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Purinergic P2X7 receptor blockade mitigates alcohol-induced steatohepatitis and intestinal injury by regulating MEK1/2-ERK1/2 signaling and egr-1 activity

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ABSTRACT

The P2X7 receptor is an ATP-binding cation channel involved in a broad range of inflammatory diseases. However, little is known about the potential role of P2X7R in alcohol-induced steatohepatitis and intestinal injury. In our study, C57BL/6 mice were intraperitoneally injected with P2X7R antagonists Brilliant Blue G and A438079 from the 4th day to the 10th day during the induction of chronic plus binge alcohol feeding model. Our results showed that alcohol feeding induced significant steatohepatitis and liver injury, which were mitigated by P2X7R blockade as evidenced by decreased serum levels of ALT, AST, T-CHO and TG, reduced lipid accumulation, and less inflammation. The increased intestinal inflammatory cytokines production and the prominent intestinal barrier disruption caused by alcohol were also modulated by P2X7R antagonism. Interestingly, alcohol feeding increased the relative abundance of phylum Bacteroidetes while decreased the number of phylum Verrucomicrobia and genus *Akkermansia* in the cecal content, which were reversed by P2X7R antagonist. Importantly, the improvement of intestinal barrier function and the restoration of partial taxonomic alterations in the gut microbiota might contribute to protect the liver from gut microbiota dysbiosis-induced second hit. Furthermore, P2X7R blockade inhibited MEK1/2-ERK1/2 phosphorylation and egr-1 expression in both liver and intestine from alcohol-fed mice. Collectively, P2X7R blockade mitigates alcohol-induced steatohepatitis and intestinal injury by inhibiting MEK1/2-ERK1/2 signaling and egr-1 expression. These studies strongly suggest that P2X7R blockade may be a promising therapeutic approach for treating alcoholic liver disease.

1. Introduction

Heavy drinking and its consequences have led to estimated 3.8% of all global deaths [1], among which, alcoholic liver disease (ALD) stands out as a significant contributor of mortality [2]. ALD is a kind of liver damage caused by excessive and chronic drinking and consists of a wide spectrum ranging from steatosis (fatty liver) to alcoholic steatohepatitis (ASH), fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC) [3]. If active prevention measures can be taken in the early phase of ALD, it is expected to prevent or even reverse ALD. Since effective therapeutic strategies for ALD are still limited, novel targeted therapies for ALD are urgently needed.

The purinergic P2X7 receptor (P2X7R) is activated by high concentrations of extracellular ATP released at multiple pathological conditions (i.e. inflammation, ischemia and hypoxia, cell necrosis), promoting the production and release of Interleukin-1β (IL-1β), Interleukin-18 (IL-18) via the NLR pyrin domain containing 3 (NLRP3) inflammasome activation [4], which is required for chronic alcohol-induced ASH [5]. A growing number of reports document that P2X7R participates in several liver diseases like non-alcoholic steatohepatitis [6], acute alcoholic fatty liver [7] and alcoholic liver fibrosis [8,9]. Recent studies have shown that gentiopicroside suppressed alcohol-induced lipid accumulation and IL-1β production by modulating P2X7R-NLRP3 inflammasome, suggesting the therapeutic potential of P2X7R blockade in ALD [10]. However, the effects of pharmacological P2X7R blockade on chronic plus binge alcohol feeding-induced liver steatosis and inflammation remain unclear.

The importance of gut-liver axis in ALD has been identified. Alcohol consumption leads to intestinal hyperpermeability and gut bacterial dysbiosis [11–13]. Gut-derived endotoxins are recognized by the toll-like receptor 4 (TLR4) complex on the surface of Kupffer cells, followed by liver damage aggravation [14]. Several studies have shown that

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alcohol-related bacterial dysbiosis promoted the development of steatohepatitis, which was alleviated after probiotics treatment [15,16]. In addition, P2X7R mediated mast cell activation and dendritic cell recruitment to infectious sites in experimental and clinical inflammatory bowel disease (IBD) [17,18]. The inhibition or deficiency of P2X7R prevented experimental colitis [19,20]. However, little is known about the potential role of P2X7R in alcohol-induced intestinal injury.

Early growth response-1 (egr-1) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) contributed to the increased tumor necrosis factor-α (TNF-α) expression in LPS-stimulated rat Kupffer cells after chronic ethanol exposure [21]. Studies of human and Caco-2 monolayers demonstrated that ethanol disrupted intestinal barrier through mitogen-activated protein kinase (MAPK) signaling [22]. Egr-1, a member of the immediate-early gene family, regulated inflammatory mediator expression like TNF-α [23], which triggered ethanol-induced jejunal mucosal injury [24]. Mitogen-activated protein kinase kinase 1/2 (MEK1/2), members of the MAPK family, lie upstream of ERK1/2. Therefore, we were intrigued whether MEK1/2-ERK1/2 signaling and egr-1 exist in chronic plus binge alcohol feeding-induced steatohepatitis and intestinal injury.

Here, we use a murine model of chronic plus binge alcohol feeding, which is close to human drinking habit and well simulates the clinical features of early steatohepatitis, with P2X7R antagonists Brilliant Blue G (BBG) and A438079 to investigate the potential role of pharmacological P2X7R blockade in early alcoholic steatohepatitis and alcohol-induced intestinal injury.

2. Materials and methods

2.1. Animals and reagents

Eight- to ten-week-old male C57BL/6 mice (body mass > 20 g) were purchased from Jinan Peng Yue Laboratory Animal Breeding Co. LTD (Jinan, China) for experiments. Lieber-DeCarli ethanol (EtOH) and control liquid diet was from TROPHIC Animal Feed High-Tech Co. LTD (Nanjing, China). The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif): P2X7R, ERK1/2, phospho-ERK1/2 (p-ERK1/2, pT202/pY204.22A), egr-1, TNF-α, mature IL-1β (17 kDa), Interleukin-6 (IL-6), Lymphocyte antigen 6G (Ly-6G), chemotactic protein-1 (MCP-1), Zonula Occludens-1 (ZO-1), and Claudin-1. MEK1/2 and phospho-MEK1/2 (p-MEK1/2, Ser218/222) were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Rabbit Anti-beta-Actin was obtained from Bioss (Beijing, China). Peroxidase-Conjugated Goat Anti-Rabbit IgG (H + L) and Peroxidase-Conjugated Goat Anti-Mouse IgG (H + L) were purchased from ZSGB-BIO (Beijing, China). Alanine aminotransferase (ALT) Assay Kit, Aspartate aminotransferase (AST) Assay Kit, Triglyceride (TG) Assay Kit and Total cholesterol (T-CHO) Assay Kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). SYBR Green™ Premix Ex Taq™ II and PrimeScript™ RT Master Mix (Perfect Real Time) were acquired from TAKARA BIO INC. (TAKARA, Japan). Brilliant Blue G (Abcam, Cambridge, MA, USA), A-438079 hydrochloride (TargetMol, USA), QIAamp DNA Stool Mini Kit (Qiagen, Germany) and Western Bright™ Sirius™ (Advansta, USA) were used.

2.2. Induction of chronic plus binge alcohol feeding model

A murine model of chronic plus binge alcohol feeding has been described by Bertola and colleagues [25]. To be specific, C57BL/6 mice were maintained in a temperature-controlled environment (24 °C) with a 12-h light/dark cycle and fed with general food and tap water ad libitum for one week. Then, mice were allowed free access to Lieber-DeCarli control liquid diet for 5 days to adapt to a liquid diet and randomly assigned to the following six groups: pair-fed group (n = 10), EtOH-fed group (n = 15), BBG-low dosage group (BBG-L, 25 mg/kg, n = 15), BBG-middle dosage group (BBG-M, 50 mg/kg, n = 15), BBG-high dosage group (BBG-H, 100 mg/kg, n = 15) [26–28] and A438079 group (100 mg/kg, n = 15) [6,29]. The EtOH-fed group and P2X7R antagonist treatment groups were all fed with Lieber-DeCarli ethanol liquid diet containing 5% (vol/vol) alcohol for ten days according to the feeding protocol. Simultaneously, the control group was pair-fed with control liquid diet during the whole process. Drug treatment groups were administrated with corresponding dosages mentioned above by intraperitoneal injection in a volume of 0.1 ml/10 g from the 4th day to the 10th day. Mice of pair-fed group and alcohol-fed group were injected with the same volume of saline. Body weight of all groups was monitored every other day. On the morning of the 11th day, animals were intragastrically administrated with either alcohol (5 g/kg body weight) or isocaloric maltose dextrin (9 g/kg body weight). Nine hours later, blood, liver tissues, small intestinal tissues and cecal contents were harvested. Liver wet weight was measured and recorded quickly. These protocols are represented in Supplementary Fig. 1. All procedures were approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences of Anhui Medical University.

2.3. Serum parameters measurements

Serum was separated from whole blood by centrifugation (3000 rpm, 30 min at 4 °C) after 3–4 h of storage at room temperature and preserved at −80 °C until use. Serum levels of ALT, AST, TG and T-CHO were measured using commercially available kits.

2.4. Histological evaluation

Fresh liver tissues were fixed in formalin followed by paraffin embedding for hematoxylin/eosin (HE) staining or P2X7R immunohistochemistry (IHC) and Ly-6G IHC. Some fresh liver tissues were immersed in optimal cutting temperature compound (OCT compound) for Oil Red O staining. Proximal small intestine was fixed and processed for HE staining and P2X7R IHC. The remaining liver and small intestine and the content of cecum were frozen immediately in liquid nitrogen and then stored at −80 °C until further analysis.

2.5. Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from the liver or intestinal tissues using Trizol™ reagent (Invitrogen, USA) following the instruction manual. RNA purity was measured by Nanodrop 2000 (Thermo Scientific, USA). Then cDNA was amplified using commercial first strand cDNA synthesis kit followed by dilution (1:9) in nuclease-free water. SYBR Green real-time qPCR was performed with PikoRreal 96 Real-Time PCR system (Thermo Scientific, USA). The primer sequences are listed in Table 1 and β-actin was used as a normalization control for $2^{-\Delta\Delta Ct}$ method of mRNA expression analysis. All primers used for RT-qPCR analysis were synthesized by Sangon Biotech (Shanghai, China).

2.6. Gut microbiota

Microbiota DNA was extracted from the frozen cecal content using a QIAamp DNA Stool Mini Kit according to the manufacturer's instruction. The relative levels of lactobacilli and bifidobacteria within the gut microbiota were detected by RT-qPCR using SYBR Green Real-Time PCR Kits as described above. The primers used here are listed in Table 1 and the relative proportions of lactobacilli and bifidobacteria were evaluated against total bacteria by $2^{-\Delta\Delta Ct}$ method.

The quality inspection and 16S rRNA sequencing of microbiota DNA were completed at BGI Genomics, BGI-Shenzhen (Shenzhen, China).

2.7. Western blot analysis

Total protein was extracted from the liver or intestinal tissues using RIPA lysis buffer (Beyotime, China) after tissue homogenization, and

Table 1
RT-qPCR primers.

Gene	Forward (5′ > 3′)	Reverse (5′ > 3′)
Total bacteria [30]	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG
Bifidobacteria [31]	GGGTGGTAATGCCGGATG	TAAGCCATGGACTTTCACACC
Lactobacilli [31]	CATCCAGTGCAAACCTAAGAG	GATCCGCTTGCCTTCGCA
β-Actin	GGGAAATCGTGCGTGAC	AGGCTGGAAAAGAGCCT
P2X7R	TGCTTTGGTGAGCGATAAGC	CCCACCCTCTGTGACATTCT
TNF-α	CAGGTCACTGTCCCAGCATCT	GAGTCCGGGCAGGTCTACTTT
IL-1β	GGTAAGTGGTTGCCCATCAGA	GTCGCTCAGGGTCACAAGAAA
IL-6	AGTCCGGAGAGGAGACTTCA	ATTTCCACGATTTCCCAGAG
ZO-1	GTTCCGGGGAAGTTACGTGC	GGGACAAAAGTCCGGGAAGC
Claudin-1	GGCTTCTCTGGGATGGATCG	TTTGCGAAACGCAGGACATC

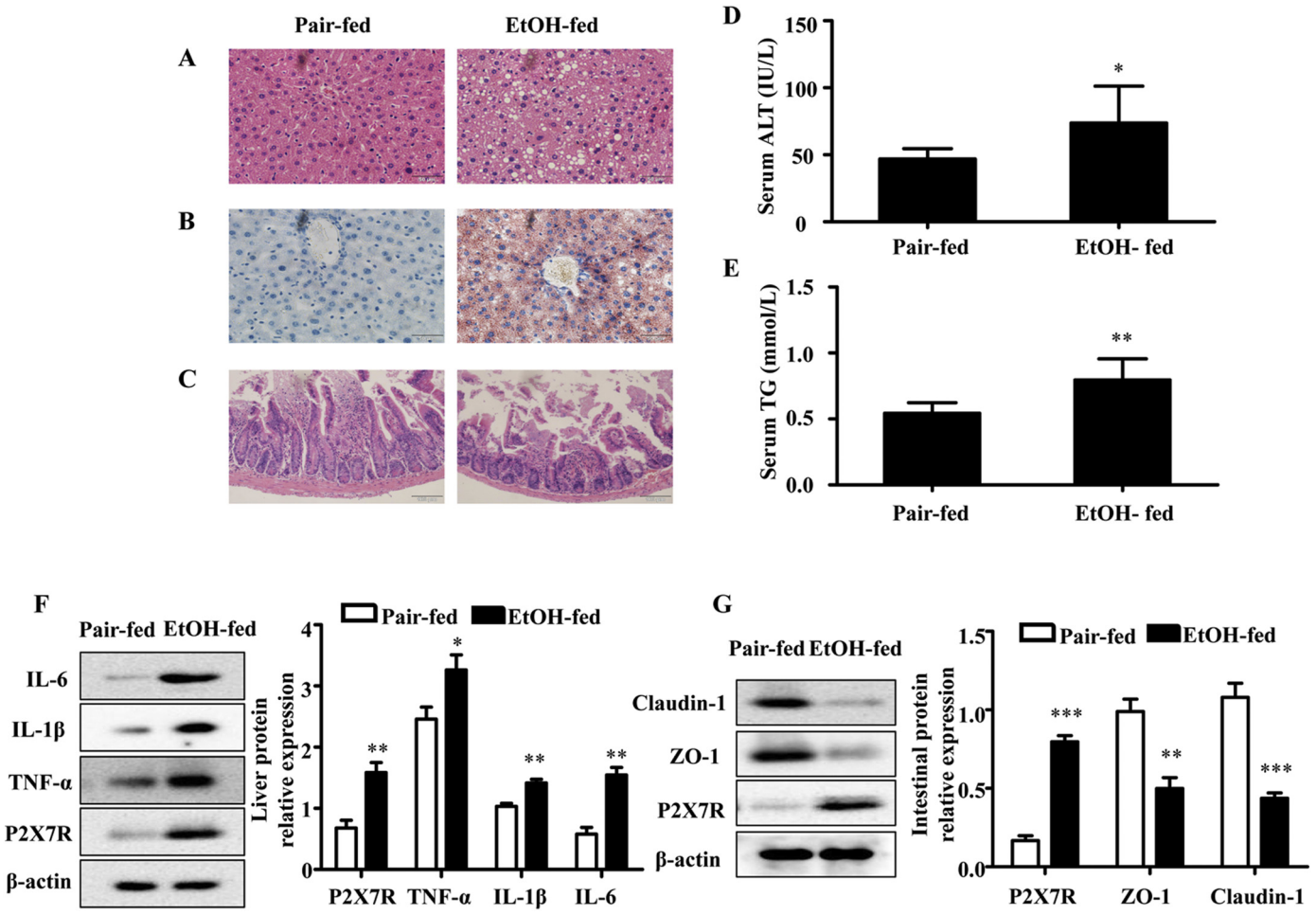

Fig. 1. P2X7R was involved in chronic-binge alcohol feeding-induced steatohepatitis and intestinal injury. (A) The representative HE staining of liver sections (original magnification: 400×, scale bar = 50 μm). (B) The representative Oil Red O staining of liver sections (original magnification: 400×, scale bar = 50 μm). (C) The representative HE staining of small intestinal tissues (original magnification: 200×, scale bar = 100 μm). Serum ALT (D) and serum TG (E) levels were measured, n = 5–6. (F) The protein expression of P2X7R, TNF-α, IL-1β and IL-6 in the liver. (G) The protein expression of P2X7R, ZO-1 and Claudin-1 in the intestine. *P < 0.05, **P < 0.01, ***P < 0.001 vs. pair-fed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein concentrations were measured utilizing a BCA protein assay kit (Beyotime, China). Protein was separated by different concentrations of SDS-PAGE gel (8%, 10% and 12%) followed by transferring to PVDF membranes (Bio-Rad, CA, USA). The membranes were blocked with 5% defatted milk for 3 h and incubated with corresponding primary antibodies at 4 °C overnight. After three washes in TBS/T (Tris Buffered Saline + 0.05% Tween 20), protein bands were incubated with secondary anti-rabbit or anti-mouse HRP conjugated antibodies for 60 min at room temperature and visualized using enhanced chemiluminescence. The relative quantity of protein was further analyzed using Image J software (NIH, Bethesda, MD, United States).

2.8. Data analysis

All results are presented as mean ± SD using GraphPad Prism (San Diego, CA). Significant differences between experimental groups were determined using one-way ANOVA test or Student's *t*-test and statistical significance was set at $P < 0.05$. Data shown are representative of at least three independent experiments.

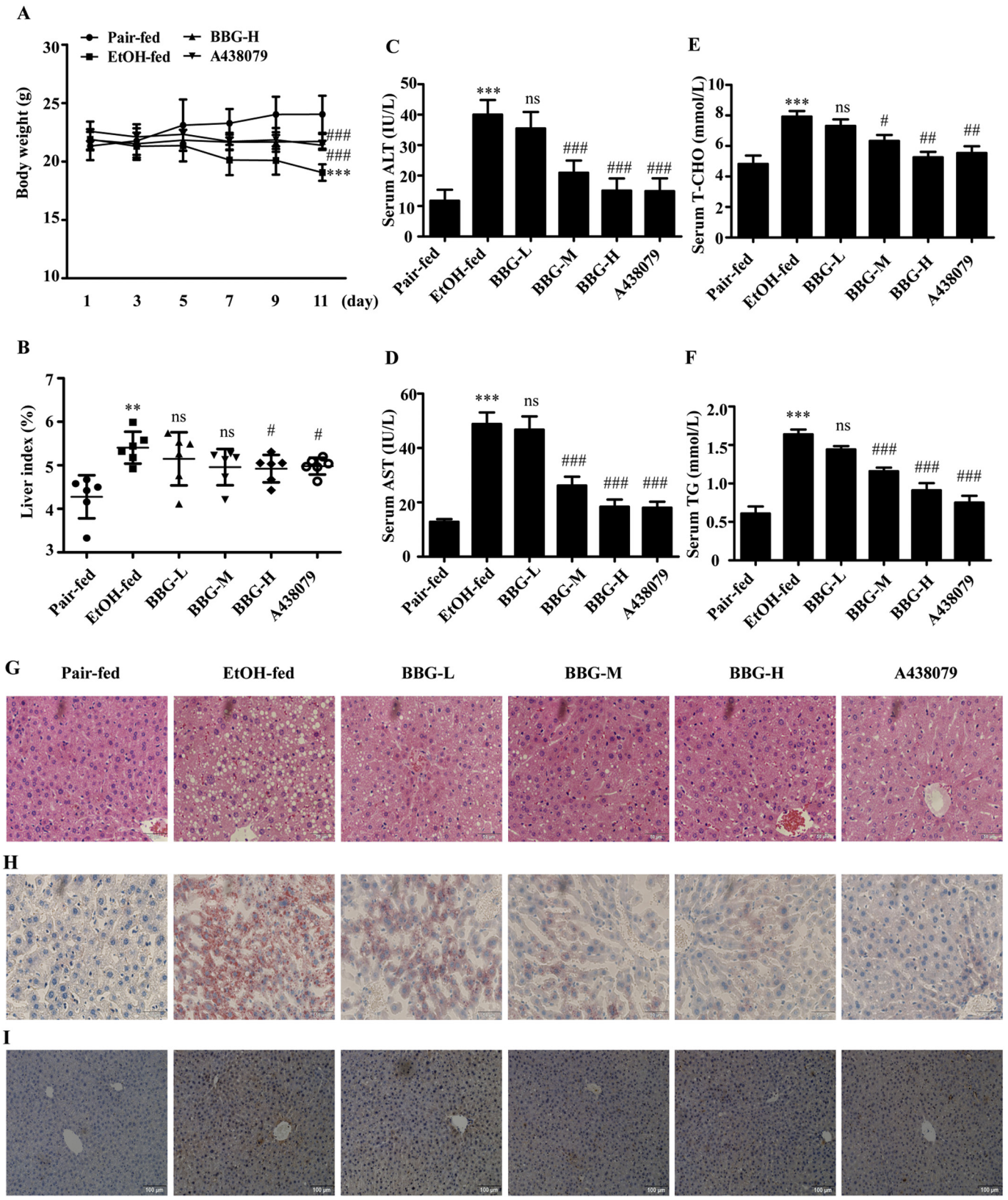

Fig. 2. Effects of P2X7R blockade on chronic-binge alcohol feeding-induced liver injury and steatohepatitis. (A) Body weight loss, n = 6. (B) Liver index changes, n = 6. Liver injury and steatosis were evaluated by measuring the serum levels of ALT (C), AST (D), T-CHO (E) and TG (F), n = 5–6. (G) The representative HE staining of liver sections (original magnification: 400×, scale bar = 50 μm). (H) The representative Oil Red O staining of liver sections (original magnification: 400×, scale bar = 50 μm). (I) The representative P2X7R immunohistochemical staining of liver sections (original magnification: 200×, scale bar = 100 μm). **P < 0.01, ***P < 0.001 vs. pair-fed; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. EtOH-fed; ns, no statistical significance vs. EtOH-fed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

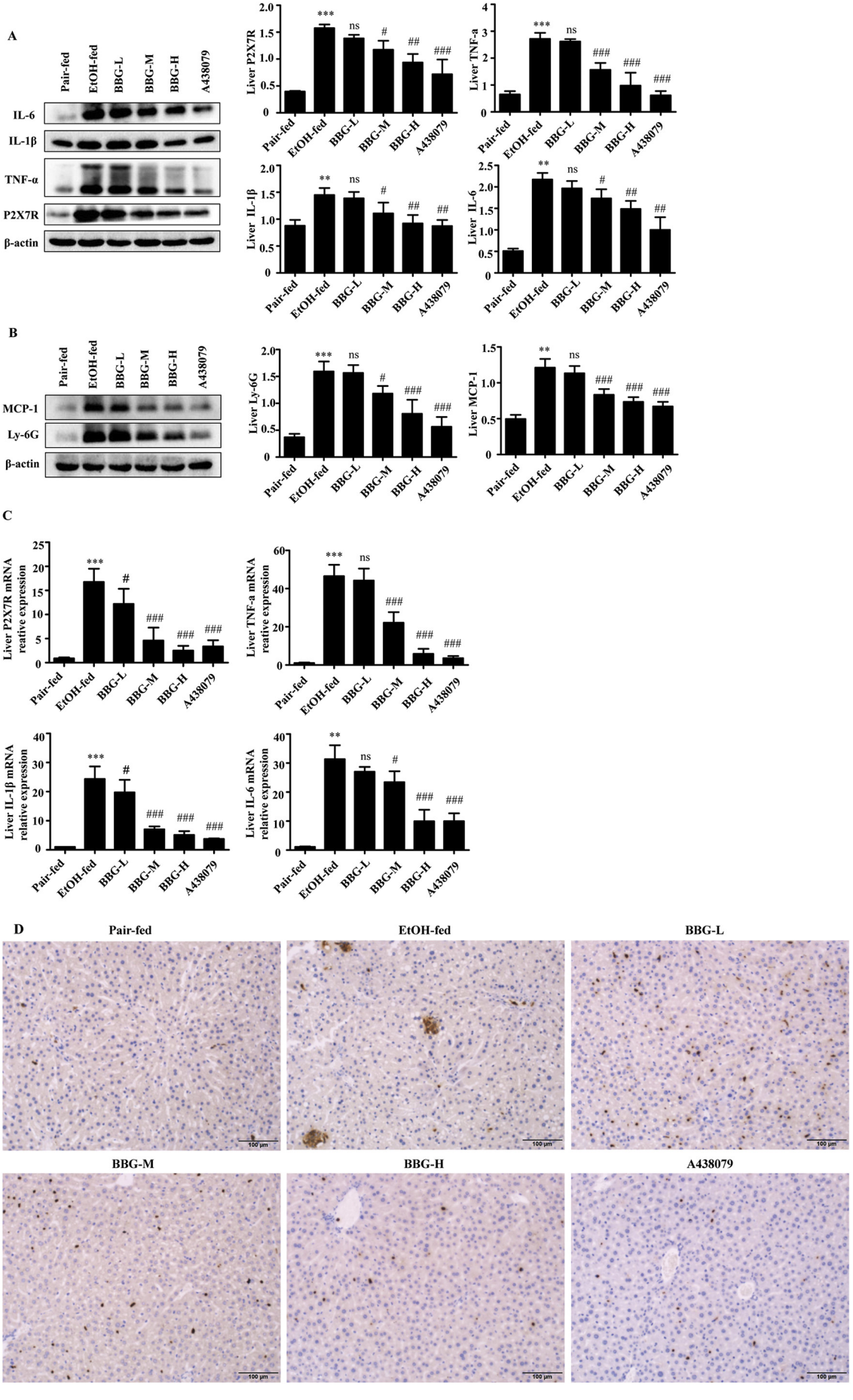

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Fig. 3. Effects of P2X7R blockade on chronic-binge alcohol feeding-induced liver inflammation. (A) The protein expression of P2X7R, TNF-α, IL-1β and IL-6 in the liver. (B) The protein expression of neutrophil marker Ly-6G and chemokine MCP-1 in the liver. (C) The mRNA expression of P2X7R, TNF-α, IL-1β and IL-6 in the liver. (D) The representative Ly-6G immunohistochemical staining of liver sections (original magnification: 200×, scale bar = 100 μm). **P < 0.01, ***P < 0.001 vs. pair-fed; #P < 0.05; ##P < 0.01, ###P < 0.001 vs. EtOH-fed; ns, no statistical significance vs. EtOH-fed.

3. Results

3.1. P2X7R was involved in alcohol-induced steatohepatitis and intestinal injury

Our present work showed that the mice from EtOH-fed group displayed fatty liver and liver damage, characterized by wide lipid droplet infiltration, hepatic cell cord derangement (Fig. 1A) and notable fat accumulation (Fig. 1B) as well as significant increases in serum ALT and TG values (Fig. 1D, E) compared to the pair-fed mice. The morphological changes in HE-stained sections of the proximal small intestine showed that the intestinal barrier was damaged during ALD induction (Fig. 1C). Moreover, the expression of P2X7R, TNF-α, IL-1β and IL-6 was significantly up-regulated in the liver of alcohol-fed mice compared to pair-fed controls (Fig. 1F). Meanwhile, there was a great increase in P2X7R expression while a dramatic decrease in the expression of two key tight junction (TJ) proteins, ZO-1 and claudin-1, in the intestinal tissues from EtOH-fed group (Fig. 1G). In view of these data, we corroborated the involvement of P2X7R in alcohol feeding-induced steatohepatitis and identified that P2X7R may play an important role in alcohol feeding-induced intestinal injury.

3.2. Blockade of P2X7R with BBG or A438079 mitigated alcohol- induced steatohepatitis and liver injury

Six mice from each group were randomly selected for the inspection of changes in body weight and liver index. As shown in Fig. 2A and B, chronic-binge alcohol consumption induced body weight loss and elevated liver index, while BBG in high dosage improved these changes. The effects of BBG were confirmed by another highly selective P2X7R antagonist A438079. Compared to pair-fed mice, alcohol exposure resulted in the significant augmentations of serum ALT, AST, T-CHO and TG levels. Treatment with BBG or A438079 reduced the serum levels of ALT (Fig. 2C), AST (Fig. 2D), T-CHO (Fig. 2E) and TG (Fig. 2F) in alcohol-fed mice. In agreement with the serum parameters of liver injury and steatosis, the liver histopathological assessment (Fig. 2G, H) showed that BBG dose-dependently ameliorated lipid droplet infiltration, hepatic cell cord derangement and fat accumulation in comparison with EtOH-fed group. A438079 treatment confirmed the protective effects of P2X7R antagonism on alcohol-induced hepatic steatosis and liver damage. The IHC of liver sections indicated that P2X7R expression was significantly inhibited by BBG in high dosage and A438079 (Fig. 2I). P2X7R antagonist also reduced the mRNA and protein levels of P2X7R, TNF-a, IL-1β and IL-6 in the liver of EtOH-fed mice (Fig. 3A, C). Furthermore, feeding mice with chronic-binge alcohol dramatically up-regulated the hepatic protein expression of neutrophil marker Ly-6G and chemokine MCP-1 compared to pair-fed group, indicating that alcohol feeding led to hepatic neutrophil infiltration (Fig. 3B), which was confirmed by Ly-6G immunohistochemical staining (Fig. 3D). The down-regulation of Ly-6G and MCP-1 levels was observed in P2X7R antagonist treatment groups, suggesting that P2X7R blockade diminished neutrophil infiltration in the liver (Fig. 3B, D). Accordingly, we concluded that pharmacological P2X7R blockade mitigated alcohol-induced steatohepatitis and liver injury.

3.3. Blockade of P2X7R with BBG or A438079 attenuated alcohol -induced intestinal injury

Compared to EtOH-fed group, the intestinal HE staining showed that BBG in high dosage and A438079 significantly reduced histological damage (Fig. 4A). There was a dramatic decrease in ZO-1 and claudin-1 expression whereas a significant rise in the levels of P2X7R, TNF-a, IL-1β and IL-6 in the intestinal tissues of EtOH-fed group compared to pair-fed mice. Importantly, the intervention of P2X7R by BBG or A438079 significantly reversed the negative effects of alcohol on the protein and mRNA expression of ZO-1 and claudin-1 (Fig. 4C, D) and ameliorated alcohol-induced intestinal P2X7R, TNF-a, IL-1β and IL-6 production (Fig. 4E). The expression of P2X7R was inhibited by BBG in a dose-dependent manner and was also markedly restrained by A438079, as shown by IHC (Fig. 4B), suggesting that pharmacological P2X7R blockade attenuated alcohol-induced intestinal barrier disruption and intestinal inflammation.

3.4. Chronic plus binge alcohol feeding induced taxonomic alterations in the gut microbiota

Bifidobacteria and lactobacilli in the gut are considered beneficial to maintaining gut micro-ecology balance. As illustrated in Fig. 4F, the relative levels of bifidobacteria and lactobacilli in EtOH-fed mice were significantly lower than those of pair-fed mice. To further evaluate and define the qualitative changes of microbiota in the cecal content, 16S rRNA sequencing was applied. It was found that there was an increase in the relative abundance of phylum Bacteroidetes while a decrease in the number of phylum Verrucomicrobia and Firmicutes in EtOH-fed group compared to pair-fed animals. Moreover, P2X7R antagonist administration reversed the taxonomic alterations of cecal Bacteroidetes and Verrucomicrobia in EtOH-fed mice (Fig. 5A, B). We also found that alcohol feeding significantly reduced the relative abundance of genus *Akkermansia*, which was up-regulated after P2X7R antagonism (Fig. 5C, D). According to the observed quantitative changes, alcohol feeding induced gut microbiota alterations leading to dysbiosis, and P2X7R blockade restored partial taxonomic alterations in the gut microbiota.

3.5. P2X7R blockade inhibited MEK1/2-ERK1/2 signaling and egr-1 expression

Further studies were conducted to ascertain the role of MEK1/2-ERK1/2 signaling and egr-1 in alcohol-induced steatohepatitis and intestinal injury. As a result, the western blot analysis showed that alcohol challenge promoted the phosphorylation of MEK1/2 and ERK1/2 and up-regulated egr-1 expression in the liver and intestinal tissues compared to pair-fed animals, which were markedly inhibited by BBG and A438079 administration (Fig. 6A, B). Accordingly, these findings indicated that the inhibition of MEK1/2-ERK1/2 signaling and egr-1 expression contributed to mitigate alcohol feeding-induced steatohepatitis and intestinal injury.

4. Discussion

Alcohol abuse is a well-recognized risk factor of liver disease and has caused lots of global public health issue. In China, with the development of economy and the improvement of people's living standards, the production and consumption of alcohol have increased year by year. As a result, the number of patients with ALD is on the rise [32]. Foreign surveys show that ALD patients are tending to be younger and the proportion of patients with alcoholic cirrhosis is high, which bring not only heavy burden to the family but also huge socio-economic loss [33,34]. Regrettably, there is a lack of effective therapies for ALD. Thereby, search for safe and efficient treatments of ALD has always been a hot topic in the academic community.

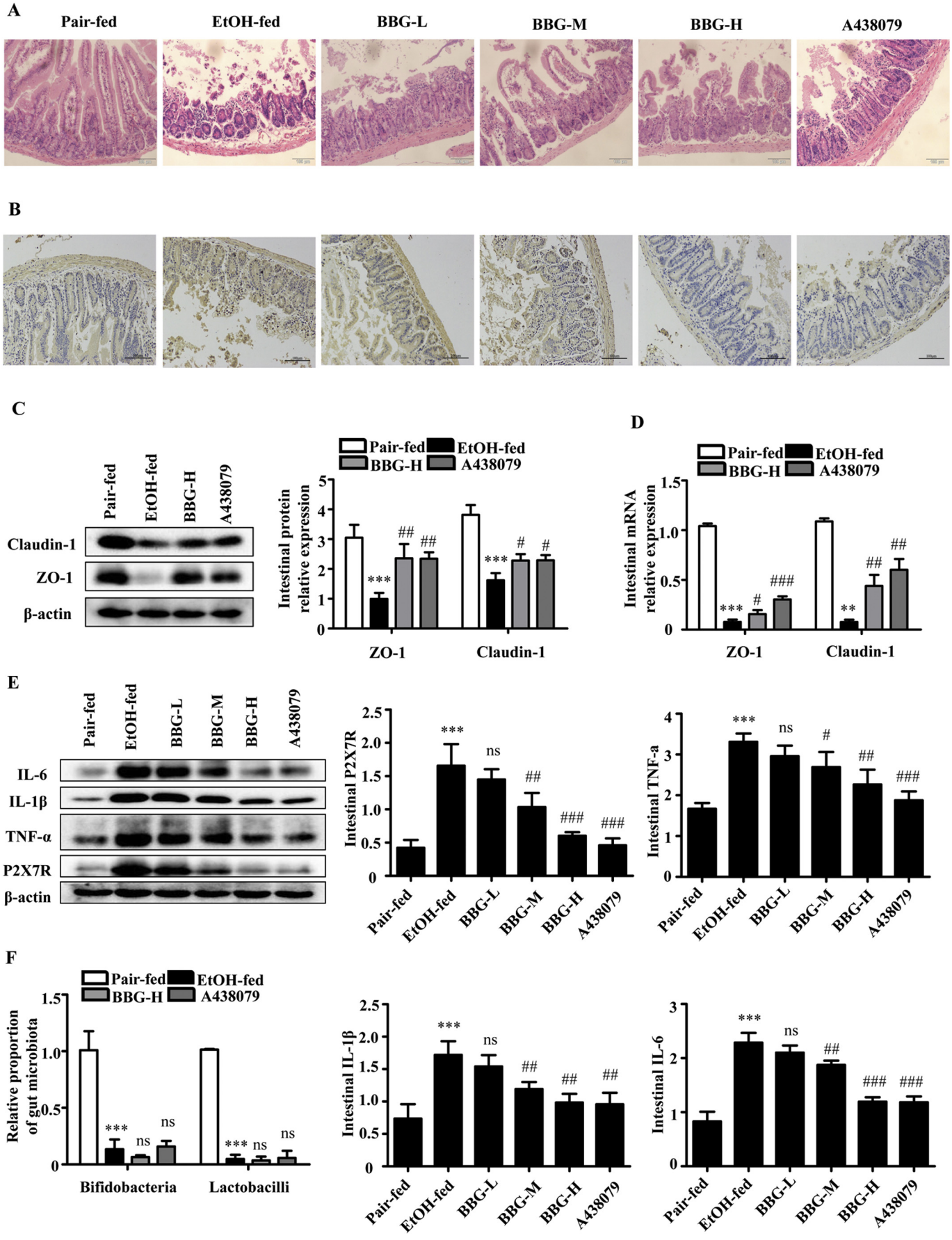

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Fig. 4. Effects of P2X7R blockade on chronic-binge alcohol feeding-induced intestinal barrier disruption, intestinal inflammation and alterations in the relative proportions of cecal bifidobacteria and lactobacilli. (A) The representative HE staining of small intestinal sections. (B) The representative P2X7R immunohistochemical staining of small intestinal sections. Original magnification: 200×, scale bar = 100 μm. (C) Western blotting and the relative quantitative analysis of ZO-1 and claudin-1 in the intestine. (D) The mRNA expression of ZO-1 and claudin-1 in the intestine. (E) The protein expression of P2X7R, TNF-α, IL-1β and IL-6 in the intestine. (F) The relative proportions of bifidobacteria and lactobacilli in the cecal content were measured by RT-qPCR. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pair-fed; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH-fed; ns, no statistical significance vs. EtOH-fed.

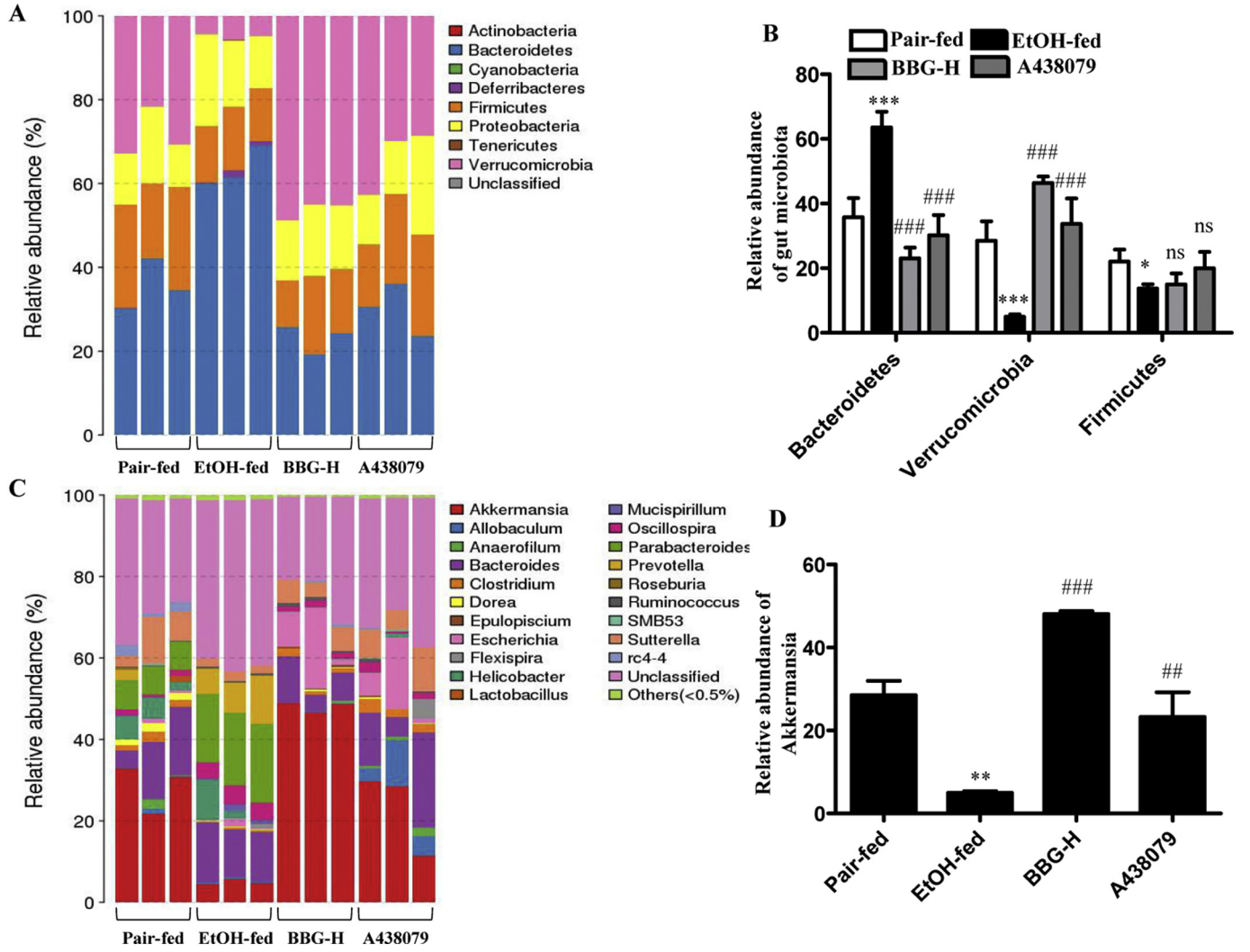

Fig. 5. Chronic-binge alcohol feeding induced taxonomic alterations in the gut microbiota. (A) The profiling barplot of gut microbiota in the taxonomic level of phylum. (B) The relative abundance analysis of Bacteroidetes, Verrucomicrobia and Firmicutes in the cecal content. (C) The profiling barplot of gut microbiota in the taxonomic level of genus. (D) The relative abundance analysis of *Akkermansia* in the cecal content. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pair-fed; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH-fed; ns, no statistical significance vs. EtOH-fed.

Previous researches revealed that the genetic or pharmacological inhibition of P2X7R significantly prevented acetaminophen-mediated hepatotoxicity [35] and dihydroquercetin improved P2X7R-dependent hepatosteatosis induced by intragastric ethanol consumption [7]. These findings led us to investigate whether P2X7R might play a role in chronic plus binge alcohol feeding-induced steatohepatitis. Although Li et al. found that P2X7R was involved in chronic-binge alcohol feeding-induced fatty liver [10], the effects of P2X7R blockade on alcoholic steatohepatitis were incompletely elucidated. Here, we corroborate that P2X7R is implicated in alcohol feeding-induced steatohepatitis and identify that P2X7R plays a pivotal role in alcohol feeding-induced intestinal injury, as evidenced by up-regulated P2X7R expression in the liver and intestinal tissues of mice with steatohepatitis and intestinal injury compared to pair-fed mice (Fig. 1).

It has been reported that chronic-binge alcohol feeding induced obvious micro- or macro-steatosis in the liver as well as elevations in the serum values of ALT, AST, T-CHO and TG, up-regulated the hepatic synthesis of TNF-α, IL-β and IL-6, and promoted hepatic neutrophil accumulation [25,36]. Our study showed that the pharmacological blockade of P2X7R protected the liver from these insults as demonstrated by decreased fat accumulation, reduced serum transaminase and serum lipid, and limitation of hepatic TNF-α, IL-1β and IL-6 synthesis relative to EtOH-fed group (Figs. 2, 3A and C), which is coincident with the beneficial influence of P2X7R deletion on chronic alcohol feeding-induced liver injury [5]. Furthermore, P2X7R blockade attenuated hepatic neutrophil infiltration based on the reduced expression of MCP-1 and Ly-6G in P2X7R antagonist-treated mice (Fig. 3B, D). This finding is consistent with previous experimental data supporting that P2X7R inhibition significantly decreased the acetaminophen-mediated hepatic neutrophil count [35]. The difference of our study from Bertola et al.

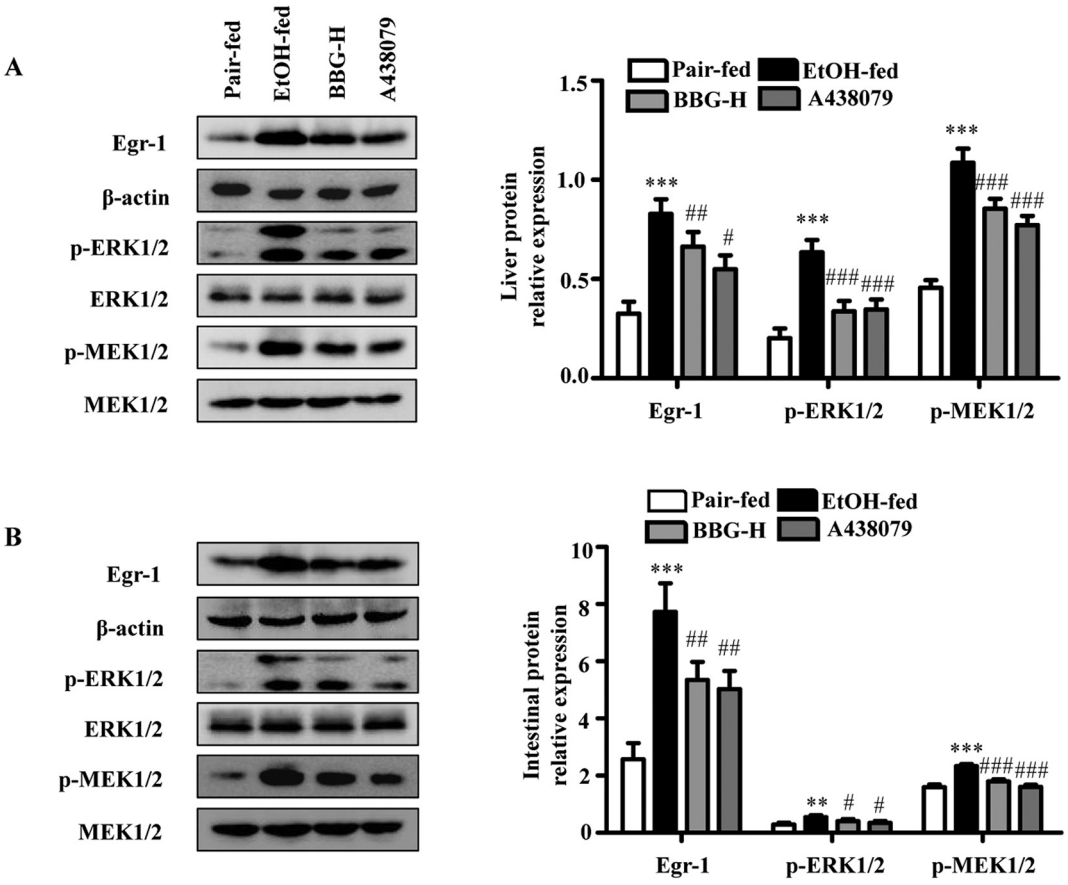

Fig. 6. P2X7R blockade inhibited MEK1/2-ERK1/2 signaling and egr-1 expression. (A) The protein expression of MEK1/2, p-MEK1/2, ERK1/2, p-ERK1/2 and egr-1 in the liver. (B) The protein expression of MEK1/2, p-MEK1/2, ERK1/2, p-ERK1/2 and egr-1 in the intestine. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pair-fed; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH-fed.

[25] is that we observed significant body weight loss and increased liver index in alcohol-fed group, which were effectively reversed by BBG and A438079 (Fig. 2A, B). It is concluded that P2X7R blockade significantly improves alcohol-induced steatohepatitis and liver injury via restraining histological damage, lipid accumulation and hepatic inflammatory reaction.

Coincident with the previous study [37], alcohol feeding caused the severe disruption of the small intestinal mucosal structure and decreased the levels of TJ proteins, claudin-1 and ZO-1, which, however, were alleviated by BBG and A438079 in our work as shown in Fig. 4A, C and D. The TJ proteins are integral factors in the intestinal barrier integrity through sealing the paracellular space between epithelial cells. Therefore, P2X7R antagonist restored the intestinal barrier dysfunction by modulating the expression of claudin-1 and ZO-1. Chessell et al. found that the deletion of P2X7R gene in mice diminished TNF-α, IL-1β and IL-6 production in chronic inflammation and neuropathic pain after mechanical stimuli [38]. Analogously, our results showed that P2X7R blockade reduced the intestinal expression of TNF-α, IL-1β and IL-6 in alcohol-fed mice (Fig. 4E). The available data suggest that P2X7R blockade mitigates alcohol-induced intestinal injury by reducing intestinal barrier disruption and intestinal inflammation.

It has been well recognized that gut microbiota and bacterial products are involved in the pathogenesis of liver disease [39]. The mice with non-alcoholic steatohepatitis exhibited much lower levels of lactobacilli and bifidobacteria in the cecal content compared to the control mice [31]. The RT-qPCR analysis of our study revealed that chronic-binge alcohol feeding dramatically reduced the number of cecal lactobacilli and bifidobacteria relative to pair-fed mice, and P2X7R blockade had no effects on such alterations (Fig. 4F). The coincidence with the cecal microbiota alterations caused by intragastric ethanol feeding [16] is that chronic-binge alcohol feeding up-regulated the relative abundance of phylum Bacteroidetes while down-regulated the number of phylum Firmicutes (Fig. 5A, B). Furthermore, we found that chronic-binge alcohol feeding decreased the number of phylum Verrucomicrobia and genus *Akkermansia* (Fig. 5), supporting that the loss of *Akkermansia* was a hallmark of alcohol-induced gut microbiota dysbiosis identified by Lowe et al. [15]. Interestingly, our team, for the first time, found that P2X7R antagonist reversed the taxonomic alterations of Bacteroidetes and Verrucomicrobia as well as *Akkermansia* in EtOH-fed mice. However, the underlying mechanism of such restoration phenomenon is unknown, deserving further exploration in the subsequent research. Taken together, chronic-binge alcohol feeding results in various taxonomic alterations in the cecum leading to gut microbiota dysbiosis. Considering the importance of gut-liver axis in ALD, P2X7R blockade might protect the liver from gut microbiota dysbiosis-induced second hit by improving intestinal barrier function and restoring partial taxonomic alterations in the gut microbiota.

The egr-1 is necessary for the development of chronic ethanol-induced hepatic steatosis [40]. However, one report addressed that egr-1 null mice developed more serious liver damage than wild-type mice after acute ethanol gavage, suggesting that egr-1 was a protective factor for alcohol-induced fatty liver [41]. Friedle et al. found that P2X7R agonist BzATP exerted neuroprotective effects via MAPK-egr pathway [42]. We performed further studies to ascertain the actual role of MEK1/2-ERK1/2 signaling and egr-1 in chronic plus binge alcohol feeding-induced steatohepatitis and intestinal injury. In contrast to pair-fed mice, alcohol feeding up-regulated egr-1 protein expression and promoted the phosphorylation of MEK1/2 and ERK1/2 in the liver

and intestinal tissues, which were significantly down-regulated by BBG and A438079 (Fig. 6). Therefore, our data support that P2X7R blockade mitigates alcohol-induced steatohepatitis and intestinal injury by inhibiting MEK1/2-ERK1/2 signaling and egr-1 expression, which will be further investigated in vitro.

5. Conclusion

Our present data provide evidence that P2X7R plays a pathogenic role in chronic plus binge alcohol feeding-induced steatohepatitis and intestinal injury. More importantly, pharmacological P2X7R blockade mitigates alcoholic steatohepatitis and alcohol-induced intestinal injury possibly by regulating MEK1/2-ERK1/2 signaling and egr-1 activity, shedding light on the development of treatments against ALD and alcohol-induced intestinal disease.

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Declarations of interest

Authors have no conflict of interest to declare.

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