International Immunopharmacology 77 (2019) 105915

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Regulation of CD39 expression in ATP-P2Y2R-mediated alcoholic liver steatosis and inflammation

Zhen-ni Liu, Wen-qian Jia, Tao Jiang, Jing-wen Dai, Chen Shuai, Xiong-wen Lv*

The Key Laboratory of Major Autoimmune Diseases, Anhui Province, Anhui Institute of Innovative Drugs, School of Pharmacy, Anhui Medical University, Hefei, China
The Key Laboratory of Anti-inflammatory and Immune Medicines, Ministry of Education, Hefei, China
Institute for Liver Diseases of Anhui Medical University, Hefei, China

ARTICLE INFO

Keywords:
ATP
P2Y2 receptor
CD39
Early alcoholic liver steatosis and inflammation

ABSTRACT

Inflammation plays a central role in the progression of alcoholic liver disease. ATP-P2Y2R signaling and CD39 play an important role in various diseases, but little is known about their role in alcoholic liver steatosis and inflammation. As a transmembrane hydrolase, CD39 hydrolyzes ATP, while the mutual regulation of CD39 and ATP-P2Y2R in alcoholic steatohepatitis is poorly understood. Here, we found that the expression of ATP, P2Y2R, and CD39 is increased significantly both in the liver of alcohol-fed mice and alcohol-induced RAW264.7 cell lines. In this study, C57BL/6 mice were intrapretationally injected with P2Y2R inhibitor suramin from day 4 until day 10 during the induction of a chronic/binge drinking model. Pharmacological blockade of P2Y2R largely prevents liver damage, lipid accumulation, and inflammation, with concomitant down-expression of CD39 in liver. We found that the inhibition of P2Y2R in vitro reduces inflammation via down-expression of interleukin 6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α), and the expression of CD39 was reduced, whereas the activation of P2Y2R showed an opposite effect. Silencing of CD39 promoted the expression of ATP and P2Y2R. These results indicate that CD39 attenuates alcohol-induced steatohepatitis by scavenging extracellular ATP to indirectly regulate the expression of P2Y2R. Interestingly, P2Y2R paradoxically boosts CD39 activity. Thus, blockade of the extracellular ATP-P2Y2R signalling represents a potential therapeutic approach against alcoholic liver disease, and CD39 is a potential therapeutic target.

1. Introduction

Alcoholic liver disease (ALD) is caused by prolonged and excessive drinking. It is the most common type of liver disease in the world and includes alcoholic fatty liver, alcoholic steatohepatitis (ASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. ALD progression is mediated via liver damage, inflammation, and liver fibrosis, with inflammation playing a central role [2,3]. Inflammation is a complex defense reaction, which reduces the injury and restores normal tissue structure and function [4]. Activated inflammatory cells including Kupffer cells (KCs), which are the resident tissue macrophages of the liver, and infiltrating macrophages promote inflammation, and activated proinflammatory macrophages secrete cytotoxic factors [5–7]. This finding demonstrates the important role played by macrophages in inflammation [8,9]. Due to the complexity of liver inflammation and fibrosis induced by ethanol mediated via multiple factors, the management of ALD continues to be an extremely challenging issue in clinical medicine [10,11].

As an important signal transducer, extracellular adenosine triphosphate (ATP) plays an important role not only in cellular energy metabolism and also serves as a “danger signal” in the pathophysiology of chronic liver diseases including ALD [12,13]. In the extracellular compartment, ATP acts as a signaling molecule primarily by activating the purinergic P2 receptor. Irachetavellve et al. found that in addition to the release of cytokines/inflammatory factors in ALD, a large amount of ATP is generated, which acts as an endogenous signal activating the inflammatory complex via the purinergic receptor pathway, thereby triggering the synthesis and release of IL-1β and amplifying the inflammatory response [14]. P2 receptors are divided into ligand-gated nonselective cationic channel receptors (P2X) and G-protein coupled receptors (P2Y). P2Y2, a subtype of P2Y receptor family, has a unique structure and function, which is activated by the ligand UTP, triggering $Ca2+$ influx, which mediates a series of cell signal transduction pathways [15]. It has been reported [16] that P2Y2R expression in bone marrow-derived cells is essential for neutrophil liver infiltration and subsequent liver injury. P2Y2 promotes TNF-α production and mediates

* Corresponding author at: School of Pharmacy, Anhui Medical University, 81 Mei Shan Road, Hefei, Anhui Province 230032, China.
E-mail address: lxw31288@aliyun.com (X.-w. Lv).

https://doi.org/10.1016/j.intimp.2019.105915
Received 24 May 2019; Received in revised form 3 September 2019; Accepted 12 September 2019
Available online 19 October 2019

hepatocyte death; however, the function and molecular mechanism of hepatitis in alcoholic steatohepatitis remain to be investigated further.

Extracellular ATP is primarily hydrolyzed to ADP and AMP by the action of the CD39/ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPD1), and AMP is primarily converted to adenosine by CD73/ecto-5′-nucleotidase. Recent studies have shown that CD39 alleviates liver damage caused by sepsis [17]. Interestingly, Beldi G et al. [13] have reported that the failure of extracellular nucleotides in VEGF receptor-2/KDR transactivation of CD39-deficient endothelial cells is associated with P2Y2 receptor desensitization. In addition, in large liver metastases of CD39 transgenic mice, the level of mRNA and protein increased significantly [18,19]. Therefore, we investigated whether CD39 can be used as a therapeutic target as well as the mutual regulation of CD39 and P2Y2 in alcoholic steatohepatitis.

Based on the aforementioned studies, we inferred that ATP-P2Y2R signaling and CD39 play a key role in ALD. To verify this assumption, the expression of ATP, P2Y2R, and CD39 was investigated in a murine model of chronic-plus-binge alcohol feeding, which is similar to alcoholism in humans and simulates the clinical features of early steatohepatitis. We also conducted a similar study in vitro using RAW264.7 cells activated by alcohol. Importantly, the expression of CD39 and P2Y2R affects each other. Given the dramatic changes in ATP, P2Y2R, and CD39 expression following alcohol stimulation, we sought to define their role in alcoholic steatohepatitis.

2. Materials and methods

2.1. Materials and reagents

Suramin hexasodium salt was purchased from Abcam (Cambridge, UK). Adenosine 5′-triphosphate (ATP) disodium salt hydrate, uridine 5′-triphosphate trisodium salt dihydrate (UTP), dimethyl sulfoxide (DMSO), and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The polyclonal antibodies for P2Y2 and β-actin were obtained from Bioss (Beijing, China). The CD39 monoclonal antibody was supplied by Abcam (Cambridge, UK). The peroxidase-conjugated Goat Anti-Rabbit IgG (H + L) was purchased from ZSGB-BIO (Beijing, China). The following assay kits were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China): alanine aminotransferase (ALT) assay kit, aspartate aminotransferase (AST) assay kit, triglyceride (TG) assay kit, and total cholesterol (T-CHO) assay kit. The enzyme-linked immunosorbent assay (ELISA) kit was purchased from Elabscience Biotechnology Co. Ltd (Wuhan, China). The ATP colorimetric/fluorometric assay kit was ordered from BioVision (Milpitas, CA, United States). SYBR Green™ Premix Ex Taq™ II and PrimeScript™ RT Master Mix were procured from TAKARA BIO INC. (TAKARA, Japan).

2.2. Animals and induction of a chronic-plus-binge alcohol feeding model

Male C57BL/6 mice, each weighing about 18–22 g and aged 6–8 weeks, were acquired from Jinan Peng Yue Laboratory Animal Breeding Co. Ltd (Jinan, China) to develop mouse models of alcoholic steatohepatitis. All animal procedures were approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences of Anhui Medical University (Hefei, Anhui). Lieber-DeCarli ethanol (EtOH) and control liquid diet were acquired from TROPHIC Animal Feed High-Tech Co. LTD (Nanjing, China). First, the mice were fed with normal diet, tap water, and drinking water ad libitum for one week, followed by adaptation to two-day liquid diet. The mice were randomly divided into pair-fed group (n = 8), EtOH-fed group (n = 16), and mice treated with suramin at doses of 5, 10, and 20 mg/kg (n = 16) [20–22]. The groups fed with EtOH and suramin were exposed to one-third and two-thirds of alcohol liquid feed on the third and fourth days, respectively, and gradually transitioned to a 5% (vol/vol) alcoholic liquid feed for routine modeling. Meanwhile, from day 8 to day 15, mice were injected intraperitoneally with 0.1 mL/10 g once daily, whereas the pair-fed and the EtOH-fed group were injected with an identical volume of physiological saline. The mice were weighed every two days. On day 16, mice were gavaged with single-binge alcohol (5 g/kg body weight) or isocaloric maltose-dextrin (9 g/kg body weight). After 9 h, the mice were sacrificed under anesthesia, and the liver tissues and blood were salvaged for the next experiment.

2.3. ALT/AST/T-CHO/TG activity

Serum was separated from the whole blood and centrifuged at 3000 rpm for 30 min. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), and total triglyceride (TG) were measured using assay kits for the relevant activity.

2.4. Histological evaluation

The fresh liver tissues were fixed with formalin for about 24 h and embedded in paraffin. The paraffin-embedded tissues were sliced into 4-μm-thick sections and stained with hematoxylin and eosin (H & E) followed by visualization with an Olympus BX-51 microscope. A few fresh liver tissues were immersed in optimal cutting temperature compound (OCT) for Oil Red O staining.

2.5. Cell culture and cell treatment

RAW264.7 (No. TCM13) cell lines were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, HyClone, USA) supplemented with 10% (v/v) heat-inactivated fatal bovine serum (FBS, Bovine, China) and incubated at 37 °C with 5% CO2. RAW264.7 cells were pre-treated with 100 mM EtOH for 12 h before treatment with ATP, UTP, or suramin for 24 h.

2.6. MTT assay

Cell viability was assayed using standard 3-(4,5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide (MTT) reagent according to the manufacturer's protocol. Absorbance was measured at 490 nm using a Thermomax microplate reader (Bio-Tek EL, USA).

2.7. Cytokine assay by ELISA

The serum was collected from mice as well as the supernatants of RAW264.7. The release of IL-6, IL-1β, and TNF-α was detected with ELISA (Elabscience, Wuhan, China) according to the manufacturer's instruction. The optical density of samples was measured using a Thermomax microplate reader at 450 nm.

2.8. Western blot analysis

Mouse liver tissues and RAW264.7 cells were lysed with RIPA (Beyotime, Shanghai, China) and PMSF lysate buffer. The protein concentration was measured using a BCA protein assay kit (Beyotime, Shanghai, China). Equal amounts of proteins from liver tissue lysates or cell lysates were separated by 10% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad, CA, USA). The membranes were blocked in TBST containing 5% skim milk and incubated for 3 h with primary antibodies diluted in a primary antibody dilution buffer (Beyotime, Shanghai, China) for 12 h at 4 °C. Rabbit polyclonal anti-β-actin was diluted at 1:500, and P2Y2 was diluted at 1:600. Rabbit monoclonal anti-CD39 was diluted at 1:600. After incubation with primary antibodies, horseradish peroxidase-conjugated anti-rabbit was used as a secondary antibody (1:5000), and the membranes were treated at room temperature for 60 min after washing three times with TBS/T (Tris-

Table 1
RT-qPCR primers.

Gene	Forward	Reverse
β-Actin	GGAGCCCACCAAGAACGATA	GTTGTCACCAGAATCAGTCC
CD39	GTGTATGTGTGGGTCTGTGC	CCAGCTTGGAAAGACTGACG
P2Y2R	ATATCAGCCCCTTTAACAAGC	CAGTCAGCAGTGACGACTCAA
IL-6	GGAGCCCACCAAGAACGATA	GTTGTCACCAGCATCAGTCC
IL-1β	AAGAGCCCATCCTCTGTGAC	AGCTCATATGGGTCCGACAG
TNF-α	GACAGTGACCTGGACTGTGG	TGAGACAGAGGCAACCTGAC

buffered saline + 0.05% Tween 20), each time for 10 min. The relative quantity of protein was visualized using enhanced chemiluminescence after washing three times with TBS/T, each time for 10 min.

2.9. Total RNA extraction and real-time q-PCR

Total RNA was extracted from the liver or RAW264.7 cells using TRIzol™ reagent (Invitrogen, USA) following the instruction manual, and RNA was quantified by Nanodrop 2000 (Thermo Scientific, USA). The first-strand cDNA was synthesized using a PrimeScript™RT Master Mix (Takara, Japan) according to the manufacturer's instructions. SYBR Green real-time qPCR was performed with a PikoRreal 96 Real-Time PCR system (Thermo Scientific, USA). The primer sequences are listed in Table 1, using β-actin as a control for the normalization of mRNA expression. All primers were synthesized by Sangon Biotech (Shanghai, China).

2.10. Small RNA interference (SiRNA) analysis

Targeted siRNA sequences for mouse CD39, P2Y2R, and negative control siRNA were purchased from Shanghai Gena Pharma Corporation (Gena pharma, China). The siRNA sequences were as follows: CD39-siRNA (sense,5′-GGGCAGAUUCACUCAGGAATT-3′, and antisense, 5′-UUCCUGAGUGAAUCUGCCCTT-3′); P2Y2R-siRNA (sense, 5′-CCGAGAGCUCUUUAGCCAUTT-3′, and antisense, 5′-AUGGCUAAAG UACUCUCGGTT-3′); and Scrambled-siRNA (sense, 5′-UUCUCCGAACG UGUCACGUTT-3′ and antisense, 5′-ACGUGACACGUUCGGAGAATT-3′). RAW264.7 cells were cultured in the indicated wells of the plate for 12 h and transfected with siRNA using Lipofectamine TM2000 (Invitrogen, USA) according to the manufacturer's protocol. After 6 h, Opti-MEM (Gibco, USA) was replaced by DMEM (10% FBS), and 100 mM EtOH was added for 36 h.

2.11. ATP measurement

According to the manufacturers' instructions, lysates of 1×10^6 RAW264.7 cells or liver tissue homogenates of mice (10 mg) were used to measure ATP concentration using an ATP Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA) based on the phosphorylation of glycerol. The absorbance at 490 nm was read using a micro-plate reader model 680 (Bio-Rad Laboratories, Hercules, CA, USA). Assays were conducted at room temperature and protected from light.

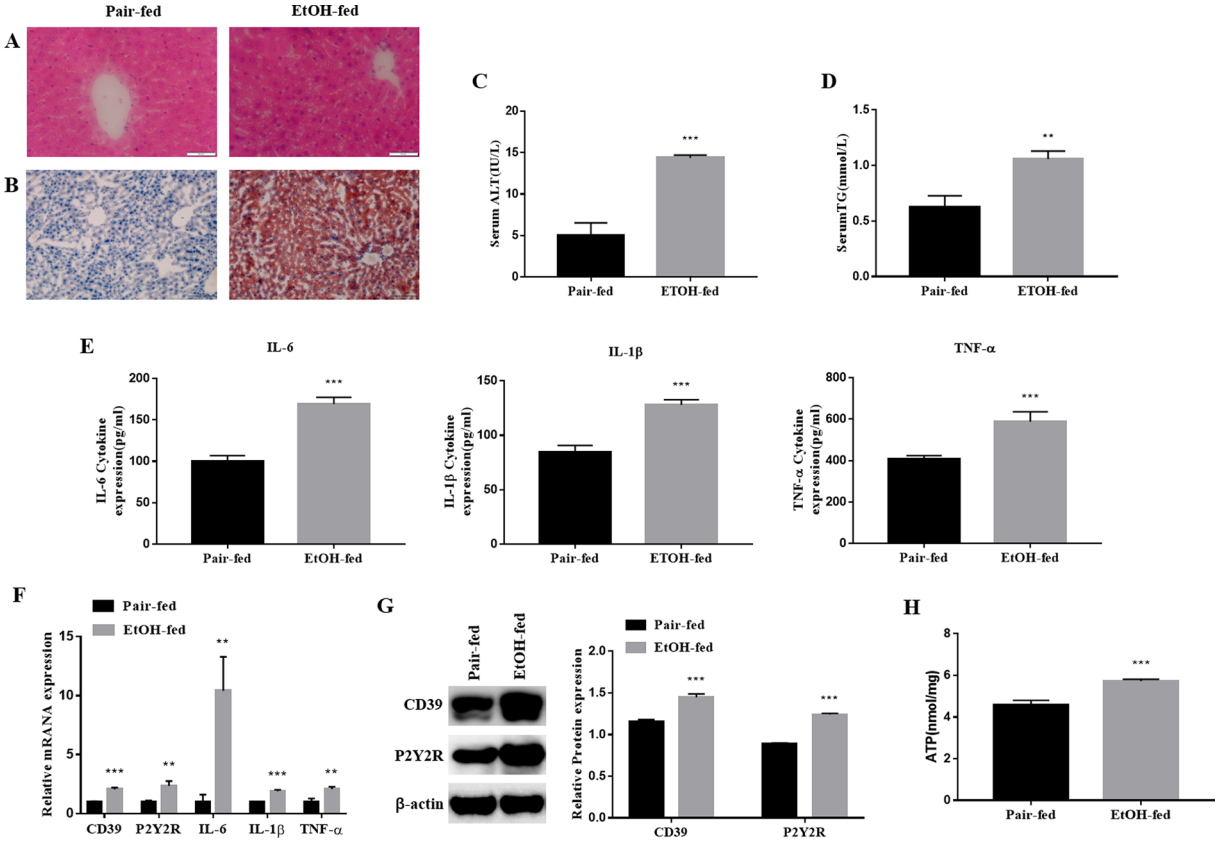

Fig. 1. The levels of ATP, P2Y2R, and CD39 increased in mice with alcohol-induced steatohepatitis. (A) Representative HE staining of liver sections (original magnification: 400×, scale bar = 50 μm); (B) Representative Oil Red O staining of liver sections (original magnification: 200×, scale bar = 100 μm); (D) and (E) Serum ALT and TG levels, n = 5–6. (E) The release of inflammatory cytokines IL-6, IL-1β, and TNF-α in mouse serum was measured by ELISA. (F) and (G) The mRNA and protein levels of CD39 and P2Y2R in the liver; (H) Levels of ATP in the liver. *P < 0.05, **P < 0.01, ***P < 0.001 compared with pair-fed group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

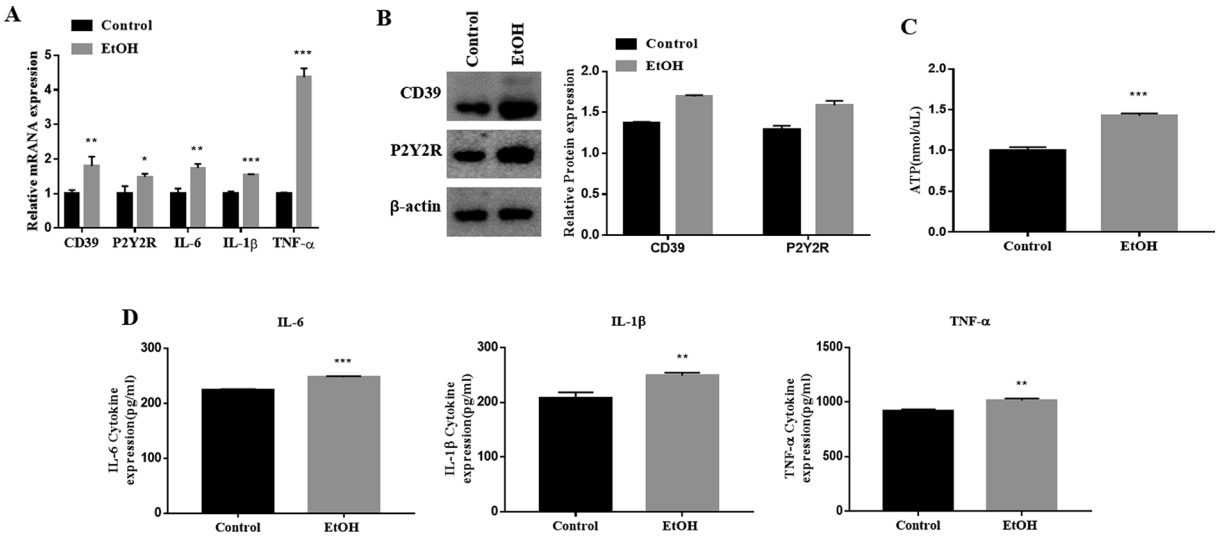

Fig. 2. ATP, P2Y2R, and CD39 levels increased in EtOH-primed RAW264.7 cells. (A) The mRNA levels of CD39, P2Y2R, IL-6, IL-1β, and TNF-α in RAW264.7 cells. (B) The protein levels of CD39 and P2Y2R in RAW264.7 cells. (C) ATP level in RAW264.7 cells. (D) The release of inflammatory cytokines IL-6, IL-1β, and TNF-α in serum from RAW264.7 cells was measured by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group.

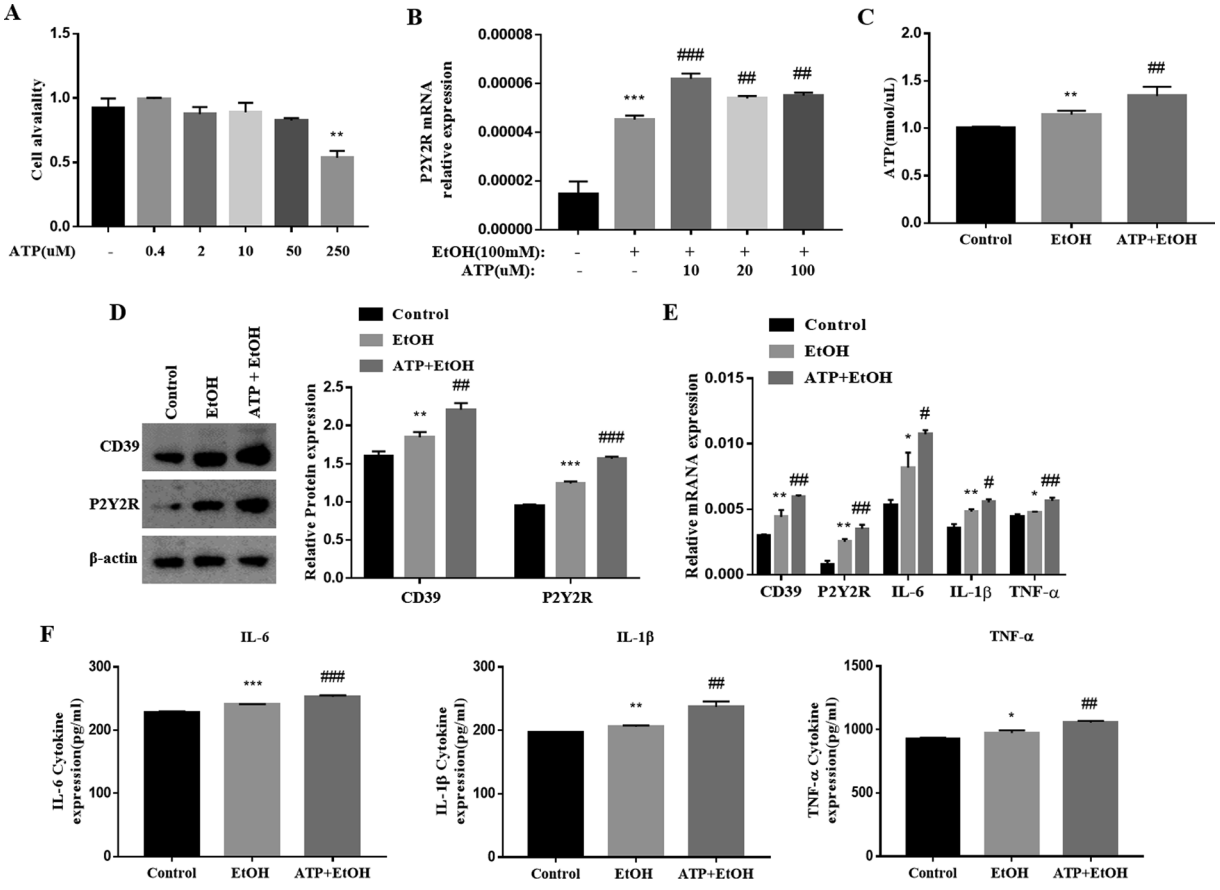

Fig. 3. Extracellular ATP acting via P2Y2R contributes to EtOH-primed inflammatory response in RAW264.7 cells. (A) MTT assay was used to select the safe dose of ATP in RAW264.7 cells. (B) RT-qPCR was used to select the appropriate dose of ATP in RAW264.7 cells. (C) The level of ATP in RAW264.7 cells. (D) The protein levels of CD39 and P2Y2R in RAW264.7 cells. (E) The mRNA levels of CD39 and P2Y2R in RAW264.7 cells. (F) The release of inflammatory cytokines IL-6, IL-1β, and TNF-α from RAW264.7 cells into the serum was measured by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with pair-fed group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with EtOH-fed group.

2.12. Statistical analysis

All results are presented as mean ± SD using GraphPad Prism (San Diego, CA). Statistical significance was performed using either the Student's *t*-test or One-Way ANOVA (LSD), and significance was denoted as follows: *P, #P < 0.05, **P, ##P < 0.01, and ***P, ###P < 0.001.

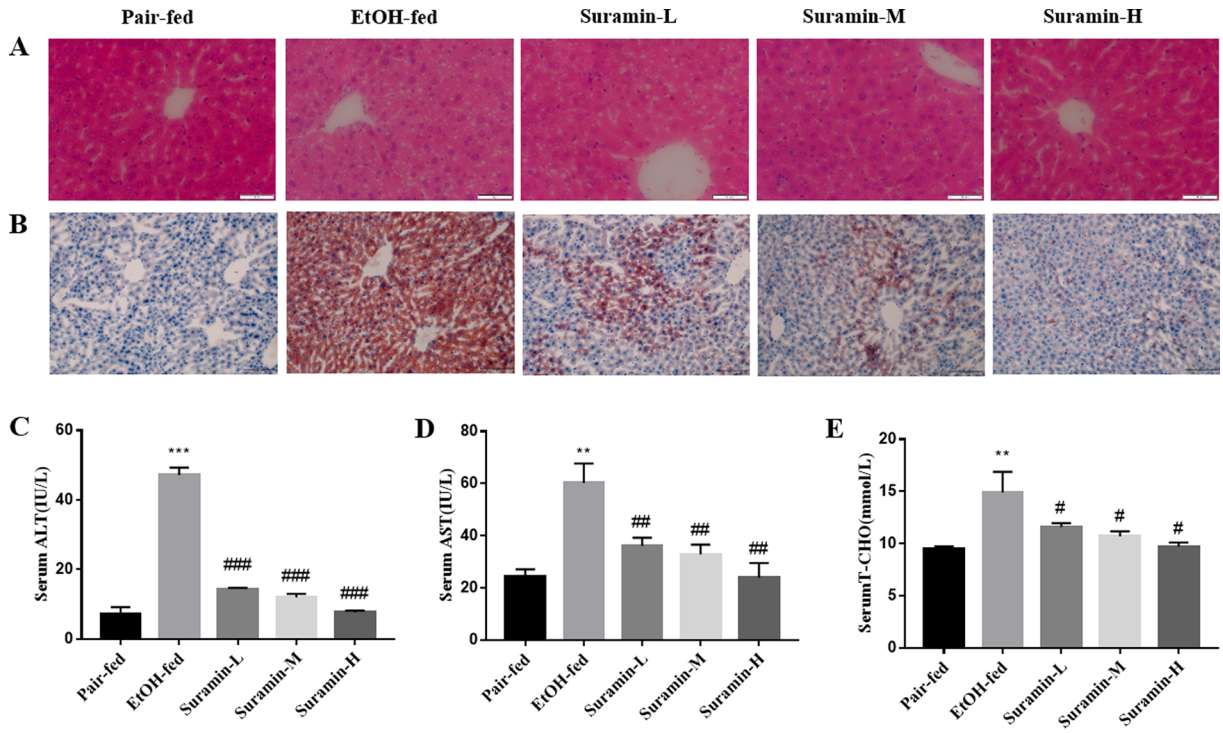

Fig. 4. P2Y2R inhibition alleviates alcoholic liver steatosis and inflammation in vivo. (A) Representative HE staining of liver sections (original magnification: 400×, scale bar = 50 μm). (B) Representative Oil Red O staining of liver sections (original magnification: 200×, scale bar = 100 μm). (C) and (D) Serum ALT and AST levels, n = 5–6. (E) and (F) Hepatic triglyceride (TG) and total cholesterol (T-CHO) levels, n = 5–6. (G) Body weight loss, n = 6. (H) Liver index changes, n = 6. (I)–(K) The mRNA levels of IL-6, IL-1β, and TNF-α in the liver. (L) The release of inflammatory cytokines IL-6, IL-1β, and TNF-α in serum from mice was measured by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with pair-fed group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with EtOH-fed group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. ATP, P2Y2R, and CD39 levels increased in mice with alcohol-induced steatohepatitis

In an attempt to identify the role of ATP, P2Y2R, and CD39 in alcohol-induced steatohepatitis, we performed histopathological and serological studies, western blot, real-time q-PCR, and ELISA assays of liver cells and tissues. Based on staining with hematoxylin and eosin (H & E) and Oil Red O, we found that the liver tissue was histologically normal in the pair-fed group. In contrast, abnormal liver cell cords and enlarged fat vacuoles as well as fat accumulation (Fig. 1A and B) were observed in the EtOH-fed group. Meanwhile, compared to the pair-fed mice, the serum levels of ALT and TG showed a significant increase in the livers of EtOH-fed mice (Fig. 1C and D). Furthermore, the release of inflammatory cytokines in the serum from mice was tested by ELISA. The results showed that EtOH exposure increased the expression of IL-6, IL-1β, and TNF-α significantly (Fig. 1E). The RT-qPCR results also showed a high level of IL-6, IL-1β, and TNF-α in EtOH-fed mice (Fig. 1F). Results of RT-qPCR and western blot demonstrated a significant upregulation of P2Y2R and CD39 expression in the livers of EtOH-fed mice compared with pair-fed mice (Fig. 1F and G). Moreover, the ATP Colorimetric/Fluorometric Assay revealed an increase in extracellular ATP concentration (Fig. 1H) in EtOH-fed mice. These results suggest that the ATP, P2Y2R, and CD39 were involved in alcohol-induced steatohepatitis.

3.2. ATP, P2Y2R, and CD39 levels increased in EtOH-primed RAW264.7 cells

To evoke an inflammatory response, 100 mM EtOH was used to stimulate RAW264.7 cells (mouse-derived macrophages). RT-qPCR and ELISA were used to determine the expression of pro-inflammatory cytokines. All the results indicate that the levels of IL-6, IL-1β, and TNF-α were enhanced after EtOH treatment (Fig. 2A and D). Western blot revealed that P2Y2R and CD39 were highly expressed in EtOH-primed RAW264.7 cells (Fig. 2B). Additionally, the mRNA levels of P2Y2R and CD39 were consistent with their corresponding protein expression (Fig. 2A). Moreover, EtOH-primed cells showed significantly higher levels of ATP compared with those of the control group (Fig. 2C). These data indicated that the ATP, P2Y2R, and CD39 mediated inflammation in vitro.

3.3. Extracellular ATP acting via P2Y2R contributes to EtOH-primed inflammation in RAW264.7 cells

According to a previous study, ATP, P2Y2R, and CD39 levels were increased in liver tissues showing alcohol-induced steatohepatitis and in EtOH-primed RAW264.7 cells. Meanwhile, ATP is one of the major natural ligands of P2Y2R, suggesting that P2Y2R activated by extracellular ATP may promote alcohol-induced inflammation. To test this hypothesis, ATP was used. First, we evaluated the safe dose of ATP in RAW264.7 cells using MTT (Fig. 3A). The result showed no cytotoxicity at concentrations up to 250 μM. Thus, we selected suramin concentrations of 10, 20, and 200 μM and found that 200 μM suramin exhibits the most inhibitory effect on P2Y2R (Fig. 3B). As shown in Fig. 3C−E, in addition to the increase in ATP, the protein and mRNA levels of P2Y2R and CD39 were clearly enhanced. Furthermore, RT-qPCR and ELISA results showed that the levels of IL-6, IL-1β, and TNF-α were obviously increased in EtOH-primed RAW264.7 cells treated with ATP compared with EtOH alone (Fig. 3E and F). Taken together, these data indicate that P2Y2R was activated by extracellular ATP, and ATP-P2Y2R signaling played a role in alcohol-induced inflammation.

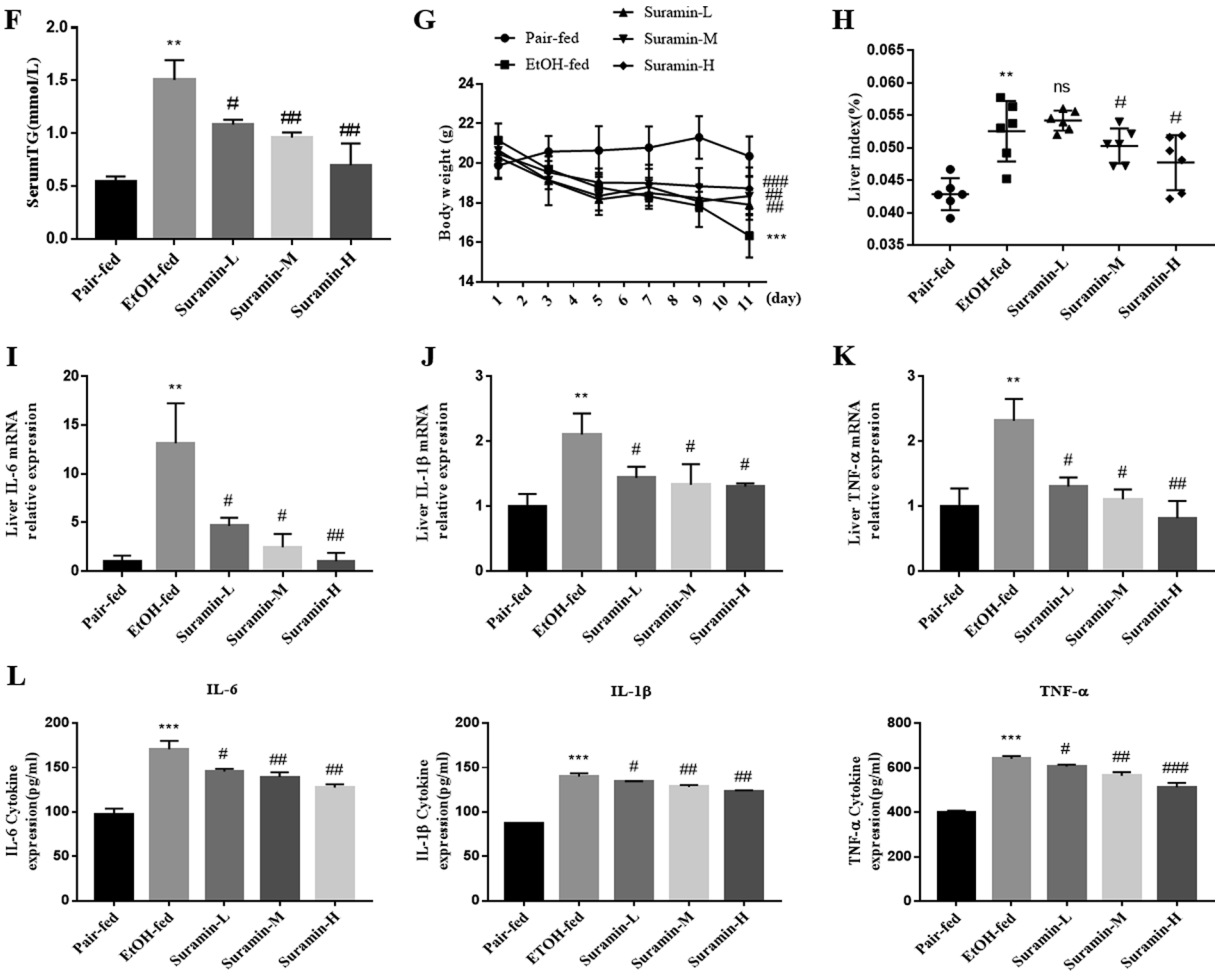

Fig. 4. (*continued*)

3.4. P2Y2R inhibition decreased CD39 activity and mitigated alcohol-induced steatohepatitis in vivo

To further confirm the effect of P2Y2R on alcohol-induced steatohepatitis, we conducted the following experiment. The pharmacological inhibition of P2Y2R using suramin significantly decreased the liver injury. First, the degree of liver injury in EtOH-fed mice was evaluated by H&E and Oil Red O. Obviously, EtOH-fed mice showed severe hepatic cord disorders as well as fat infiltration, and this effect was reversed by pharmacological blockade of the P2Y2R (Fig. 4A and B). These results were further confirmed by the levels of ALT, AST, TC, and TG (Fig. 4C − F). Meanwhile, chronic-binge-alcohol consumption induced body weight loss and elevated liver index, whereas exposure to high-dose suramin improved these changes obviously (Fig. 4G and H). Treatment with P2Y2R inhibitor also reduced the expression of IL-6, IL-1β, and TNF-α, which were tested by RT-qPCR and ELISA of the livers in EtOH-fed mice (Fig. 4I − L). Western blot and RT-qPCR results showed that treatment with suramin concentrations of 5, 10, and 20 mg/kg markedly inhibited P2Y2R in a dose-dependently manner. Interestingly, we further observed a significant decrease in CD39 production in the suramin groups (Fig. 5A − C). Therefore, we conclude that P2Y2R inhibition attenuates alcohol-induced steatohepatitis, and CD39 activity is modulated by P2Y2R.

3.5. P2Y2R aggravates EtOH-primed inflammation as well as modulates the activity of CD39 in vitro

Emerging evidence supports the pro-inflammatory effect of P2Y2R and the modulation of CD39 by P2Y2R in EtOH-primed RAW264.7 cells. Suramin and UTP were used to activate and inhibit P2Y2R, respectively. MTT assays showed that treatment with 600 μM suramin and 240 μM UTP had a limited suppressive effect on the cell viability of RAW264.7 cells (Fig. 6A and B). Finally, we selected the most appropriate concentration based on the results of RT-qPCR (Fig. 6C and D). The RT-qPCR and Western blot data consistently demonstrated an obvious decrease in P2Y2R in the suramin + EtOH-primed group. Conversely, the levels of P2Y2R were increased in the UTP + EtOH-primed group, and the CD39 level was consistent with the increase (Fig. 7A and B). In addition, RAW264.7 cells expressed higher levels of IL-6, IL-1β, and TNF-α after stimulation with UTP and EtOH, compared with EtOH-primed RAW264.7 cells. Conversely, suramin exhibited a markedly anti-inflammatory activity in EtOH-primed RAW264.7 cells (Figs. 6E and 7B). These findings also suggest that P2Y2R aggravates EtOH-primed inflammation and controls CD39 activity.

3.6. SiRNA-mediated knockdown of P2Y2R aggravates EtOH-primed inflammation and decreases CD39 expression in vitro

To further investigate the effect of P2Y2R on CD39, P2Y2R-siRNA was used in RAW264.7 cells. As shown in Fig. 8A and B, the down-regulation of P2Y2R in EtOH-primed RAW264.7 cells decreased the protein, and mRNA levels of CD39. P2Y2R-siRNA also decreased the expression of IL-6, IL-1β, and TNF-α (Fig. 8B). These results further implicate P2Y2R in the aggravation of EtOH-primed inflammation and control of CD39 activity.

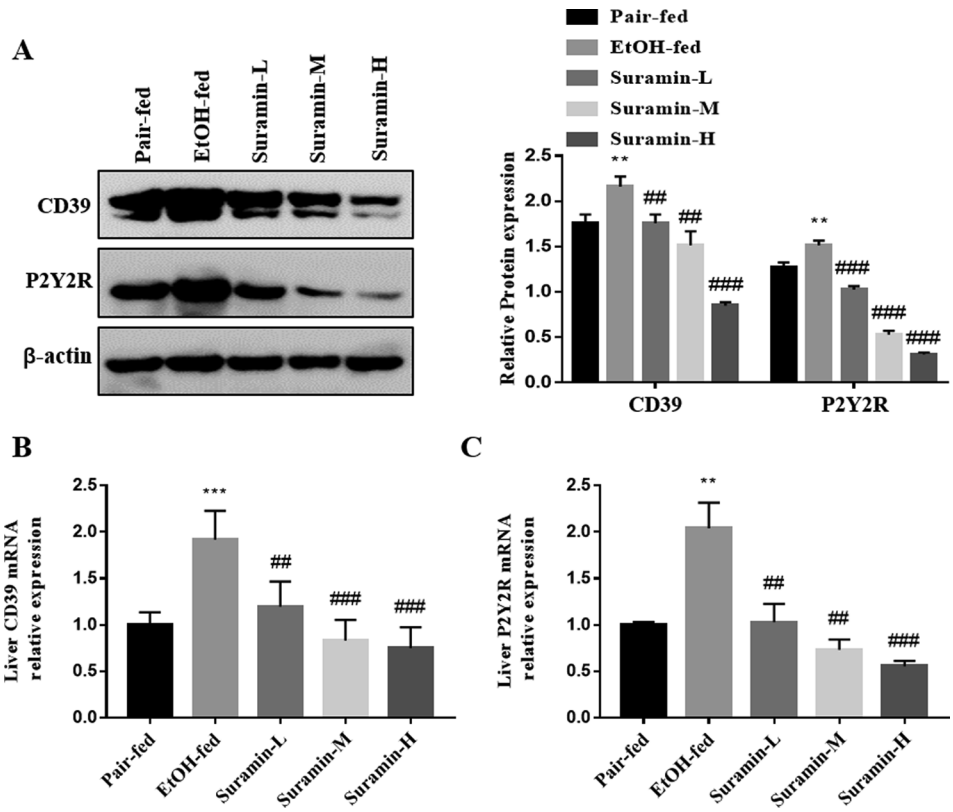

Fig. 5. P2Y2R inhibition decreased CD39 activity in vivo. (A) The protein levels of CD39 and P2Y2R in the liver. (B) and (C) Hepatic levels of CD39 and P2Y2R mRNA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with pair-fed group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with EtOH-fed group.

3.7. SiRNA-mediated knockdown of CD39 contributes to P2Y2R activation by increasing extracellular ATP concentration and exacerbates EtOH-primed inflammation

Based on the increased levels of CD39 and ATP-P2Y2R upon EtOH treatment in vivo and in vitro, we inferred that CD39 may be associated with alcoholic liver injury, and CD39 may modulate P2Y2R activity via ATP indirectly. Therefore, siRNA was used to knock down CD39 in EtOH-primed RAW264.7 cells (Fig. 10A and B). As shown in Fig. 10C, the extracellular ATP levels were upregulated in CD39-siRNA-transfected cells compared with scrambled-siRNA. Consistent with previous studies, the expression of P2Y2R was increased both at the protein and mRNA levels (Fig. 10A and B). This increase indicated that CD39 limits P2Y2R response by hydrolyzing extracellular ATP. Notably, the down-regulation of CD39 in EtOH-primed RAW264.7 cells increased the levels of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α (Figs. 9A and 10B). These data validate the anti-inflammatory effect of CD39.

4. Discussion

ALD is an inflammatory reaction induced directly or indirectly by ethanol and its derivatives during metabolism [23,24]. Steatosis is the earliest response to heavy drinking. During ALD pathogenesis, liver inflammation drives the progression of steatosis to steatohepatitis, a more severe type of inflammatory liver injury [25]. Studies have shown that ALD has become more common in many parts of Asia, while its incidence is decreasing in Western Europe [26]. ALD is a huge concern in China. Nonetheless, no FDA-approved drugs or nutritional therapies are available for the treatment of patients with ALD [27]. The only treatments available include behavioral interventions and drug interventions for alcohol withdrawal [28].

In recent years, purinergic signaling has attracted increased attention due to the complexity of signal transduction and the diversity of biological effects. It is involved in the pathophysiological regulation of various diseases, including liver disease [29,30]. Purinergic signaling is mediated mainly via extracellular nucleotides, nucleotide hydrolases, and specific receptors [31]. The current study showed the release of high concentrations of nucleotides (such as ATP) from intracellular to extracellular environment upon exposure to external stimuli such as ethanol. Inflammatory disorders are associated with extracellular release of nucleotides [32]. Several lines of evidence suggest extracellular ATP produces multiple biological effects when combined with P2 receptors in different diseases or different processes of the same disease [14,33,34]. P2Y2 receptors are widely expressed and play a key role in multiple domains. Neutrophil migration is associated with ATP-P2Y2R, and P2Y2R is associated with hepatocyte death [16,35,36]. Alcoholic hepatitis is characterized by hepatocyte death and neutrophil infiltration around the liver cells. Furthermore, conclusive evidence indicated that purinergic signaling promotes the release of pro-inflammatory cytokines such as IL-6 and TNF-α via P2YR, activates Kupffer cells, and mediates liver injury [16,37]. However, the effects of ATP-P2Y2R signaling on liver steatosis and inflammation induced by chronic-plus-binge alcohol feeding remain unclear.

Here, we demonstrate that ATP promotes inflammation by activating P2Y2R. The role of ATP-P2Y2R signaling in chronic-plus-binge alcohol feeding-induced liver steatosis and inflammation was evidenced by upregulated ATP and P2Y2R expression in the liver as well as RAW264.7 cells following EtOH stimulation compared with the control group. In order to confirm the effects of P2Y2R blockade on alcohol-induced liver steatosis and inflammation, suramin was used in vivo and in vitro. Our study showed that suramin significantly reduced fat accumulation, serum transaminase, and serum lipids as well as inhibited the release of inflammatory cytokines IL-6, IL-1β, and TNF-α. Importantly, suramin reversed the body weight loss and increased the liver index in EtOH-fed mice. Conversely, stimulation with UTP or ATP

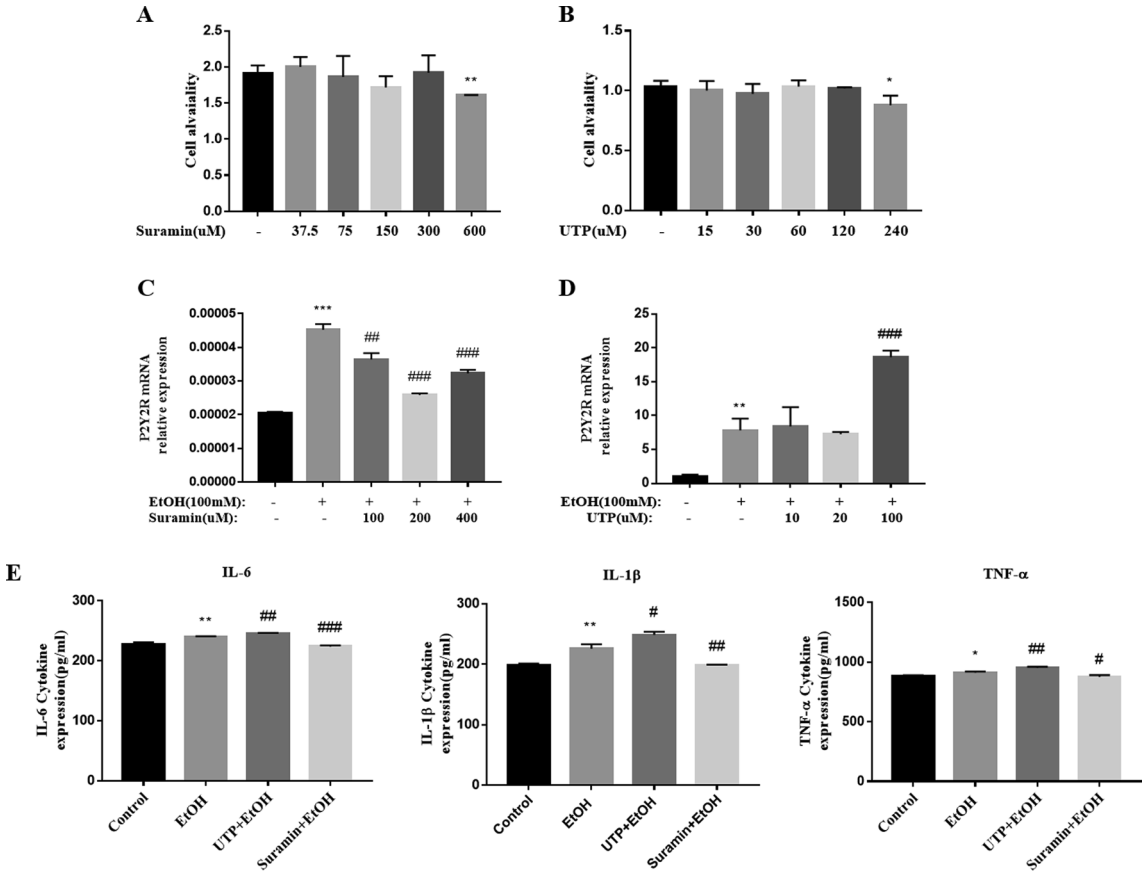

Fig. 6. The effect of P2Y2R on EtOH-primed RAW264.7 cells. (A) and (B) MTT assay was used to select the safe dose of suramin and UTP in RAW264.7 cells. (C) and (D) RT-qPCR was used to select the correct dose of suramin and UTP in RAW264.7 cells. (E) The release of inflammatory cytokines IL-6, IL-1β, and TNF-α in serum from RAW264.7 cells was measured by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with EtOH-primed group.

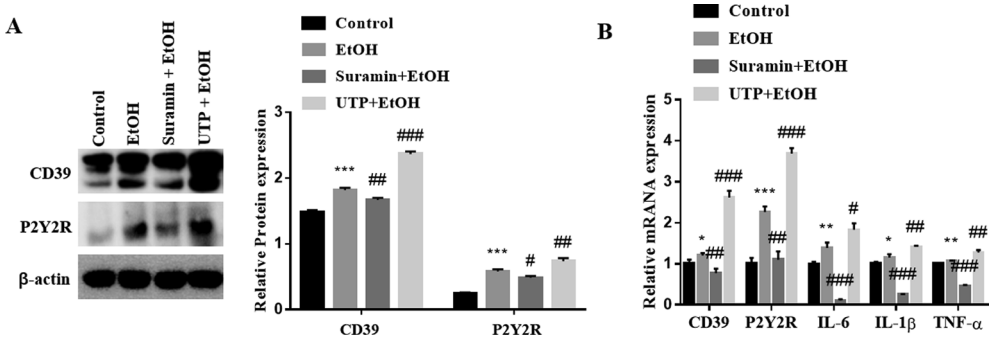

Fig. 7. P2Y2R modulates CD39 activity in vitro. (A) The protein levels of CD39 and P2Y2R in RAW264.7 cells. (B) and (C) The mRNA levels of CD39, P2Y2R, and pro-inflammatory cytokines in RAW264.7 cells. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with EtOH-primed group.

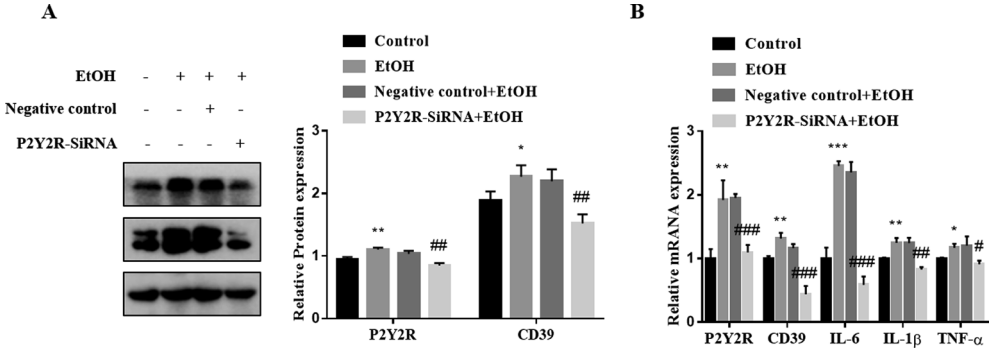

Fig. 8. SiRNA-mediated knockdown of P2Y2R aggravates EtOH-primed inflammation and inhibits CD39 expression. (A) The protein levels of CD39 and P2Y2R in RAW264.7 cells. (B) The mRNA levels of P2Y2R, CD39, and pro-inflammatory cytokines in RAW264.7 cells. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with scrambled-siRNA group.

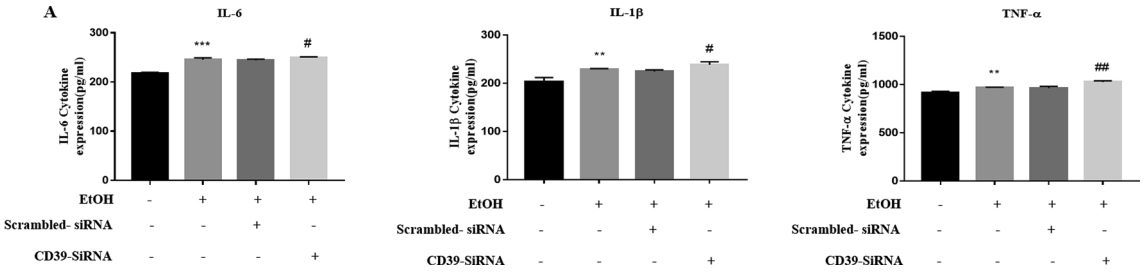

Fig. 9. SiRNA-mediated knockdown of CD39 aggravates EtOH-primed inflammation. (A) The release of inflammatory cytokines IL-6, IL-1β, and TNF-α from RAW264.7 cells into serum was measured by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with EtOH-primed group.

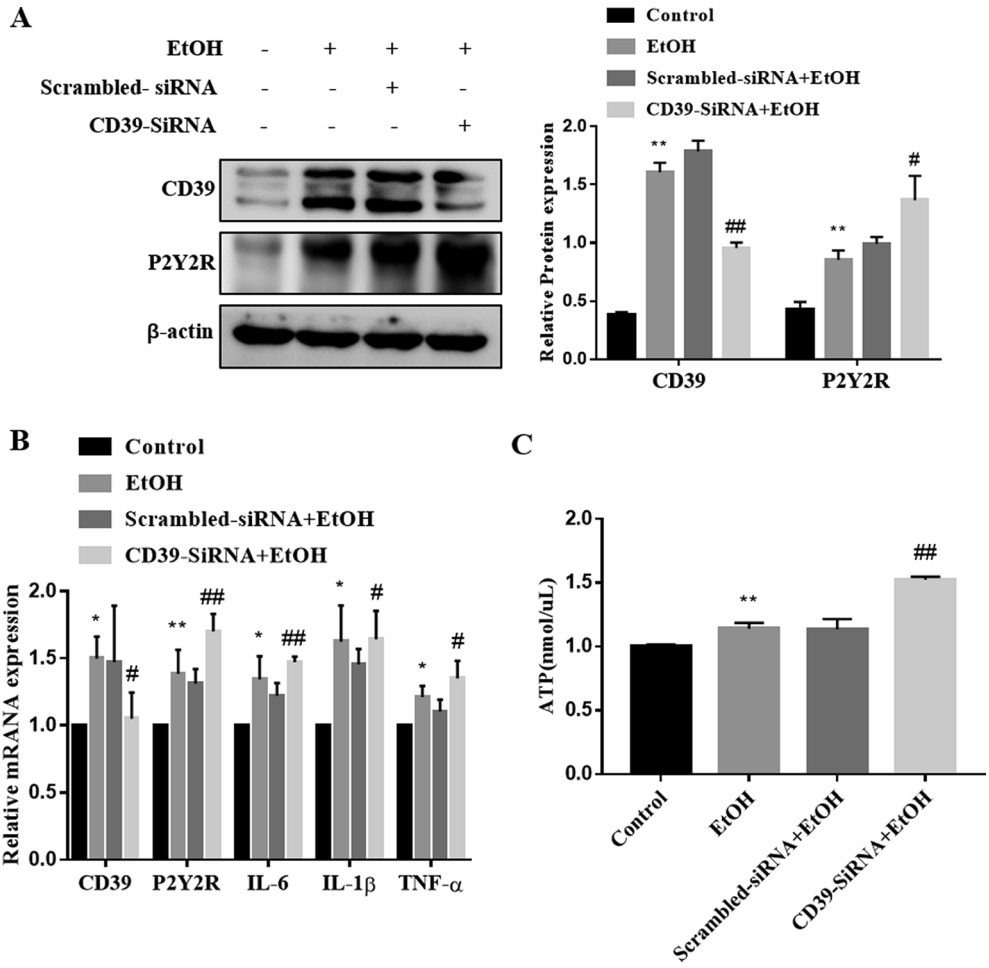

Fig. 10. CD39 modulates P2Y2R activity by hydrolyzing ATP in RAW264.7 cells. (A) The protein levels of CD39 and P2Y2R in RAW264.7 cells. (B) The mRNA levels of CD39, P2Y2R, and pro-inflammatory cytokines in RAW264.7 cells. (C) The ATP level in RAW264.7 cells. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with scrambled-siRNA group.

increased the expression of P2Y2R remarkably as well as aggravated liver inflammation. Treatment with P2Y2R-siRNA further demonstrated the role of P2Y2R in alcoholic inflammation. In conclusion, these results suggested that the inhibition of ATP-P2Y2R signaling with suramin may be a therapeutic strategy to limit ALD progression and manage alcoholic liver steatosis and inflammation.

CD39 is the rate-limiting enzyme in the extracellular nucleotide-to-adenosine phosphate hydrolysis cascade, which regulates the activation of purinergic receptors and inflammation in the liver [38,39]. Several lines of evidence reveal the protective role of CD39 in kidney, and lung [40–42]. Although Tak et al. found that CD39 effectively mediated the prevention and treatment of TAA-induced liver injury [43], the effects of CD39 on alcohol-induced liver steatosis and inflammation were incompletely elucidated. Our current study found that silencing of CD39 in RAW264.7 cells enhanced IL-6, IL-1β, and TNF-α expression, which aggravated inflammation. Meanwhile, the lack of CD39 raised extracellular ATP levels. Taken together, the dysregulation of the CD39-mediated protection mechanism in the body leads to disease, suggesting that CD39 can be used as a therapeutic target.

CD39 maintains the balance between nucleotide P2 receptor and adenosine P1 receptor signaling in inflammation and liver disease [29]. Künzli et al. found correlations between CD39 and P2R levels in tumor matrix of human PaCa (pancreatic cancer) [44]. Our findings suggest that the level of CD39 was decreased in mice with alcoholic steatohepatitis treated with suramin and in RAW264.7 cells primed with suramin and EtOH compared with EtOH treatment alone. Treatment with

P2Y2R-siRNA showed similar results. Interestingly, the deletion of CD39 in RAW264.7 cells enhanced P2Y2R production compared with the negative control group. Taken together, our findings indicate that P2Y2R boosts CD39 activity directly, and CD39 modulates P2Y2R levels based on the levels of extracellular ATP. Thus, CD39 represents a potential therapeutic target to limit P2Y2-dependent inflammation.

5. Conclusion

Given the pivotal role of ATP-P2Y2R and CD39 in alcoholic liver steatosis and inflammation, further studies are needed to elucidate the mechanism underlying ALD. Furthermore, P2Y2R may modulate the functionality of CD39, and CD39 may limit P2Y2R activation by hydrolyzing the extracellular ATP. Furthermore, these data suggest that ATP-P2Y2R signaling blockade and simulation of ectonucleotidase activity are possible therapeutic approaches.

Acknowledgments

This study was supported by National Natural Science Foundation of China (grant 81970518) and National Natural Science Foundation of China (grant 81270498). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

References

[1] S. Stewart, D. Jones, C.P. Day, Alcoholic liver disease: new insights into mechanisms and preventative strategies, Trends Mol. Med. 7 (9) (2001) 408–413.
[2] B. Gao, R. Bataller, Alcoholic liver disease: pathogenesis and new therapeutic targets, Gastroenterology 141 (5) (2011) 1572–1585.
[3] H.J. Wang, B. Gao, S. Zakhari, et al., Inflammation in alcoholic liver disease, Annu. Rev. Nutr. 32 (1) (2012) 343–368.
[4] H. Kiyoshi, P. Amanda, V. Golnaz, et al., Mechanisms underlying helper T-cell plasticity: implications for immune-mediated disease, J. Allergy Clin. Immunol. 131 (5) (2013) 1276–1287.
[5] Y. Iimuro, R.M. Gallucci, M.I. Luster, et al., Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat, Hepatology 26 (6) (2010) 1530–1537.
[6] P. Jan, B. Shashi, C. Timea, et al., IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice, J. Clin. Invest. 122 (10) (2012) 3476.
[7] M. Yin, M.D. Wheeler, H. Kono, et al., Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice, Gastroenterology 117 (4) (1999) 942–952.
[8] T. Zeng, C.L. Zhang, M. Xiao, et al., Critical roles of Kupffer cells in the pathogenesis of alcoholic liver disease: from basic science to clinical trials, Front. Immunol. 7 (2016) 10.
[9] C.J. Mcclain, D.B. Hill, Z. Song, et al., Monocyte activation in alcoholic liver disease, Alcohol 27 (1) (2002) 53–61.
[10] B. Saberi, A.S. Dadabhai, Y.Y. Jang, et al., Current management of alcoholic hepatitis and future therapies, J. Clin. Transl. Hepatol. 4 (2) (2016) 113–122.
[11] A.K. Singal, V.H. Shah, Therapeutic strategies for the treatment of alcoholic hepatitis, Semin. Liver Dis. 36 (01) (2016) 56–68.
[12] M. Ishimaru, N. Yusuke, M. Tsukimoto, et al., Purinergic signaling via P2Y receptors up-mediates IL-6 production by liver macrophages/Kupffer cells, J. Toxicol. Sci. 39 (3) (2014) 413–423.
[13] J. Petrasek, A. Iracheta-Vellve, B. Saha, et al., Metabolic danger signals, uric acid and ATP, mediate inflammatory cross-talk between hepatocytes and immune cells in alcoholic liver disease, J. Leukoc. Biol. 98 (2) (2015) 249–256.
[14] A. Irachetavellve, J. Petrasek, A. Satishchandran, et al., Inhibition of sterile danger signals, uric acid and ATP, prevents inflammasome activation and protects from alcoholic steatohepatitis in mice, J. Hepatol. 63 (5) (2015) 1147–1155.
[15] R.C.Y. Choi, G.K.Y. Chu, N.L. Siow, et al., Activation of UTP-sensitive P2Y2 receptor induces the expression of cholinergic genes in cultured cortical neurons: a signaling cascade triggered by Ca2+ mobilization and extracellular regulated kinase phosphorylation, Mol. Pharmacol. 84 (1) (2013) 50–61.
[16] C.K. Ayata, S.C. Ganal, B. Hockenjos, et al., Purinergic P2Y 2 receptors promote neutrophil infiltration and hepatocyte death in mice with acute liver injury, Gastroenterology 143 (6) (2012) 1620–1629.
[17] S. Leb, A.M.P. De, V.R. Figliuolo, et al., CD39 limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury, J. Hepatol. 67 (4) (2017).
[18] G. Beldi, Y. Wu, X. Sun, et al., Regulated catalysis of extracellular nucleotides by vascular CD39/ENTPD1 is required for liver regeneration, Gastroenterology 135 (5) (2008) 1751–1760.
[19] B.M. Künzli, S. Rath, S. Käser, et al., Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer, Purin. Signal 7 (2) (2011) 231–241.
[20] D. Sahu, S. Sharma, R.K. Singla, et al., Antioxidant activity and protective effect of suramin against oxidative stress in collagen induced arthritis, Eur. J. Pharmaceut. Sci. Off. J. Eur. Feder. Pharmaceut. Sci. 101 (2017) 125–139.
[21] L. Han, J.C. Hou, X.M. Fang, et al., Inhibitory effect of suramin on inflammatory response in pulmonary tissue and peripheral blood in LPS-induced septic mice, Zhejiang Da Xue Xue Bao Yi Xue Ban 44 (5) (2015) 553–558.
[22] A. Alyoussef, Suramin attenuated inflammation and reversed skin tissue damage in experimentally induced atopic dermatitis in mice, Inflam. Allergy Drug Targets 13 (6) (2015).
[23] S.W. French, Mechanisms of alcoholic liver injury, Canad. J. Gastroenterol. 14 (4) (2000) 327–332.
[24] L. Alexandre, M. Philippe, Alcoholic liver disease: mechanisms of injury and targeted treatment, Nat. Rev. Gastroenterol. Hepatol. 12 (4) (2015) 231–242.
[25] C. Benoit, E.M. Lucie, A.T. Gewirtz, Microbiota-liver axis in hepatic disease, Hepatology 59 (1) (2014) 328–339.
[26] S. Liangpunsakul, P. Haber, G.W. Mccaughan, alcoholic liver disease in Asia, Europe, and North America, Gastroenterology 150 (8) (2016) 1786–1797.
[27] N.A. Osna, T.M. Donohue Jr, K.K. Kharbanda, Alcoholic liver disease: pathogenesis and current management, Alcohol Res. Curr. Rev. 38 (2) (2017) 147.
[28] S. Wang, J.Q. Wang, X.W. Lv, Exosomal miRNAs as biomarkers in the diagnosis of liver disease, Biomark. Med. 11 (6) (2017) 491–501.
[29] B.P. Vaughn, S.C. Robson, M.S. Longhi, Purinergic signaling in liver disease, Dig. Dis. 32 (5) (2014) 516–524.
[30] G. Burnstock, B. Vaughn, S.C. Robson, Purinergic signalling in the liver in health and disease, Purin. Signal. 10 (1) (2014) 51–70.
[31] A. Verkhratsky, G. Burnstock, Biology of purinergic signalling: its ancient evolutionary roots, its omnipresence and its multiple functional significance, BioEssays 36 (7) (2014) 697–705.
[32] M. Dosch, J. Gerber, F. Jebbawi, et al., Mechanisms of ATP release by inflammatory cells, Int. J. Mol. Sci. 19 (4) (2018) 1222.
[33] B. Csóka, Z.H. Németh, G. Törő, et al., Extracellular ATP protects against sepsis through macrophage P2X7 purinergic receptors by enhancing intracellular bacterial killing, FASEB J. 29 (9) (2015) 3626–3637.
[34] J.B. Seo, S.R. Jung, B. Hille, et al., Extracellular ATP protects pancreatic duct epithelial cells from alcohol-induced damage through P2Y1 receptor-cAMP signal pathway, Cell Biol. Toxicol. 32 (3) (2016) 229–247.
[35] T. Müller, B. Robaye, R.P. Vieira, et al., The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation, Allergy 65 (12) (2010) 1545–1553.
[36] I. Yoshiaki, C. Yu, M.I. Hirsh, et al., A3 and P2Y2 receptors control the recruitment of neutrophils to the lungs in a mouse model of sepsis, Shock 30 (2) (2008) 173–177.
[37] I. Makiko, Y. Negishi, T. Mitsutoshi, et al., Purinergic signaling via P2Y receptors up-mediates IL-6 production by liver macrophages/Kupffer cells, J. Toxicol. Sci. 39 (3) (2014) 413.
[38] S.C. Robson, J. Sévigny, H. Zimmermann, The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance, Purin. Signal. 2 (2) (2006) 409–430.
[39] B.P. Vaughn, S.C. Robson, G. Burnstock, Pathological roles of purinergic signaling in the liver, J. Hepatol. 57 (4) (2012) 916–920.
[40] B.K. Kishore, S.C. Robson, K.M. Dwyer, CD39-adenosinergic axis in renal pathophysiology and therapeutics, Purin. Signal 14 (1) (2018) 1–12.
[41] Z.W. Peng, S. Rothweiler, G. Wei, et al., The ectonucleotidase ENTPD1/CD39 limits biliary injury and fibrosis in mouse models of sclerosing cholangitis[J], Hepatol. Commun. 1 (9) (2017) 957.
[42] Z. Lazar, N. Müllner, M. Lucattelli, et al., NTPDase1/CD39 and aberrant purinergic signalling in the pathogenesis of COPD, Eur. Respir. J. 47 (1) (2015) 254.
[43] E. Tak, D.H. Jung, S.H. Kim, et al., Protective role of hypoxia-inducible factor-1α-dependent CD39 and CD73 in fulminant acute liver failure, Toxicol. Appl. Pharmacol. 314 (2017) 72–81.
[44] B.M. Künzli, P.O. Berberat, G. Thomas, et al., Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease, Am. J. Physiol. Gastrointest. Liver Physiol. 292 (1) (2007) 223–230.