1α levels were unchanged (Figure 1B; 1C; 2B; 2C). Furthermore, we observed a greater IL-1β production in the LPS + BzATP and LPS + MSU conditions, relative to LPS alone (Figure 1A; 2A). When BzATP had been added, the presence of 300 mM ethanol was associated with lower cell viability. When MSU had been added, the presence of 1000 mM ethanol was associated with lower cell viability (Figure 1D; 2D). Considering that IL-1β production was first inhibited at a non-toxic concentration of ethanol, our results show that

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ethanol's effect was not associated with cell death. Furthermore, given that others cytokines levels (IL-6 and IL-1α), regardless produced from NLRP3 inflammasome, were not affected by ethanol, the effect of this alcohol can be considered selective for the NLRP3 inflammasome pathway.

Ethanol's inhibition of IL-1β production disappears after 48 h

Identification of the lowest concentration of ethanol capable of inhibiting the NLRP3 inflammasome enabled us to investigate the time course of IL1α, IL-1β and IL-6 production. After 7 h of ethanol exposure at 100 mM or 300 mM, only IL-1β production was inhibited in MdMs stimulated with LPS overnight and then with 300 µg/mL MSU for 6 h (Figure 3A). Levels of IL-6 and IL-1α were unchanged (Figure 3C, 3E). Forty-eight hours of ethanol exposure at 100 mM or 300 mM did not significantly inhibit the production of any of the ILs, regardless of the cell stimulus (3B, 3D, 3F). The MdMs' viability was not impaired by exposure to 300 mM ethanol for 48 h, followed by activation with 100 ng/mL LPS overnight and then with 300 µg/mL MSU for 6 h (data not shown). These results show that the inhibition of IL-1β production associated with acute ethanol exposure (7 h) is no longer present after 48 h of exposure.

Ethanol does not influence the mRNA expression of inflammasome components or interleukins 1β, 1α and 6

To investigate the mechanism whereby the inflammasome NLRP3 pathway is inhibited by 7 h of ethanol exposure and to establish why this inhibition is no longer present after 48 h of ethanol exposure, we measured the mRNA expression of inflammatory cytokines (*IL-1β, IL-1α* and *IL-6*) and inflammasome components *(NLRP3, CASP1 and ASC*) after 7 h of exposure to ethanol. There were no significant changes, relative to a control experiment in the absence of ethanol (Figure 4). These results show that the phenomena observed after 7 and 48 h of ethanol exposure were not related to the induction or inhibition of NLRP3 inflammasome components or the pro-inflammatory cytokines IL-1β, IL-1α and IL-6.

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Ethanol promotes the mRNA expression of P2X7R and P2X4R

Given that NLRP3 inflammasome is mainly activated by P2X receptors, we measured the mRNA expression levels of P2X1R, P2X4R and P2X7R after 7 h of exposure to ethanol or medium alone in MdMs treated with 100 µg/L MSU or 250 mM BzATP. We found that ethanol exposure was associated with an induction of P2X4R and P2X7R mRNA expression but not P2X1R mRNA expression, regardless of whether the cells had been treated with MSU or BzATP (Figure 5A; B).

Ethanol promotes P2X7R and P2X4R expression

In order to confirm that the induction of P2X4R and P2X7R mRNA expression corresponded to higher cell surface levels of these receptors, we used flow cytometry to study MdMs incubated in the presence of absence of 100 mM ethanol for 24 h. The cells were incubated with 100 ng/mL LPS overnight and then with 300 µg/mL MSU for 6 h. A shift in fluorescence was observed for P2X7R but not P2X4R - indicating that P2X7R (but not P2X4R) was overexpressed in ethanol-treated cells (Figure 6).

DISCUSSION

Our present results show that prolonged exposure of MdMs to ethanol promoted activation of the NLRP3 inflammasome and upregulation of the P2X7R, which are known be involved in the pathophysiology of alcohol abuse. It has been well established that alcohol abuse is a leading cause of inflammatory diseases, including fibrosis, cirrhosis, chronic obstructive pulmonary disease, diabetes, and neuroinflammation (5,10,38–43). It is also known that ethanol is a preventable risk factor for certain cancers, including colon cancer (44), hepatocellular carcinoma (45), breast cancer (46), and esophageal, pancreatic and prostate cancers (47). *Via* activation of the NLRP3 inflammasome pathway and ROS generation, purinergic receptors are involved in the pathogenesis of many of these diseases - including liver fibrosis (48), diabetes (49), and neuroinflammation (50). The overexpression of purinergic receptors (especially P2X7R) is thought to be involved in tumorigenesis (36,51–55).

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Studies of ethanol's immunomodulatory effect on macrophages have recently highlighted the involvement of the NLRP3 inflammasome. Moreover, ethanol has a dual, contrasting effect on inflammation, depending on the concentration and exposure time. Considering that BzATP is the most potent P2X7R agonist available, we decided to use this compound to study selective P2X7R activation in the NLRP3 inflammasome pathway. Monosodium urate is also known to be a potent danger signal that activates the NLRP3 inflammasome pathway through cathepsin B, ATP release, and autocrine purinergic signaling (56). As we have reported previously, LPS + BzATP stimulation selectively enhanced IL-1β production (25). Furthermore, LPS + MSU stimulation was found to enhance IL-1β, IL-1α and IL-6 production. After stimulation with BzATP or MSU, acute (7h) ethanol exposure selectively inhibited IL-1β secretion (from 30 mM ethanol for BzATP and from 100 mM for MSU) but did not inhibit IL-6 and IL-1 α levels (Figure 1A-C; Figure 2A-C). These results were consistent with Hoyt et al.'s (2017) report whereby acute ethanol exposure strongly and selectively inhibits the NLRP3 inflammasome pathway. Interestingly, IL-1α levels were greater after 10mM of ethanol exposure for 7 h, but not after exposure of a higher concentration of ethanol, when MdMs were stimulated with BzATP (Figure 1B). Redox imbalance, known to induce IL-1α expression (57), is induced by ethanol (58). Thus, we hypothesized that ethanol-induced oxidative stress is responsible of the greater amount of this cytokine. Same results were found with IL-6 (Figure 1C). These results are consistent with those of Chen et al. (59) and Hong et al. (60), reporting elevated amount of IL-6 during acute exposure of ethanol. Furthermore, we think that the disappearance of the greater amount of IL-1α and IL-6 with higher concentration of ethanol is linked to the inhibition of IL-1β by ethanol from 30mM. These results are supported by those of Cahill et al. (61) and Fettelschoss et al. (62), reporting that IL-1β secretion induces IL-6 production and is required for IL-1α secretion. Taken as a whole, these results indicate that our model is a relevant one for studying activation or inhibition of the NLRP3 inflammasome.

Forty-eight hours exposure to ethanol was enough to suppress the previously observed inhibitory effect of acute ethanol exposure on IL-1β secretion (Figure 3A-B). This observation indicates that only 48 h of ethanol exposure is enough to promote a response capable of activating the NLRP3 inflammasome. It has been shown that ethanol's effect on the NLRP3 inflammasome pathway is associated with greater inducible nitric oxide synthase expression, NO production, and mitochondrial dysfunction (ROS generation). Moreover, ethanol metabolites (including acetaldehyde) were found to be probable activators of the

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NLRP3 inflammasome (9). However, toxicological monitoring of acetaldehyde is challenging, given the very low concentrations produced after alcohol consumption in humans (63–65). Indeed, acetaldehyde is rapidly cleared from blood and metabolized into acetic acid and other compounds (66,67). Although the blood acetaldehyde concentration is higher in alcoholics than in non-alcoholics, the mean plasma concentration measured in the former group was only 3.74 µM (68). This acetaldehyde concentration is still far lower than those used in studies of NLRP3 inflammasome activation (9). Furthermore, acetaldehyde is highly volatile in cell cultures at 37°C. Considering that acetaldehyde is also metabolized through an oxidative pathway, it is possible that oxidative stress is more involved than acetaldehyde *per se* in NLRP3 inflammasome activation. We therefore hypothesize that chronic ethanol exposure triggers another pathway and thus suppresses an inhibitory effect of acute ethanol exposure on the NLRP3 inflammasome pathway.

The literature data show that two weeks of ethanol treatment leads to the hypersecretion of inflammasome compounds, including NLRP3, caspase-1 and ASC (9). Conversely, our experiments on the THP-1 cell line (data not shown) and on MdMs demonstrated that 48 h of ethanol exposure does not modify the expression of IL-1α, IL-1β, IL-6 or the inflammasome components NLRP3, caspase-1 and ASC (Figure 4). These results suggest that ethanol does not increase NLRP3 inflammasome activity by increasing intracytoplasmic levels of this complex. We therefore decided to study the expression of proteins involved in the NLRP3 inflammasome activation pathway. As mentioned above, purinergic receptors are involved in NLRP3 inflammasome activation. Remarkably, we found that ethanol exposure is associated with greater mRNA expression levels of P2X4R and P2X7R but not P2X1R (Figure 5). For 100 mM and 300 mM ethanol, the same dose-dependent results were found with THP-1 (data not shown). Considering that MSU is known to upregulate P2X7R (56), we first hypothesized that ethanol potentiates this effect but does not have its own specific role. This hypothesis was rejected because P2X7R expression was induced with ethanol after BzATP stimulation - making ethanol the only common factor. These findings were confirmed by our flow cytometry experiments; P2X7R fluorescence was shifted when cells were exposed to ethanol (Figure 6). Taken as a whole, these results show for the first time that ethanol induces P2X7R expression independently of other treatments.

There is a growing body of evidence to show that P2X7R activation leads to mitochondrial toxicity, oxidative stress, and activation of the NLRP3 inflammasome pathway (69–71). In parallel, overexpression of purinergic receptors has been observed in several

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disease processes. Here, we found that ethanol induced P2X7R protein expression in an MdM model. Thus, given that chronic use of alcohol is a leading cause of inflammatory diseases and cancer, and that purinergic receptor (particularly P2X7R) are involved in the pathogenesis of many of these ethanol-related diseases, it is therefore conceivable that ethanol amplifies inflammation activation and pathophysiological processes through mechanisms involving the upregulation of purinergic receptors. In the inflammatory environment, ethanol-induced overexpression of P2X7R in peripheral macrophages can trigger a greater fixation of agonistic danger signals of purinergic receptors, such as endogenous ATP. Therefore, the severity of inflammatory ethanol-related diseases can be directly linked to the upregulation of P2X7R. The observed upregulation of P2X7R indicates that it is especially worthwhile to test antagonists *in vitro* of this putative target in the context of ethanol-associated pathologies.

Several disease models involving ethanol are now being used to examine the effects of P2X7R antagonists. In HepG2 cells, it has been shown that decreasing the expression of P2X7R and the NLRP3 inflammasome pathway by treatment with dihydroquercetin reduces alcoholic liver steatosis through the inhibition of lipogenesis (72). Similarly, inhibiting P2X7R with gentiopicroside enhances alcoholic liver steatosis, while IL-1β secretion aggravates it (34). In *ex vivo* cultures of vessels with carotid plaques, the selective P2X7R antagonist A740003 decreased IL-1β secretion without involving the NLRP3/caspase-1 pathway; high IL-1β levels are usually associated with carotid plaque instability (73). These findings suggest that targeting P2X7R might be of value in the treatment of atherosclerosis (73). In the field of neurology, it has been shown that ethanol dose-dependently induces P2X4 and P2X7 expression in BV2 microglial cells (37). Justifiably, P2X7 antagonists are now starting to be studied as drug candidates for the inhibition of neuroinflammation (74).

In conclusion, we found that the NLRP3 inflammasome pathway was inhibited by 7 h of exposure to ethanol and stimulated after 48 h of exposure to ethanol. Furthermore, ethanol induced P2X7R expression in MdMs. It is therefore conceivable that ethanol amplifies inflammation activation pathways and the pathophysiological processes through purinergic receptor upregulation - making macrophages react more strongly to danger signals. Our present results highlight a new putative mechanism for inflammation activation in ethanol-induced diseases. Hence, P2X7R antagonists might constitute a novel class of anti-inflammatory molecules with potential value in the treatment of ethanol-related inflammatory diseases.

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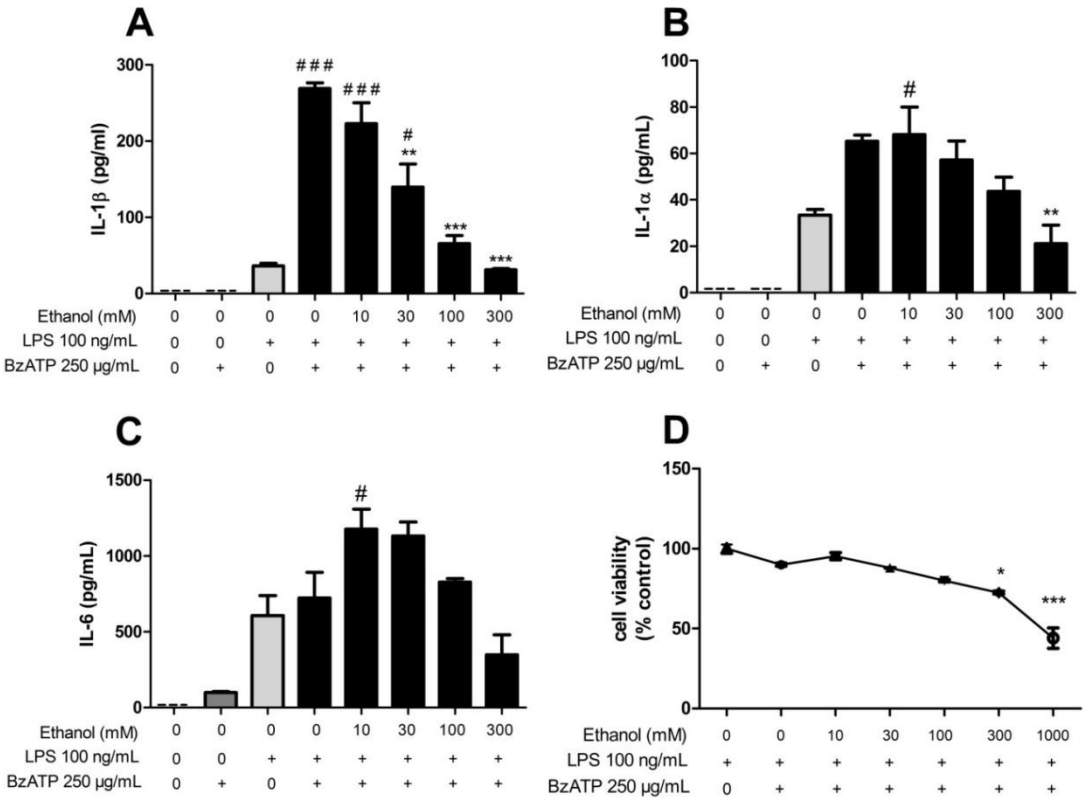

Figure 1: Acute exposure of MdMs to ethanol dose-dependently inhibits IL-1β hypersecretion after cell stimulation with LPS + BzATP. Secretion of IL-1β, IL-1α, and IL-6 into the cell culture supernatant was assessed (using an ELISA) after activation of MdMs with 100 ng/mL LPS overnight or medium, followed by a 6 h incubation with 250 µM BzATP or medium (A-C). Cell viability was measured in an MTT assay and expressed relative to the value determined after LPS treatment alone (set arbitrarily to 100%) (D). The data are quoted as the mean ± SEM from one representative experiment performed in triplicate. ***p<0.001; **p<0.01: LPS + BzATP in the absence of ethanol, compared with LPS + BzATP in the presence of various concentrations of ethanol. ###p<0.01; # p<0.05: LPS compared with LPS + BzATP in the presence or absence of ethanol.

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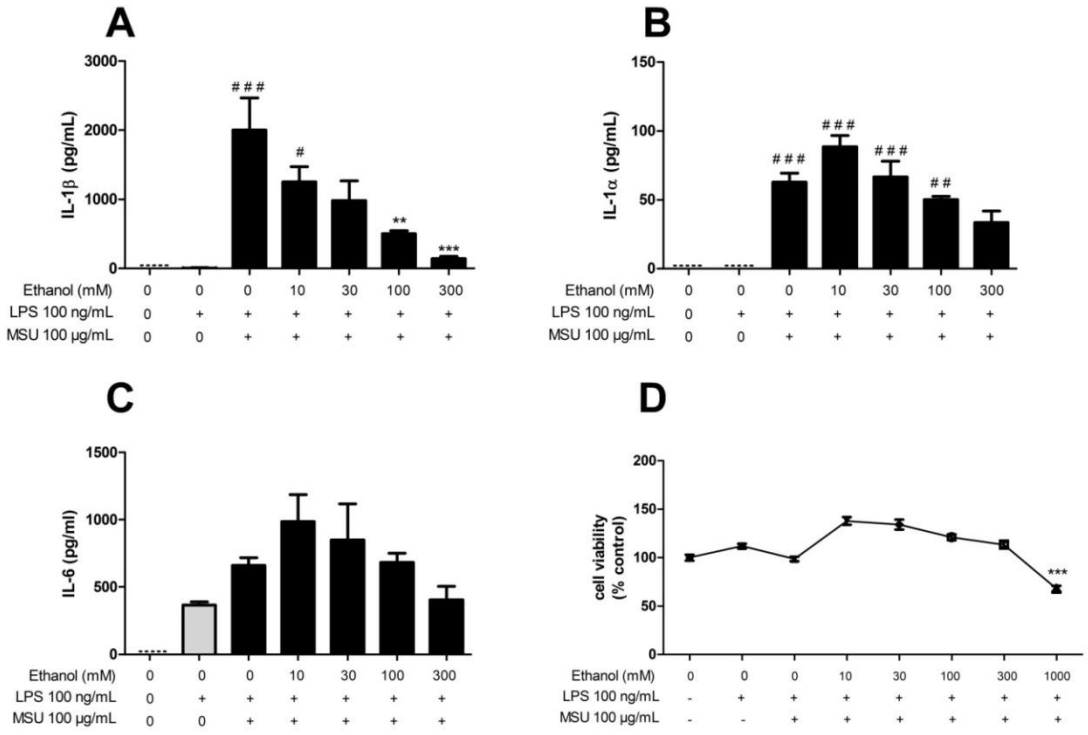

Figure 2: The acute exposure of MdMs to ethanol dose-dependently inhibits IL-1β hypersecretion after LPS + MSU stimulation. Secretion of IL-1β, IL-1α, and IL-6 into the culture supernatant was assessed by ELISA after cell activation with 100 ng/mL LPS overnight or medium, followed by 6 h of treatment with 100 µg/mL MSU or medium (A-C). Cell viability was measured in an MTT assay and expressed relative to the value determined after LPS treatment alone (arbitrary set to 100%) (D). The data are quoted as the mean ± SEM from one representative experiment performed in triplicate. ***p<0.001; **p<0.01: LPS + MSU in the absence of ethanol, compared with LPS + MSU in the presence of ethanol at various concentrations. ## p<0.01; ### p<0.001: LPS compared with LPS + MSU in the presence or absence of ethanol.

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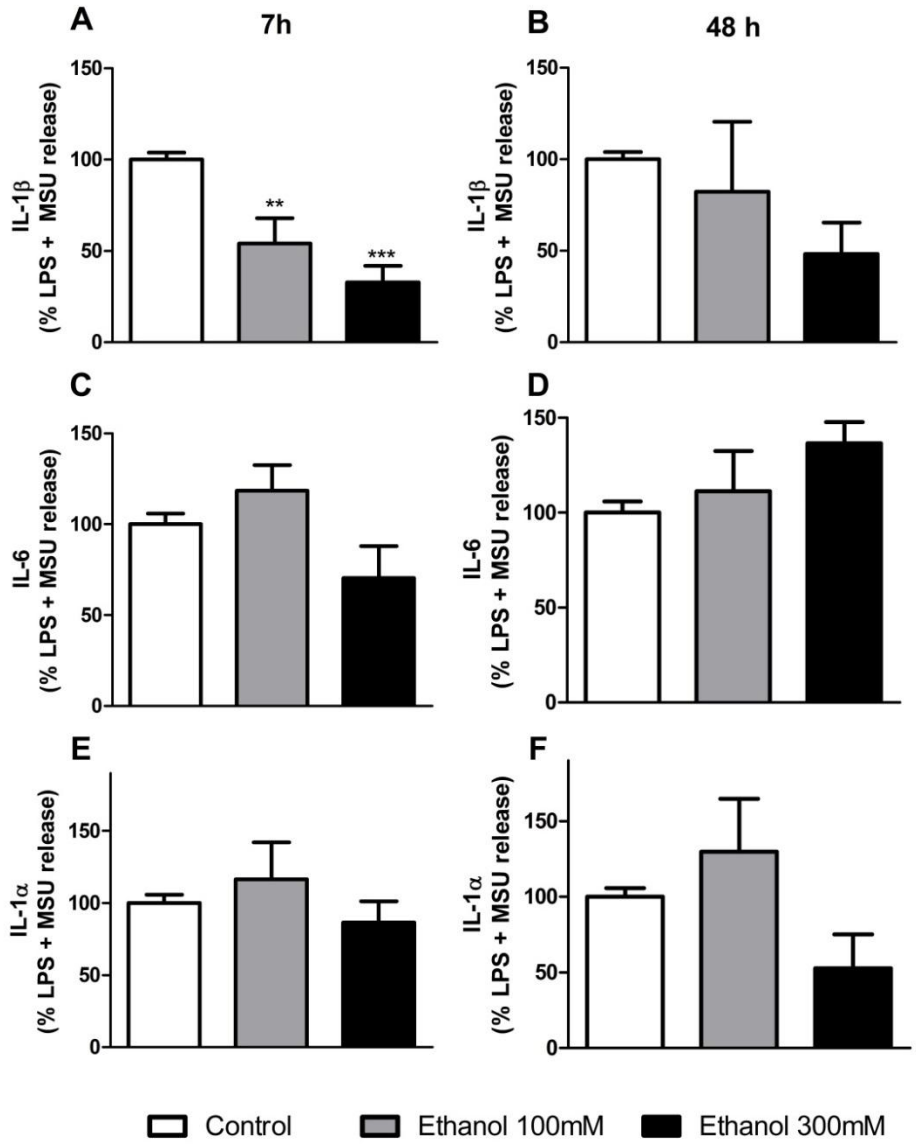

Figure 3: Ethanol's inhibition of IL-1β production disappears after 48 h. Secretion IL-1β, IL-1α, and IL-6 into the culture supernatant was assessed by ELISA in the cell culture supernatant of MdMs after activation with 100 ng/mL LPS overnight, followed by 6 h of treatment with 300 µg/mL MSU (A-F). Ethanol at 100 mM or 300 mM was added 1 h (A; C; E) or 42 h (B; D; F) before MSU treatment, and was present throughout the treatment period. The data are quoted as the mean ± SEM from three independent experiments performed in triplicate. ***$p<0.001$; **$p<0.01$; *$p<0.05$: LPS + MSU in the absence of ethanol, compared with LPS + MSU in the presence of ethanol at various concentrations.

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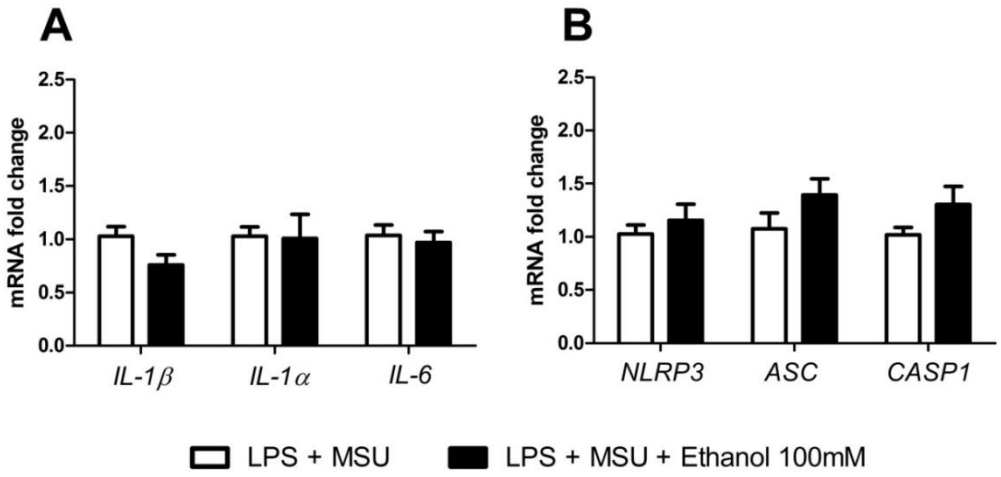

Figure 4: Ethanol does not influence the mRNA expression of inflammasome components or IL-1β, IL-1α and IL-6 after LPS + MSU stimulation of MdMs. The MdMs were incubated overnight with 100 ng/mL LPS, and then stimulated with 100 µg/mL MSU for 6 h. Ethanol (at 100 mM) was added 1 h before MSU, and was present throughout the treatment period. Cell lysates were collected, and the mRNA expression levels of *IL-1β, IL-1α, IL-6* (A) and inflammasome components (*NLRP3, CASP1, and ASC*) (B) were determined using RT-qPCR. The results were normalized against *GAPDH* gene expression. The data are quoted as the mean ± SEM from three independent experiments performed in triplicate. ***$p<0.001$; **$p<0.01$; *$p<0.05$ for ethanol treatment compared with the control treatment, for each receptor.

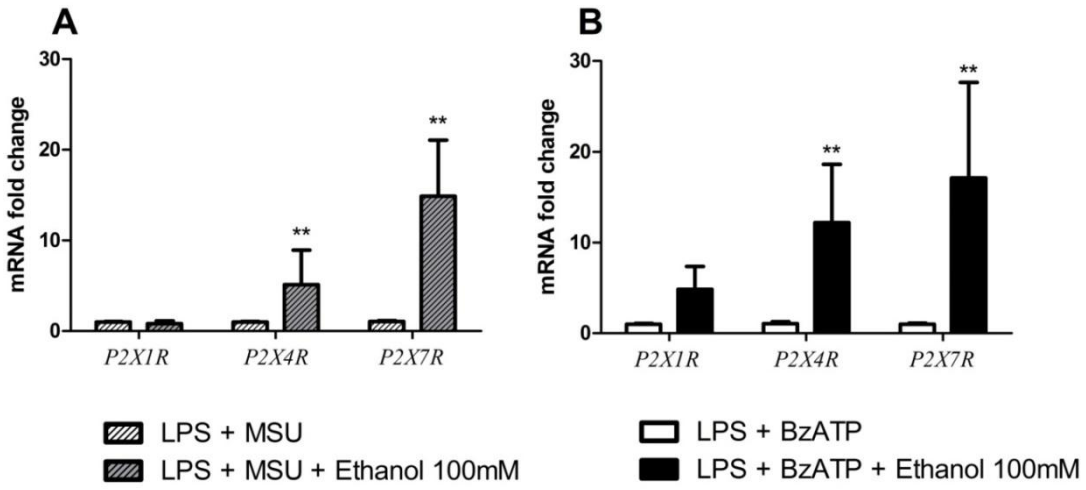

Figure 5: Ethanol promotes the mRNA expression of the purinergic receptors P2X7 and P2X4 following stimulation of MdMs with LPS + MSU. The MdMs were incubated overnight with 100 ng/mL LPS, followed by treatment with 100 µg/mL MSU or 250 mM BzATP for 6 h. Ethanol (100 mM) was added 1 h before MSU or BzATP, and was present throughout the treatment period. Cell lysates were collected, and mRNA expression levels of purinergic receptors (P2X1, P2X4, and P2X7) were determined using RT-qPCR (A-B). The results were normalized against *GAPDH* gene expression. The data are quoted as the mean ± SEM of at least three independent experiments performed in triplicate. **p<0.01: treatment with ethanol, compared with the control treatment for each receptor.

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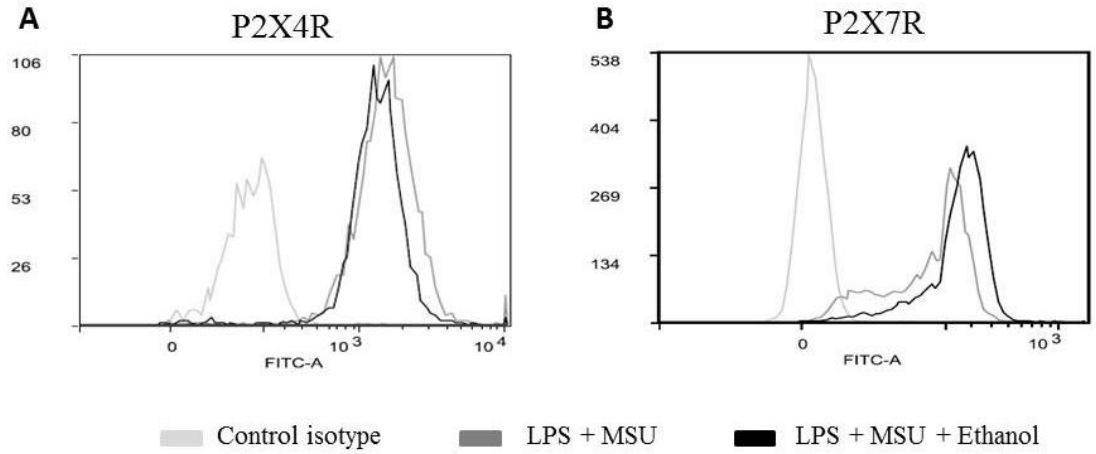

Figure 6: Ethanol increases mRNA expression levels of purinergic receptors in MdMs. The MdMs were obtained by incubating monocytes with rhGM-CSF (50 ng/mL) in medium. After 8 d, the cells were incubated overnight with 100 ng/mL LPS and then with 300 µg/mL MSU for 6 h. Ethanol (at 100 mM) or medium was added 18 h before MSU, and was present throughout the treatment period. Expression of P2X7R and P2X4R on the cell surface was studied using flow cytometry. The data correspond to the fluorescence values for one experiment performed in triplicate.