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Macrophages-derived p38α promotes the experimental severe acute pancreatitis by regulating inflammation and autophagy

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ABSTRACT

Background: Severe acute pancreatitis (SAP) is a common threat to human health. In the present study, we aimed to investigate the underlying mechanisms by which p38α in macrophages contributes to SAP. We used conditional knockout of p38α in macrophages and p38 MAPK inhibitors to understand the effects of p38α in macrophages on caerulein-induced inflammatory responses in SAP mice models.

Methods and materials: Wild-type (WT) mice were randomly divided into three groups: a control group, SAP group, and SAP + p38MAPK inhibitor (SB203580) group, and mice with a conditional knockout (KO) of p38α in macrophages were included in a KO + SAP group. We evaluated pancreatic pathology and ultra-structure by hematoxylin and eosin staining and transmission electron microscopy. The pulmonary wet-to-dry weight ratio was calculated. The serum levels of TNF-α and IL-1β were determined by ELISA. The mRNA and protein expression of inflammatory cytokines TNF-α, IL-1β, IL-17, IL-18, MIF, and MCP-1 in pancreatic tissues were tested by qRT-PCR and immunohistochemistry analysis. The protein expression of p38, caspase-1, ULK1, LC3B and p62 in pancreatic tissues was examined by Western blotting.

Results: The results indicated that the severity of SAP as well as the expression of the cytokines TNF-α, IL-1β, IL-17, IL-18 and MCP-1 were higher in the SAP group than those in the control group, but were lower in the SAP + SB203580 and KO + SAP groups as compared with the SAP group. The protein expression of p38, caspase-1, LC3B and p62 was increased in the SAP group than that in the control group, but this result was reversed in the SAP + SB203580 and KO + SAP groups as compared with the SAP group. In addition, the ULK1 level was significantly lower in the SAP group than that in the control group, but was increased in the SAP + SB203580 and KO + SAP groups as compared with the SAP group.

Conclusions: Our findings demonstrated that, macrophage derived p38α promoted the experimental severe acute pancreatitis by regulating inflammation and autophagy.

1. Introduction

Acute pancreatitis (AP) is a poorly understood inflammatory disease with an increased incidence worldwide each year [1]. Severe acute pancreatitis (SAP), a severe form of AP, is always accompanied by impaired function or multiple system and multiple organ failure [2]. Currently, the clinical prognosis of SAP is poor and the treatment options for SAP are limited. Understanding the mechanisms underlying the pathogenesis of SAP is essential for the development of effective therapies [3,4].

Monocytes/macrophages play a central role in the pathogenesis of AP, and the activation of macrophages is associated with the severity of AP. Macrophages induce the activation of acinar cells and the systemic generation of inflammatory mediators during AP [5]. The release of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, is associated with activated macrophages [6]. There is increasing evidence that proinflammatory cytokines and oxidative stress are involved in the occurrence of systemic complications of SAP [7].

The activation of inflammation-related NF-κB and mitogen-activated protein kinases (MAPKs) pathways gives rise to the production of proinflammatory cytokines [8]. p38 MAPK can be triggered by inflammatory cytokines [9] and participate in inflammatory responses

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[10]. p38α regulates cell cycle, cell differentiation, cell death, tumorigenesis and immune responses [10–12]. Previous studies showed that LPS in macrophages induces the activation of p38α and in turn activates the downstream cascade of proinflammatory cytokines TNFα, IL-1 and IL-6 [10,13–16]. In the present study, we used specific deletion of p38α in macrophages or its inhibitor SB203580 to conform the essential role of p38α in SAP.

Autophagy acts an anti-inflammatory role by regulating innate immune and inflammasome [17]. Basal autophagy maintains pancreatic acinar cell homeostasis [18]. However, impaired autophagy mediates acinar cell vacuole formation and trypsinogen activation in AP models and aggravates the progress of pancreatitis [19]. Excessive cell vacuolization and the accumulation of the lysosomal markers microtubule-associated protein 1 light chain 3 (LC3B) and p62/sequestosome1 (SQSTM1) are indicators of impaired autophagy [20]. A recent study showed that p38α relieves autophagic control in response to inflammatory signals by inhibiting UNC51-like kinase-1 (ULK1) kinase activity during microglial inflammation [21]. However, whether p38α in macrophages affects autophagy and inflammatory activity in SAP is still unclear.

2. Materials and methods

2.1. Chemicals and reagents

Caerulein and LPS were obtained from Yeasen (Shanghai, China). SB203580 was obtained from MCE (Shanghai, China). Anti-p38 and anti-caspase-1 antibodies were obtained from Beyotime (Shanghai, China); anti-ULK1, anti-LC3B and anti-p62 antibodies were obtained from ABclonal (Shanghai, China); and anti-TNF-α, anti-IL-1β, anti-Monocyte chemotactic protein-1 (MCP-1) and anti-macrophage migration inhibitory factor (MIF) antibodies were obtained from Absin (Shanghai, China). TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience (San Diego, CA, USA).

2.2. Animals

Fifteen healthy male C57BL/6 mice weighing 20–22 g were provided by the animal center of Shanghai Sixth People's Hospital. Mice with a macrophage-specific deletion of p38α were provided by Xiamen University. The p38α floxed allele was generated by homologous recombination in embryonic stem cells (lexicon), and the first exon (containing ATG) was flanked by two loxP sites as previously described [10,22]. Animals were housed in cages under a controlled temperature of 22 ± 1 °C and 12 h light-dark cycles and were provided by standard laboratory chow and water ad libitum for acclimatization for one week.

2.3. Experimental SAP mice models

Wild-type (WT) mice were randomly divided into three groups: control group (n = 11), SAP group (n = 15), SB203580 + SAP group (SB, n = 11), and mice with a conditional knockout (KO) of p38α in macrophages were included in a KO + SAP group (KO, n = 12). Then, the mice received 7 hourly intraperitoneal (IP) injections of 50 μg/kg caerulein (Sigma) in sterile saline followed by an IP injection of LPS (10 mg/kg) immediately, while the control mice were given saline. The SB203580 + SAP group was given by IP injection of SB203580 (10 mg/kg) for 30 min before the injection of caerulein. Animals were sacrificed 24 h after the injection of LPS, and the blood and tissue samples were collected. The blood samples were stored at −20 °C until use. Pancreas and lung tissue samples were frozen and stored at −80 °C. The other portions were fixed in 4% paraformaldehyde for histopathological examination. The experiment was approved by the Ethical and Research Committee of Shanghai Sixth People's Hospital.

Table 1
Histologic Scoring for Acute Hemorrhagic Necrotizing Pancreatitis.

Conditon	Score	Description
Edema	0	Absent
	1	Focally increased between lobules
	2	Diffusely increased between lobules
	3	Tense acini, widely separated lobules
	4	Gross lobuar separation
Inflammation	0	Absent
	1	Around ductal margins
	2	In parenchyma (< 50% of lobules)
	3	In parenchyma (51–75% of lobules)
	4	Massive collection, abscesses
Hemorhage	0	Absent
	1	Blood in parenchyma (< 25%)
	2	Blood in parenchyma (25–50%)
	3	Blood in parenchyma (50–75%)
	4	Blood in 100% of lobules
Necrosis	0	Absent
	1	Periductal parenchymal destruction
	2	Focal parenchymal necrosis (< 20%)
	3	Diffuse loss of lobules (20–50%)
	4	Severe loss of lobules (> 50%)

2.4. Biochemical analysis

Blood was collected from mice via intracardiac puncture, and the serum was isolated for detection of subsequent amylase and lactate dehydrogenase (LDH) levels in a diagnostic laboratory. Pancreas and lung tissue samples were collected for the detection of myeloperoxidase (MPO) using commercial kits (Biovision, San Francisco, USA) following the manufacturer's guidelines.

2.5. Inflammatory cytokine assay

The concentrations of serum inflammatory cytokine TNF-α and IL-1β were assayed by ELISA kits (eBioscience, San Diego, USA). The measurements were performed according to the manufacturer's instructions.

2.6. Calculation of pulmonary wet-to-dry weight (W/D) ratios

The blood and water on the surface of the lung were absorbed with filter paper. The wet weight (W) was immediately measured, and then the tissue was placed in an oven for 72 h at 70 °C to obtain a constant weight [the dry weight (D)]. The W/D ratio of the lung tissue was measured to assess pulmonary edema.

2.7. qRT-PCR analysis

Total RNA was extracted from pancreatic tissues using TRIzol reagent (Invitrogen Life Technologies, CA, USA), and cDNAs were generated using a first-strand cDNA synthesis kit (Takara, Tokyo, Japan). qRT-PCR was performed with the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Shanghai, China) using a SYBR Green PCR kit (Applied Biosystems, CA, USA). The amplification reaction conditions were as follows: 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 60 sec. This procedure was repeated for 30 cycles. Relative mRNA expression was calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The primer sequences were listed as follows: TNF-α forward, 5′-CCAAAGGGATGAGAAGTTCC-3′ and reverse, 5′-CTCCACTT GGTGGTTTGCTA-3′; IL-1β forward, 5′-TTCAGGCAGGC AGTATCA-3′ and reverse, 5′-GTC ACAACCAGCAGGTTA-3′; IL-18 forward, 5′-ACAACTTTGGCCGAC TTCAC-3′ and reverse, 5′-GGGTTCAG GCACTTTGAT-3′; IL-17 forward, 5′-GCTCCAGA AGGCCCTCAGA-3′ and reverse, 5′-AGCTTTCCCTCCGCCATTGA-3′; MIF forward, 5′-CCCAGAA

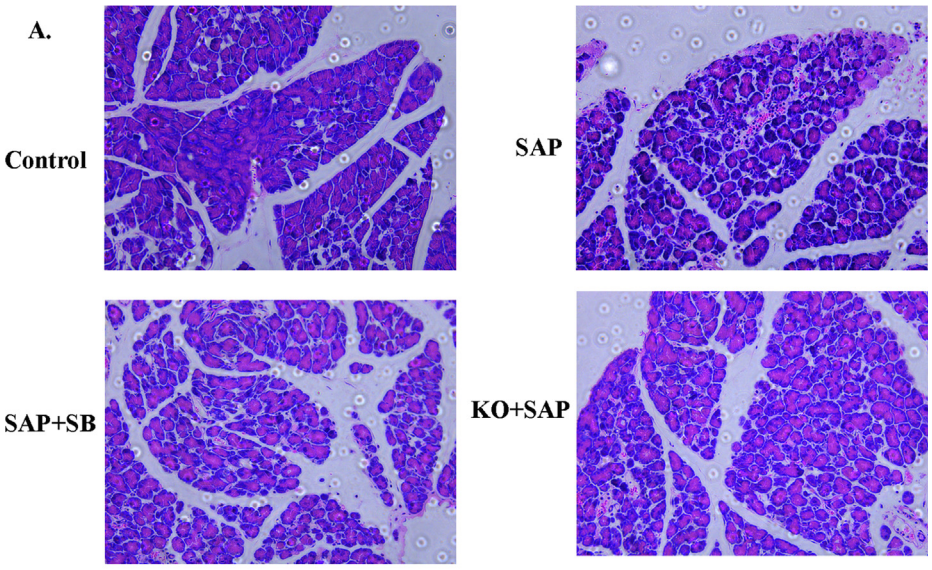

Fig. 1. p38α KO in macrophages and SB203580 improve pancreatic histopathological injury. (A) Histological changes and pathological scores of pancreatic tissue samples from mice in the NC, SAP, SAP + SB, and SAP + KO groups at the 24-h time point (200×). (B) Pathological scores at the 24-h time point. (C). The ratio of the wet weight to the dry weight of the same lung (W/D). *$P < 0.05$ vs. the control group. #$P < 0.05$ vs. the SAP group.

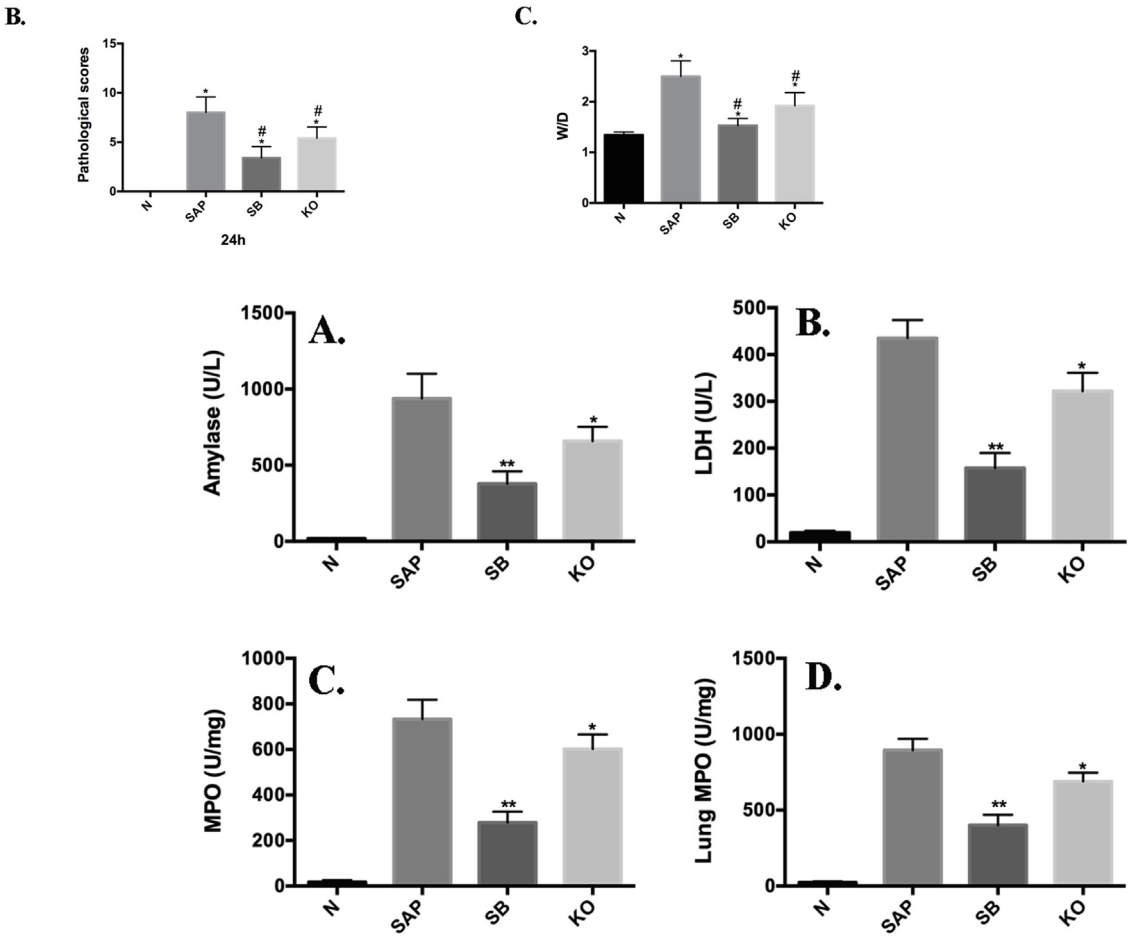

Fig. 2. p38α KO in macrophages and SB203580 ameliorate experimental SAP. (A) Plasma amylase activity, (B) pancreatic MPO activity, (C) lung MPO activity, and (D) plasma lactate dehydrogenase (LDH) activity at 24 h following administration of caerulein. *$P < 0.05$ vs. the control group. **$P < 0.01$ vs. the control group.

CCGCAACT ACA-3′ and reverse, 5′-GAGCGAGGCTCAAAGAAC-3′; MCP-1 forward, 5′-CCCTATTCCTG ATGGCACT-3′ and reverse, 5′-CTATGA GAAACCCAC CACATCT-3′; and GAPDH forward, 5′-TGTGTCCGTCGT GGATCTGA-3′ and reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′.

2.8. Western blotting analysis

Tissues from each mouse were homogenized in an ice-cold lysis buffer containing a cocktail of protease inhibitors. The supernatant fluid of the lysate was collected by centrifugation (4 °C, 12,000 rpm, 10 min). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). The membrane was then rinsed with a blocking solution of 5% nonfat milk for 60 min and incubated overnight at 4 °C with primary antibodies. Dilutions for

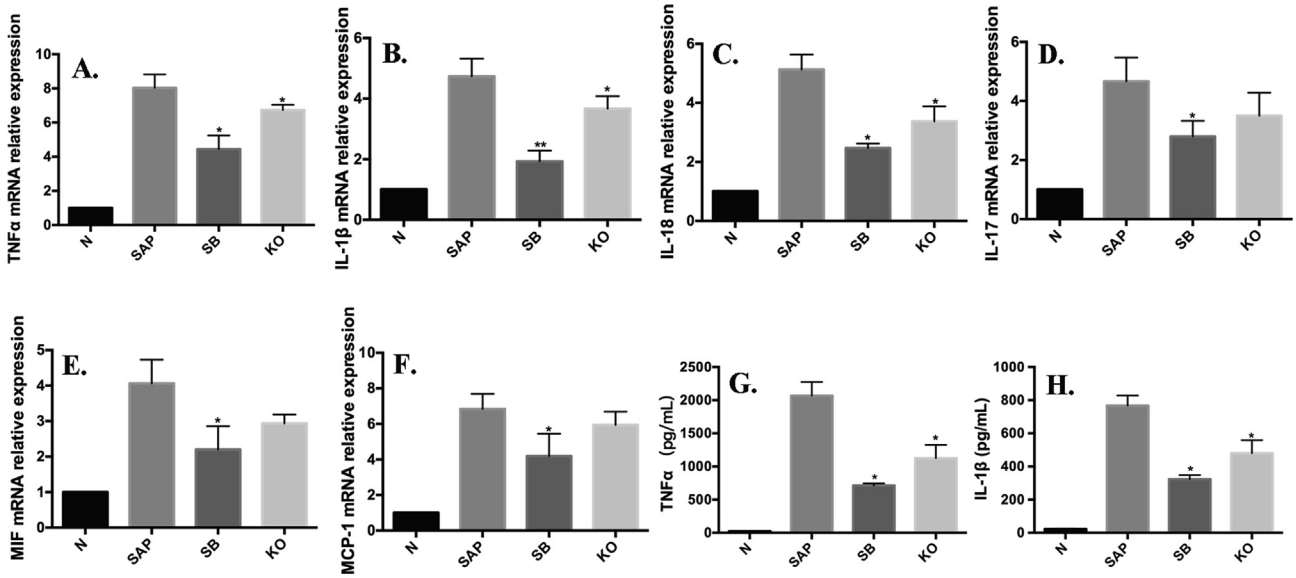

Fig. 3. p38α KO in macrophages or SB203580 suppresses the production of proinflammatory cytokines in experimental SAP. (A-F) The mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, IL-18, IL-17, MCP-1 and MIF) in pancreatic tissue was tested by qRT-PCR assay. (G-H) The serum levels of proinflammatory cytokines were tested by ELISA. *$P < 0.05$ vs. the SAP group. **$P < 0.01$ vs. the SAP group.

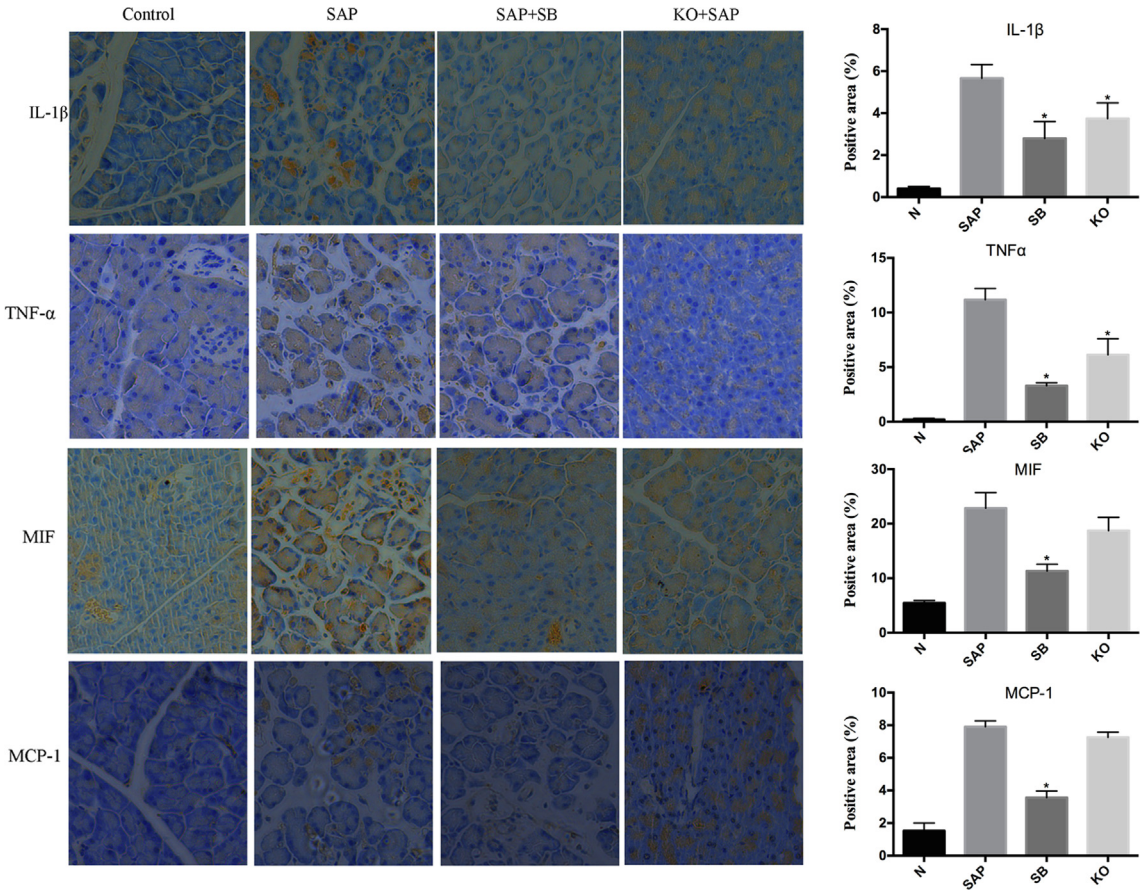

Fig. 4. p38α KO in macrophages or SB203580 suppresses the production of proinflammatory cytokines in experimental SAP. The protein expression levels of proinflammatory cytokines (TNF-α, IL-1β, IL-18, IL-17, MCP-1 and MIF) in pancreatic tissue were tested by IHC. *$P < 0.05$ vs. the SAP group. **$P < 0.01$ vs. the SAP group.

primary antibody were as follows: anti-p38 MAPK (1:1000), anti-caspase-1 (1:1000), anti-LC3B (1:500), anti-ULKl (1:1000) and p62 (1:1000), and was followed by an incubation with secondary antibodies at room temperature for 1 h. The proteins were visualized using an enhanced chemiluminescence (ECL) system (Thermo Scientific). Protein expression levels were normalized to the level of GAPDH.

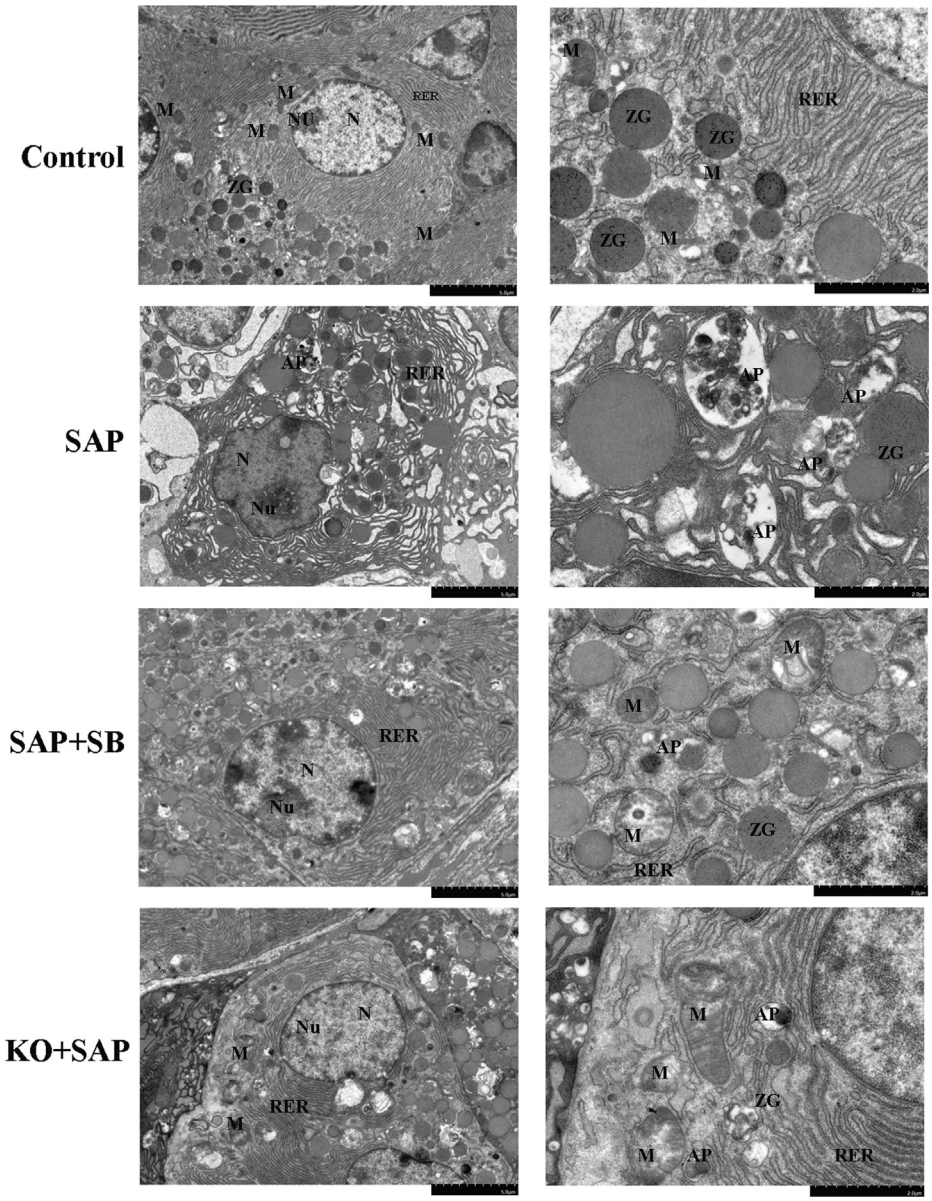

Fig. 5. p38α KO in macrophages or SB203580 alters the morphological structure of pancreatic cells in SAP. Electron micrograph of pancreatic acinar cell in control group showed normal mitochondria [M], nucleus [N], nucleolus [Nu], zymogen granules [ZG], rough endoplasmic reticulum [RER]. The pancreatic acinar cell in SAP group showed severe edema, the nucleus [N] was pyknotic, the number of mitochondria [M] was small, and most of mitochondrial cristae were disappeared, the RER was severely expanded, the size of ZG was varied and impaired autophagy was increased, including abnormal aggregation of autophageosomes. The pancreatic acinar cell in SB and KO groups showed relatively mild acinar cell damage, normal nuclear structure, obvious swelling of mitochondria, a small amount of RER expansion, uniform size of ZG, and a small amount of damaged autophagy. Scale bar = 5 μm and Scale bar = 2 μm.

2.9. Histopathological examination

Pancreatic tissue from each mouse was incised and fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Two experienced pathologists scored the pancreatic tissue samples on a scale from 0 to 4 for the degrees of edema, inflammation, hemorrhage, and necrosis. We applied the scoring system as shown in Table 1, and the final scores of each histological examination were added together [23].

2.10. Immunohistochemistry (IHC) analysis

IHC analysis was performed to examine protein expression levels of TNF-α, IL-1β, MIF and MCP-1 in pancreatic tissue samples according to our previous report [24].

2.11. Transmission electron microscopy (TEM)

TEM was performed according to the previous report [25].

2.12. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The significance of differences in histopathological scores was assessed by the Kruskal-Wallis test. Other continuous data were analyzed by Student's *t* test and factorial design ANOVA. All statistical analyses were carried out using GraphPad Prism 7 (La Jolla, CA, USA) with statistical significance set at $P < 0.05$.

3. Results

3.1. p38α KO in macrophages or SB203580 improves pancreatic and pulmonary injury in experimental SAP

We used caerulein plus LPS to establish a mouse SAP model and found that the mortality in the SAP group at 24 h was 33.3% (5/15). All mice in the control, SB203580 group and KO group survived at 24 h. Pancreatic injury, as determined by the histological scores, was significantly higher in SAP, SB and KO groups than that in the control group. However, the histological scores in the SB and KO groups were lower than those in the SAP group (Fig. 1A, B). Pulmonary injury, as

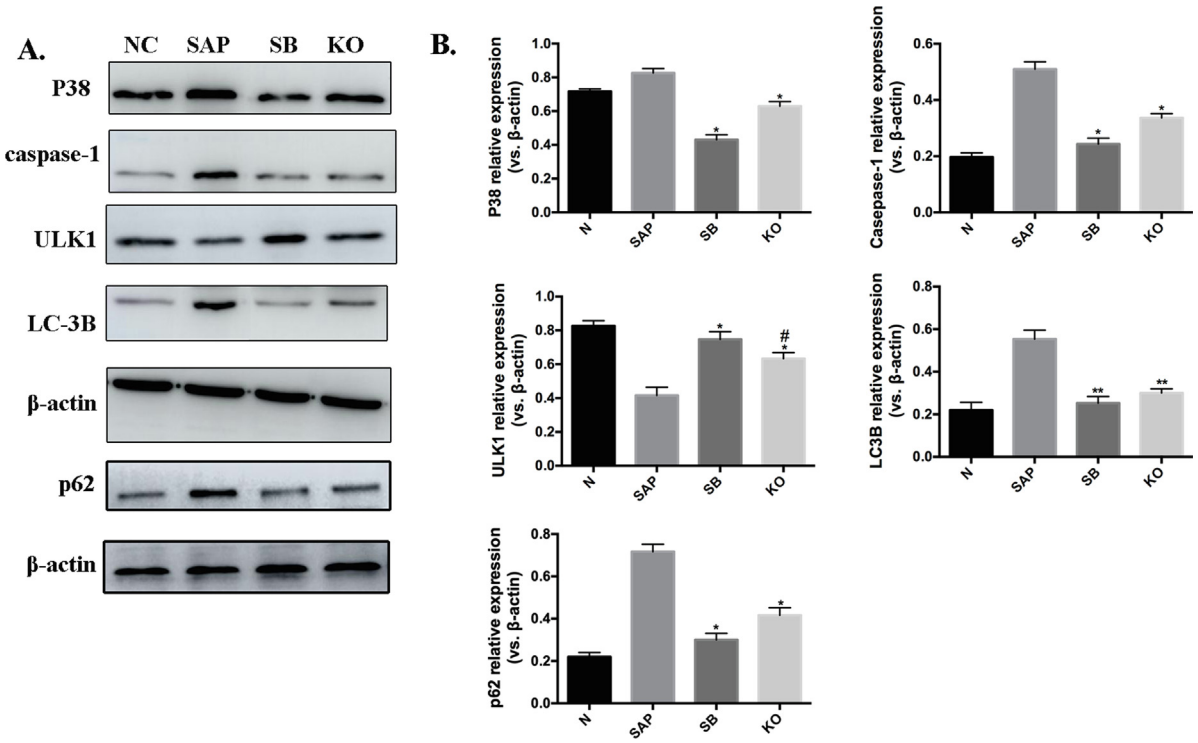

Fig. 6. p38α KO in macrophages or SB203580 inhibits inflammation and regulates autophagy in experimental SAP. (A. B) Pancreatic p38, caspase-1, ULK1, LC3B and p62 protein expression was determined by Western blotting. *$P < 0.05$ vs. the SAP group. **$P < 0.01$ vs. the SAP group. #$P < 0.05$ vs. the SB group.

determined by the W/D ratio, was also higher in SAP, SB and KO groups than that in the control group. However, the W/D ratios in the SB and KO groups were significantly lower than that in the SAP group (Fig. 1C).

3.2. p38α KO in macrophages or SB203580 ameliorates experimental SAP

We then found that p38α KO in macrophages or SB203580 dramatically suppressed the elevation of serum amylase and LDH levels as compared with the SAP group (Fig. 2A, B). In addition, p38α KO in macrophages or SB203580 protected against pancreatic injury and pancreatitis-associated lung injury, as shown by decreased pancreatic and lung MPO levels as compared with the SAP group (Fig. 2C, D).

3.3. p38α KO in macrophages or SB203580 suppresses the production of proinflammatory cytokines in experimental SAP

We found that p38α KO in macrophages or SB203580 reduced the mRNA expression of proinflammatory cytokines TNF-α, IL-1β and IL-18 in the pancreas as compared with the SAP group (Fig. 3A–C). In addition, SB203580 decreased the levels of the proinflammatory cytokines IL-17, MIF and MCP-1 as compared with the SAP group (Fig. 3D–F). We also found that p38α KO in macrophages or SB203580 lowered the serum levels of the proinflammatory cytokines TNF-α and IL-1β as compared with the SAP group (Fig. 3G, H).

In addition, we assessed the protein expression of TNF-α, IL-1β, MIF and MCP-1 by IHC and found that p38α KO in macrophages or SB203580 also decreased the protein levels of TNF-α and IL-1β, but only SB203580 inhibited the protein expression of MIF and MCP-1 as compared with the SAP group in pancreatic tissues (Fig. 4).

3.4. p38α KO in macrophages or SB203580 inhibits inflammation and facilitates autophagy in experimental SAP

As demonstrated in Fig. 5, ultra-structurally, as compared with the control group, SAP group showed severe pancreatic acinar cell damage, for example, the nucleus was pyknotic, most of mitochondrial cristae were disappeared, rough endoplasmic reticulum (RER) was severely expanded, the size of zymogen granules (ZG) was varied and impaired autophagy was increased, such as abnormal aggregation of autophageosomes. However, SB and KO groups showed relatively mild acinar cell damage, normal nuclear structure, obvious swelling of mitochondria, slight RER expansion, uniform size of ZG, and a small amount of damaged autophagy.

To further evaluate the potential mechanisms of SAP, the protein expression levels of p38, caspase-1, ULK1, LC3B and p62 were detected by Western blotting analysis. We found that p38, caspase-1, LC3B and p62 levels were increased, but the ULK1 level was decreased in the SAP group than those in the control group (Fig. 6A, B). Intriguingly, p38α KO or SB203580 reduced the activity of caspase-1, LC3B and p62, but increased ULK1 expression as compared with the SAP group (Fig. 6A, B).

4. Discussion

Patients with SAP suffer from severe pancreatic hemorrhage, necrosis, and systemic inflammatory response syndrome, and these features are accompanied by lung injury, acute renal injury and intestinal mucosal injury. However, little is known about the pathogenesis of SAP and its effective treatment [20,26]. In the present study, we found that SB203580 and p38α KO in macrophages reduced pancreatic and lung injury (Figs. 1 and 2) and the release of proinflammatory cytokines TNF-α, IL-1β and IL-18 (Figs. 3 and 4). Caspase-1 is an effector protein involved in inflammatory responses. Inhibition of caspase-1 and its subsequent proinflammatory cytokines IL-1β and IL-18 alleviates lung injury, acute renal injury and intestinal mucosal injury in SAP [27–29]. In our study, SB203580 and p38α KO in macrophages downregulated caspase-1 expression, and in turn inhibited the production of inflammatory cytokines TNF-α, IL-1β and IL-18.

Autophagy and inflammation are highly intertwined cellular processes [30]. Autophagy is a key protective mechanism that enables cell survival and adaptation to fluctuations in environmental conditions

[31]. Impaired autophagy is recognized as a major pathogenic event in pancreatitis [32,33], increases the accumulation of reactive oxygen species (ROS) and results in defective clearance of damaged mitochondria and activate oxidative stress in AP [31–34]. A recent study suggested that autophagy is activated in experimental AP, but its completion (lysosomal degradation) is inhibited [35]. However, the understanding of the role of autophagy in pancreatitis appears to be incomplete [36]. ULK1 is known as autophagy-related gene 1 (ATG1) in yeast. Autophagy initiation is controlled by the ULK1/ATG1-mediated complex [37]. p38 MAPK can phosphorylate ULK1 and destroy ULK1/ATG13 complex, causing disrupted autophagy in response to proinflammatory signals [21]. p38 MAPK inhibitor SB203580 can alleviate pancreatitis [38], but the effect of p38α in macrophages on SAP has not been reported. In the present study, we found that autophagy was impaired in SAP, but B203580 and p38α KO in macrophages reduced damaged autophagy by upregulating ULK1 and downregulating caspase-1, LC3B and p62 in SAP.

In addition, mitochondrial dysfunction and consequent energy deficit may be causally related to pancreatic cell injury. We found that SB203580 and p38α KO ameliorated the morphological injury of pancreatic cells and facilitated the autophagy in SAP. Thus, p38α-ULK1 axis might be involved in regulating the inflammatory responses in SAP, but the precise regulatory mechanisms between them in SAP need be further explored.

In conclusion, our findings demonstrated that, macrophage derived p38α promoted the experimental severe acute pancreatitis by regulating inflammation responses and autophagy.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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