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Research article

Sodium butyrate restricts neutrophils migration and NETs formation through reducing macrophage-derived CXCL16 in calculous cholecystitis

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ABSTRACT

Background: Neutrophil extracellular traps (NETs) havebeen demonstrated to initiate gallstone formation. Cholecystitis is a common complication of gallstones. As short-chain fatty acids (SCFAs), Butyrate acid has anti-inflammatory effects and alleviates cholesterol gallstones. However, the role of Butyrate acid in NETs of calculous cholecystitis and the molecular mechanism remains unclear. The effect of Sodium butyrate on neutrophil migration and NETs formation involved in macrophages polarization and exosomalCXCL16 in calculous cholecystitis was explored in our study.

Methods: The number of neutrophils and NETs, macrophages polarization and exosomal CXCL16 level were analyzed in clinic samples from patients. Exosomes were obtained and verified by gradient centrifugation, transmission electron microscopy, NanoSight analysis and Western blotting. Transwell, immunofluorescence and ELISA were used to detect neutrophil migration and NETs formation.

Results: Our results demonstrated that a large number of neutrophils and NETs, as well as M1 macrophages and exosomal CXCL16, were found in the blood of gallstones patients, especially patients with acute calculous cholecystitis. Exosomal CXCL16 was upregulated in plasma of calculous cholecystitis patients or Lipopolysaccharide induced macrophages, and promoted neutrophil cell migration and NETs formation. Sodium butyrate reduced exosomal CXCL16 secretion through the inhibition of M1 macrophage polarization to suppress neutrophils migration and NETs formation.

Conclusion: Our study suggested that Sodium butyrate may inhibit neutrophils migration and NETs formation to alleviate calculous cholecystitis by reducing exosomal CXCL16 secretion from macrophage and macrophage polarization.

General significance: Our finding may provide a link between exosomes and neutrophils to serve as a potential therapeutic intervention in calculous cholecystitis.

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1. Introduction

Gallstone is one of the most common diseases of the digestive tract with an increasing incidence rate in recent years [1,2] and a highly prevalent and severe disease as one of the leading causes of hospital admissions worldwide [3]. As reported in a previous study, the total prevalence of gallstones is up to 20 % worldwide [4], as well as the prevalence of gallstones in China is 3 %–11 % [5]. The long-term existence of the gall bladder calculi not only lead to the increased incidence of chronic or acute cholecystitis [6], but also cause obstruction of the cystic duct, which gradually increased the incidence of acute cholecystitis. The morbidity and mortality of acute cholecystitis were more than chronic cholecystitis. Therefore, decreasing the incidence of gallstones can effectively reduce the occurrence of acute cholecystitis to improve patient survival.

Neutrophils, part of the first line of defense against invaders, are rapidly recruited to sites of inflammation, and eradicate pathogens through extensive release of neutrophil extracellular traps (NETs) [7]. NETs are web-like structures that are composed of de-condensed chromatin and anti-microbial granule proteins, including neutrophil elastase (NE) and myeloperoxidase (MPO) [8]. NETs formation has been reported to activate the innate immune system to contribute to the development and growth of gallstones [3]. Neutrophils contact with calcium or cholesterol crystals to externalize chromatin decorated with granular proteins and formed aggregated NETs (aggNETs) [9,10] to precipitate small nidi promptly to initiate gallstone formation and development [3]. Inhibition of NETs formation or stunning of neutrophils effectively inhibited gallstone formation [3].

Gallstones are associated with acute or chronic cholecystitis. Increased macrophages were found in gallbladder tissue of patients with cholecystitis [11,12]. Macrophages can release tumor necrosis factor (TNFα) and interleukin 1β (IL-1β) when induced by inflammation. In inflammatory conditions, tissue macrophages modulate neutrophil trafficking and activation [13]. CXCL16 is a kind of chemokines, which tended to be increased in injury liver accompanied gallstones. CXCL16 was also found upregulated in xanthogranulomatous lesions of xanthogranulomatous cholecystitis [14]. CXCL16 secreted by macrophages is not only involved in the inflammatory response, but also regulates the migration of neutrophils and macrophages, thus accelerating the release of inflammatory mediators and magnification of local and systemic inflammatory responses [15,16]. Moreover, CXCL16 can communicate between cells through exosomes [17].

Gut microbiota correlates with gallstone formation and development [18–20]. Butyrate acid is one of the short-chain fatty acids (SCFAs) fermented from non-digestible carbohydrates by gut microbiota [21]. Many studies have shown that Butyrate acid exhibits promising effects in various diseases, such as inflammatory diseases [22], diabetes [23], colorectal cancer etc [24,25]. However, further research on the effect and possible molecular mechanisms of Butyrate acid in gallstone is required to explore.

In the present study, the effect of Sodium butyrate on neutrophil migration and NETs formation involved in macrophages and exosomal CXCL16 in calculous cholecystitis was explored. Our finding may serve as a potential intervention for gallstone as well as cholecystitis.

2. Materials and methods

2.1. Reagents and antibodies

Human (P9040) and mouse (P8550) Neutrophil Isolation Kits were purchased from Solarbio (China). Antibodies of CD86 (sc-28347) and CD206 (sc-376232) were supplied by Santa Cruz (USA). Antibody of CD63 (10628D) was purchased from Invitrogen (USA), Antibody of TSG101 (ab125011) was bought from Abcam (UK), antibody of HSP70 (4872) was obtained from Cell Signaling (USA), antibody of CXCL16 (bs-1441R) was purchased from Bioss (China), and antibody of CXCR6 (822171) was supplied by ZEN BIO (China). All primers were synthesized and obtained from Invitrogen.

2.2. Gall bladder samples collection

The samples of gall bladder were collected from patients with gallstones confirmed by B-type ultrasonography and undergoing operation (male/female: 22/18, GS group). 40 total gall bladder tissues and blood samples include 20 cases of chronic cholecystitis and 20 cases of acute cholecystitis. 20 normal blood samples were obtained from healthy volunteers. All studies followed the ethics committee of The Second Affiliated Hospital of Baotou Medical College (Approval # LW-012 #), and all the participants wrote informed consent.

2.3. Neutrophil analysis and isolation

Human peripheral venous blood samples were collected into K2 EDTA tubes. Percentage analysis of neutrophils in human blood was performed using Blood cell analyzer (UniCel® DxH 600 Coulter®) according to the standard operating procedure.

The neutrophils in human blood were isolated and purified using Human peripheral blood neutrophil isolation fluid kit based on the manufacturer's instructions. Briefly, blood was layered over separation solution and centrifuged at 500×*g* for 30min. After centrifugation, the neutrophil layer was collected and washed with 5 mL cleaning solution twice. Neutrophils were gathered by centrifugation at 250×*g* for 10 min. For mouse bone marrow neutrophil isolation, according to the directions, single-cell suspension of bone marrow prepared by 1 mL 10 % FBS, and other steps remain the same.

2.4. NETs quantification assay

The NETs in human blood and cell culture supernatant were quantified according to previous study [26,27]. PicoGreen dsDNA Quantification Kit (Invitrogen, Carlsbad, CA, USA) and capture Elisa kits of human or mouse MPO (Roche, Indianapolis, IN, USA) were used to quantify NETs followed the instruction. The concentration of dsDNA and MPO-DNA complex is indicated by optical density (OD) according to the instructions of Cell Death Detection ELISA (Roche, Indianapolis).

2.5. RAW264.7 cells culture and treatment

RAW264.7 cell line was supplied by the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM. Cells were seeded into 6-well plates at 2×10^5 cells/well, stimulated with LPS (100 ng/mL, No.L2630, Sigma-Aldrich, USA) for 6 h to generate M1 macrophage and then subsequently co-cultured with Sodium butyrate (1 mM, 5 mM; Sigma Aldrich, NO. V900464, USA) for 24 h. Exosomes were collected for the further experiments.

2.6. Exosome isolation and characterization

Exosomes in the plasma of patients and cell culture were isolated according to the previous study [17]with slight modification. In brief, exosomes were isolated by differential centrifugation and the size of which was analyzed by a nanoparticle tracking analysis (NTA) using a Zetasizer Nano S90 system (Malvern Instruments, England). Exosome morphology was observed using a transmission electron microscope (TEM) (JEM-1200EX; JEOL, Tokyo, Japan). Exosome was identified using markers of protein as CD63, TSG101 and HSP70 in the exosomes pellet fraction assayed by Western blot.

2.7. Immunohistochemistry

The tissue of gall bladder was fixed in 4 % paraformaldehyde and then embedded in paraffin. 5 μm sections were made for immunohistochemical staining. The slices were incubated with primary human antibodies of NE (Abcam, ab68672, 1:100), CXCL16 (bioss, bs-1441R, 1:200), CXCR6 (ZEN BIO, 822171, 1:100) overnight at 4 °C, and then incubated with a horseradish peroxidase-conjugated secondary antibody (CST, USA). After incubated with 3,3′-Diaminobenzidine tetrahydrochloride (Sigma, USA), photographs were taken under a microscope (Olympus, Japan).

2.8. Immunofluorescence assay

The slices of gall bladder tissue were made similarly to the immunohistochemistry assay. The sections were incubated with primary antibodies of CD86 (Santa Cruz, sc-28347) and CD206(Santa Cruz , sc-376232) then with different fluorescent labeled secondary antibodies (Cy3 labeled and FITC labeled, Beyotime, China). DAPI solution (MBD0015, Sigma, USA) was used to stain nuclei. Photographs were taken under a fluorescence microscope.

For RAW264.7 cells immunofluorescence assay, the cells with/without treatment were fixed using 4 % paraformaldehyde and then incubated with primary antibodies of CD86 (Santa Cruz, sc-28347) and CD206 (Santa Cruz,sc-376232), and the next steps are similar to immunofluorescence staining of tissue.

To identify NETs formation, MPO-immunofluorescence assay was also performed. 1×10^5 cells/well neutrophils were plated onto 24-well culture plates containing glass coverslips treated with collagen type I solution (Corning, New York, USA). Then neutrophils were cultured with exosomes obtained from a human blood sample or conditional medium of RAW264.7 (LPS, LPS + NaB, LPS + Anti-CXCL16). The cells were fixed and stained according to the above method for immunofluorescence assay of RAW264.7 cells with primary antibody of MPO (bioss, bs-4943R, 1:100).

2.9. Neutrophils transwell migration assays

Neutrophils migration assay was performed in our study [28]. Briefly, the neutrophils were stained with calcein-AM (2 μg·mL-1, C3099, Invitrogen) for 15 min at 37 °C and seeded on the upper chamber of the transwell inserts with 3.0 μm pore-size transparent PET membranes (FALCON-353096) and co-cultured with RAW264.7 cells or exosomes derived from treatment RAW264.7 cells or CXCL16 neutralizing antibody (0.25 μg; R&D Systems, USA)/recombinant CXCL16 (100 ng/mL; MedChemExpress, USA) in the lower chamber for 1 h at 37 °C according to the instructions. Then the upper chamber was removed and photographs of the cells in the lower chamber were taken under a microscope (IX73, Olympus) and the number of migrated neutrophils was analyzed.

2.10. Western blotting

The protein expression of CD63, TSG101 and HSP70 in the exosomes from the plasma of patients or culture supernatant was measured. Total proteins were obtained throughprotein lysis, the concentration of which was detected by BCA protein concentration Detection Kit (Thermo Fisher, 23227). Different proteins were isolated by SDS-PAGE and then transferred to PVDF membrane, which was followed by incubation with primary antibodies such as CD63, TSG101 and HSP70 and corresponding secondary antibodies. Photographs were obtained under a gel imaging development (TOCAN240, LCNJ2021076623).

2.11. Real-time quantitative PCR (qRT-PCR) detection

qRT-PCR assay was performed to measure the mRNA expression of TNF-α, IL-1β and IL-10 in tissues or cells. Briefly, total RNAs were isolated using Trizol (Molecular Research Center) as the volume ratio of sample to Trizol is 1–2. Reverse transcription was performed according to the instructions of the Reverse transcription kit (Promega), products of which as cDNA was used for fluorescent quantitative PCR. Primer 5.0 was used to design the gene primers for detection (Table 1 primers), and the internal control gene was GAPDH. The relative expression of the target gene was calculated by $2^{-\triangle\triangle CT}$ method.

2.12. Statistical analysis

Results were analyzed using GraphPad Prism 9.0 (USA). Measurement data with normal distribution were expressed as mean ± standard deviation (SD), which were obtained from at least three samples or results of at least three independent experiments. Differences between the two groups were analyzed using the Student's *t*-test, and among three or more groups using one-way ANOVA with Bonferroni correction. It is considered statistically significant when p value is less than 0.05.

3. Results

3.1. NETs formation is enhanced in neutrophils from patients with calculous cholecystitis

To explore the role of neutrophils and NETs formation in calculous cholecystitis with different pathological classifications, neutrophils amount and NETs formation in peripheral blood of gallstones patients with chronic or acute cholecystitis were detected compared with normal volunteers (Control). Results were shown in Fig. 1A that, the neutrophils amount in the blood of gallstones patients was higher than that of control; moreover gallstones patients with acute cholecystitis have even more neutrophils than patients with chronic calculous cholecystitis. It was similar to neutrophils in blood that NETs formation significantly increased in gallstones patients with the aggravation of inflammatory pathology (Fig. 1B) which was further confirmed by dsDNA concentration (Fig. 1C) and MPO-DNA complex assay (Fig. 1D). An ELISA analysis was performed to confirm the concentration of CXCL16 in serum, the results (Fig. 1E) showed CXCL16 was higher in serum of gallstones patients than that of healthy volunteers, and the concentration of CXCL16 was positively associated with neutrophils amount (Fig. 1F). NE detected by immunohistochemical staining was upregulated significantly in gallbladder tissue of gallstones patients with acute cholecystitis compared with gallstones patients with chronic cholecystitis (Fig. 1G). The immunohistochemical analysis revealed that CXCL16 was highly expressed in acute calculus cholecystitis (Fig. 1H).

Enhanced NETs formation is positively correlated with M1 macrophages secreted proinflammatory cytokines in calculous cholecystitis.

As reported in the previous study, increased macrophages were observed in gallbladder tissue of patients with cholecystitis [11,12]. Macrophage polarization was investigated in gallbladder tissue of patients with cholecystitis in our study. As shown in Fig. 2A, both M1 and M2 macrophages, especially M1 macrophages were significantly increased in gallbladder tissue of patients with acute cholecystitis compared with chronic cholecystitis. The expression of inflammation-related factors, such as TNF-α and IL-1β was significantly upregulated in gallbladder tissue of patients with acute cholecystitis compared with chronic cholecystitis (Fig. 2B–C). To explore the relationship between NETs formation and macrophages-mediated inflammation in calculus cholecystitis, correlation analysis indicates that the MPO-DNA complexes was positively correlated with the expression of TNF-α ($r = 0.425$) and IL-1β ($r = 0.514$) (Fig. 2D–E).

3.2. CXCL16 was upregulated in exosomes from calculous cholecystitis

The exosomes in the plasma of gallstones patients and normal volunteers (Control) were separated. The morphology of exosomes obtained from TEM was shown in Fig. 3A; particle size distribution was in Fig. 3B, and the vast majority of exosomes have particle sizes less than 200 nm. Compared with normal control, patients with calculous cholecystitis have higher exosomes concentration, especially in the plasma of patients with acute cholecystitis (Fig. 3C). It was worth noting that the total protein in exosomes from patients with calculous cholecystitis was positively correlated with plasma MPO-DNA complexes ($r = 0.788$) (Fig. 3D). The protein content of exosomes markers such as CD63, TSG101 and HSP70, as well as CXCL16 was significantly upregulated in exosomes from calculous

Table 1
Primer sequences for real-time PCR used in the study.

Gene	Primer (5′-3′)
TNF-α	F: GCCAACTCCCTCTGTTTATGT
	R: GACACCTTGACCTCCTGAATAA
IL-10	F:CCCATTCCTCGTCACGATCTC
	R: TCAGACTGGTTTGGGATAGGTTT
IL-1β	F: CTATTCTCTCCAGCCAACCTTC
	R: CTCGTCACTGTAGTAAGCCATC
GAPDH	F:GGGTCATCATCTCTGCACCT
	R:GGTCATAAGTCCCTCCACGA

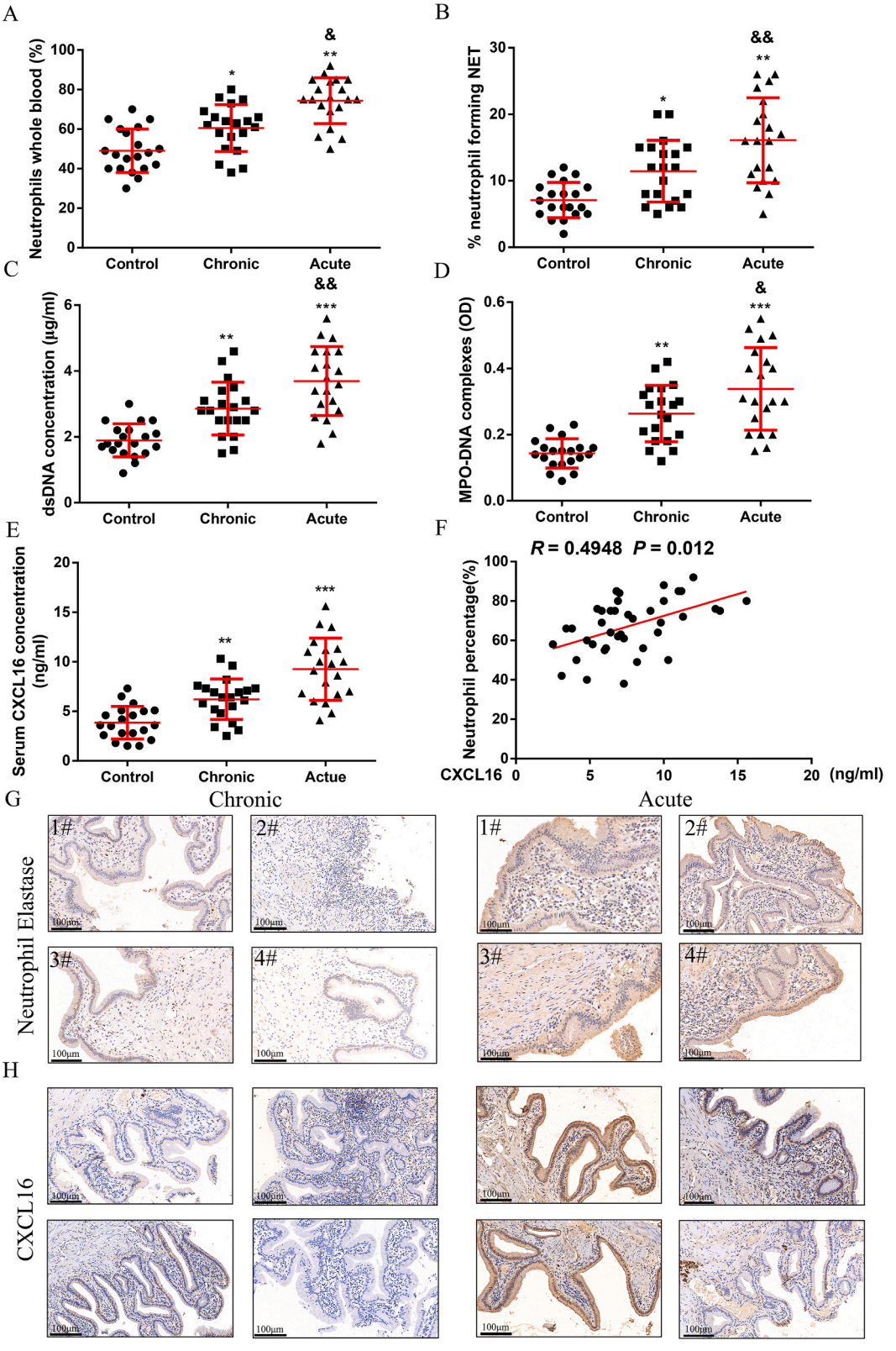

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Fig. 1. The increased NETs formation is associated with pathological classification of calculous cholecystitis. (a) Blood neutrophil percentage count of normal control, chronic cholecystitis and acute cholecystitis (n = 20). (b) Proportion of neutrophils forming NETs in patients with control, chronic cholecystitis and acute cholecystitis (n = 20). (c–d) Quantification of plasmatic dsDNA and MPO-DNA in healthy controls, patients with chronic cholecystitis and acute cholecystitis (n = 20). (e) Concentration of serum CXCL16 in healthy controls, patients with chronic cholecystitis and acute cholecystitis (n = 20). (f) Correlation analysis between CXCL16 and neutrophil amount. (g) Immunohistochemical staining for Neutrophil Elastase in cholecyst sections from patients with chronic cholecystitis (n = 20) and acute cholecystitis (n = 20). (g) Immunohistochemical staining for CXCL16 in cholecyst sections from patients with chronic cholecystitis (n = 20) and acute cholecystitis (n = 20).

cholecystitis patients compared with normal control, especially in acute calculous cholecystitis patients ($P < 0.05$, Fig. 3E–F). To further verify the expression of CXCL16 receptor C-X-C motif chemokine receptor 6 (CXCR6) in gallbladder tissues, immunohistochemical staining showed an increase in CXCR6 expression in acute calculous cholecystitis patients compared to the chronic patients (Fig. 3G).

CXCL16-riched exosomes derived from calculous cholecystitis patients promote neutrophil migration and induce NETs formation.

The role of exosomes derived from plasma of patients with calculous cholecystitis in neutrophil migration and NETs induction was further investigated. It was shown in Fig. 4A that the migration ability of human neutrophils significantly increased by exosomes derived from plasma of patients with calculous cholecystitis, especially exosomes from acute calculous cholecystitis patients. NETs formation induced by exosomes from calculous cholecystitis patients was elevated significantly compared with normal control indicated by immunofluorescence staining of MPO, dsDNA concentration assay and MPO-complexes detection shown in Fig. 4B–C. The expression of CXCL16 receptorCXCR6 was upregulated significantly in exosomes from calculous cholecystitis patients compared with normal control (Fig. 4D). In addition, exosomes from gallstones patients with acute cholecystitis induced much more CXCR6 expression than that from chronic calculous cholecystitis patients (Fig. 4D).

3.3. Sodium butyrate suppressed LPS-induced exosomal CXCL16 secretion in RAW264.7 cells

Butyrate acid was reported to be correlated with gallstone formation and development [18–20], and tended to be associated with inflammation involving macrophages polarization. Therefore, we investigated the effect of Sodium butyrate on LPS-induced M1 macrophages polarization in RAW264.7 cells. As shown in Fig. 5A–B, LPS induced both M1 and M2, especially M1 macrophages increased with CD86 and CD206 upregulation in RAW264.7 cells, which was inhibited by Sodium butyrate in a dose-dependent manner. Results of qPCR assay indicated that the mRNA expression of inflammatory factors TNF-α was significantly upregulated and anti-inflammatory factor IL-10 downregulated in LPS-induced cells. It is important that Sodium butyrate reversed the LPS induction in RAW264.7 (Fig. 5C–D). Sodium butyrate might antagonize the induction of LPS on macrophage polarization.

Inflammation-mediated exosomal CXCL16 secretion in macrophages accompanied with Sodium butyrate treatment were explored further. Exosomes secreted by RAW264.7 cells treated with LPS with/without Sodium butyrate were isolated firstly (Fig. 6A).The morphology of exosomes was observed by TEM (Fig. 6B) and particle size distribution detected by NTA (Fig. 6C), which semblable to those from plasma of patients with gallstones. The expression of exosomes markers such as CD63, TSG101 and HSP70 along with CXCL16 induced by LPS was inhibited by Sodium butyrate in a dose-dependent manner (Fig. 6D–E). All the above results suggested that Sodium butyrate suppressed inflammation-mediated exosomal CXCL16 secretion in macrophages.

Sodium butyrate suppressed neutrophil migration and NETs formation by regulating exosomal CXCL16 in LPS induced RAW264.7 cells The effect of Sodium butyrate on neutrophil migration and NETs formation mediated by inflammation-induced macrophages was investigated in our followed study. Results showed that LPS induced neutrophil migration, NETs formation and CXCR6 expression was inhibited by Sodium butyrate dose-dependently when co-cultured with RAW264.7 cells (Supplementary Fig. 1) or exosomes (Fig. 7A–D). The inhibitory effect of Sodium butyrate was also preliminarily verified in a gallbladder gallstones mouse model *in vivo* (Supplementary Fig. 2). It's worth noting that CXCL16 antagonist recapitulated, whereas recombinant CXCL16 alleviated the inhibitory effects of Sodium butyrate, suggesting the effect of Sodium butyrate on neutrophil migration and NETs formation is mediated via macrophages exosomal CXCL16.

In this study, we found NETs formation is upregulated in calculous cholecystitis, which is positively correlated with proinflammatory cytokines secreted by M1 macrophages. Additionally, exosomes derived from the plasma of patients with calculous cholecystitis promote neutrophil migration and induced NETs formation. *In vitro* experiments demonstrated Sodium butyrate reduced NETs formation by inhibiting LPS-induced exosomal CXCL16 secretion in RAW264.7 cells.

4. Discussion

As one of the most common gallbladder-related diseases worldwide, gallstones have increased incidence in recent years due to living conditions, changes in diet, and other factors [5,29,30]. Although gallstones don't threaten life, with increased incidence of nausea, vomiting, along with feelings of fullness after meals, the patients' quality of life is affected by upper right abdominal pain [5]. Therefore, it is necessary to conduct further investigation of the molecular mechanism of gallstone disease.

NETs were initially discovered to be secreted by activated neutrophils to capture and eradicate pathogens [7]. However, emerging evidence suggested aberrant release of proinflammatory mediators and NETs indeed exacerbate inflammation [31]. Moreover, NETs was reported to correlate with gallstones development by activating the innate immune system [3]. NETs formed aggNETs by interacting with calcium or cholesterol crystals to externalize chromatin decorated with granular proteins to precipitate small nidi promptly to initiate gallstone formation and development [3,9,10]. In the present study, NETs formation was increased in patients with

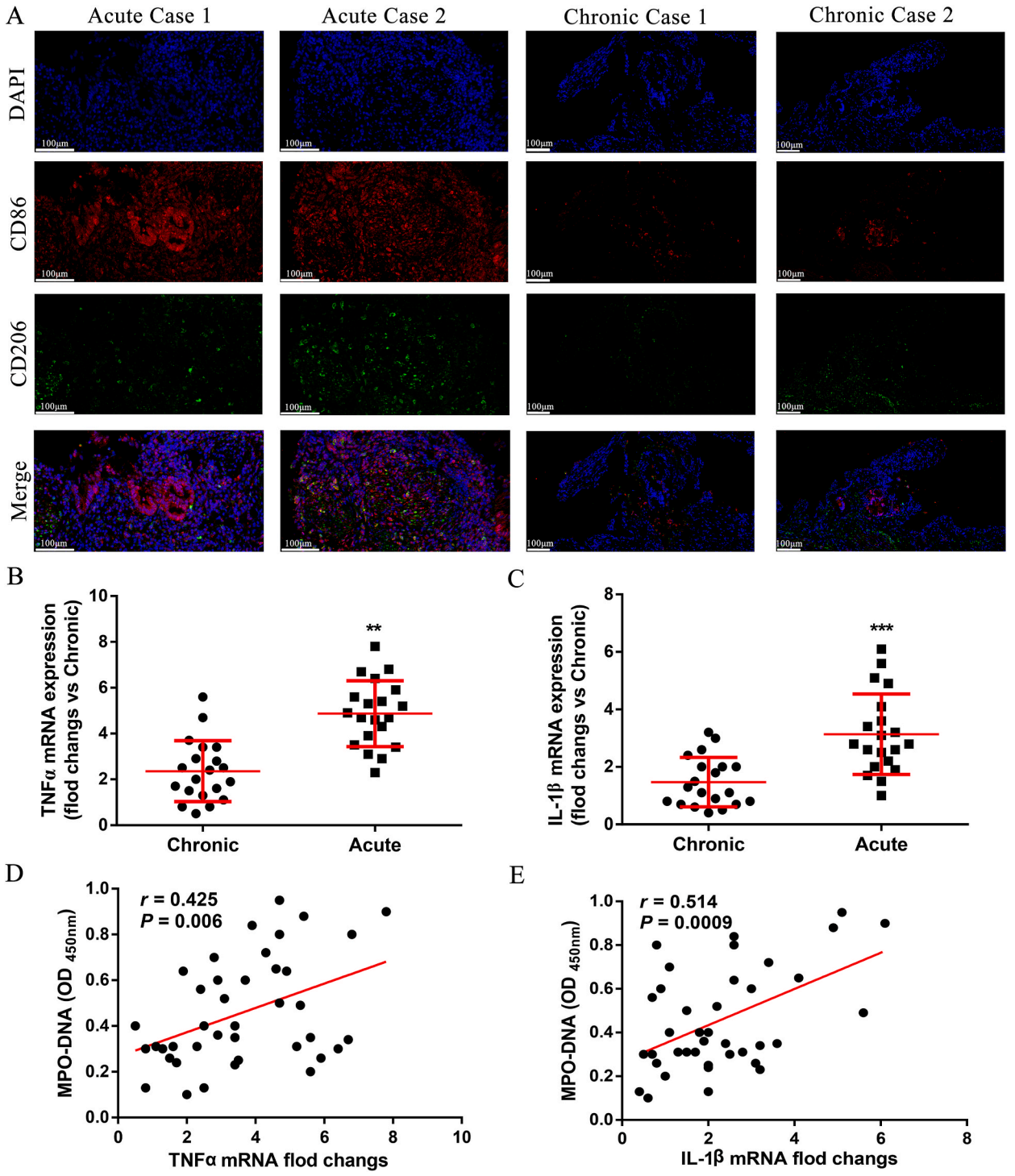

Fig. 2. NET formation is positively related to proinflammatory cytokines secreted by M1 macrophages. (a) Representative immunofluorescence images of M1 macrophage marker (CD86, red) and M2 macrophage marker (CD206, green) in cholecyst sections from patients with chronic cholecystitis (n = 20) and acute cholecystitis (n = 20). (b–c) Inflammatory markers mRNA expression in gallbladder tissue from patients with chronic cholecystitis (n = 20) and acute cholecystitis (n = 20). (d–e) Correlation analysis between MPO-DNA concentration and TNF-α as well as IL-1β. Data are presented as the mean ± SD. **and*** denote $P < 0.01$ and $P < 0.001$ compared to the Chronic group, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

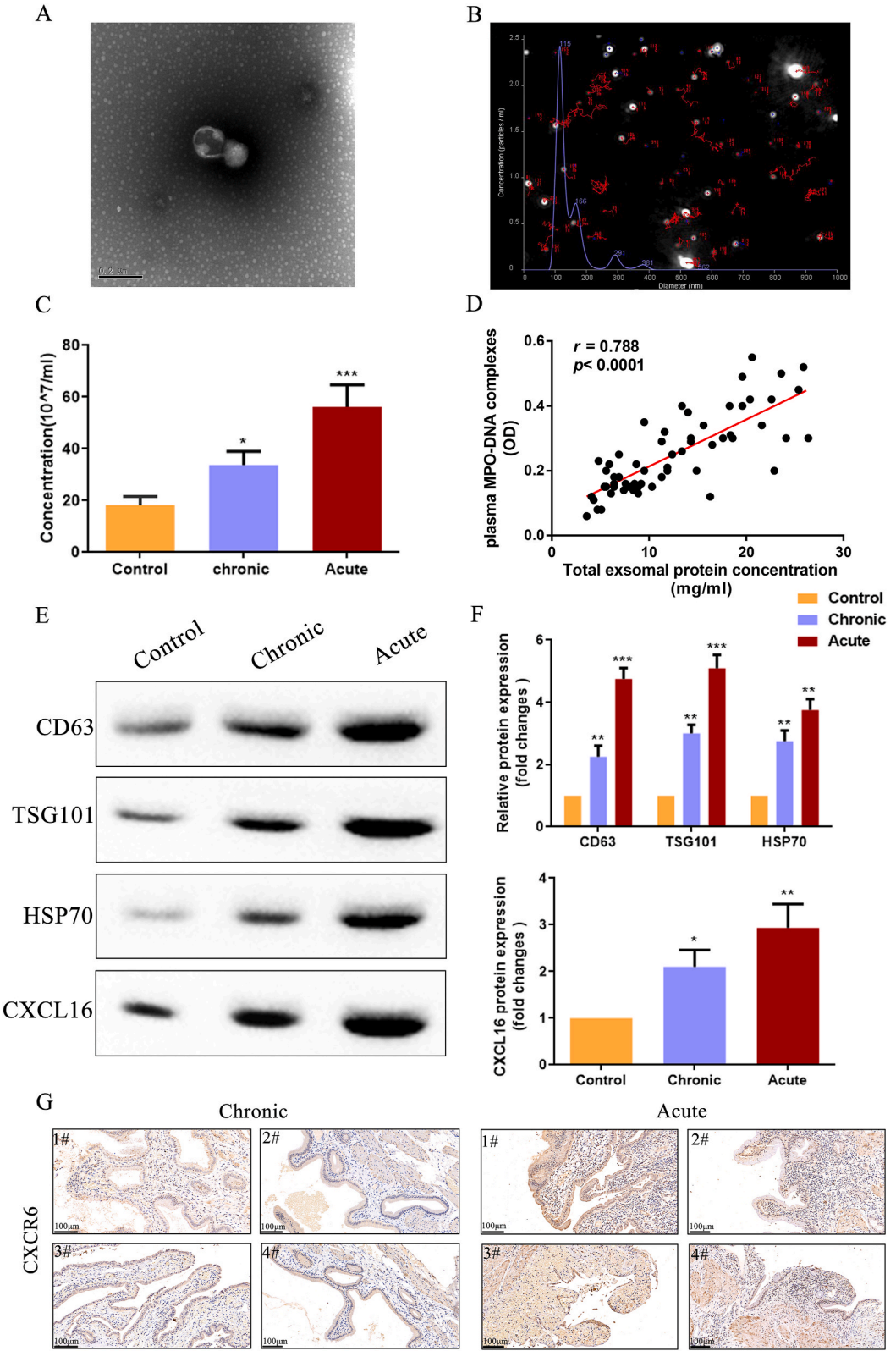

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Fig. 3. The release of exosomes containing CXCL16 in plasma was elevated in calculous cholecystitis. (a) EVs obtained from plasma of patients with calculous cholecystitis were observed by TEM. Scale bar 200 nm. (b–c) The distribution and concentration of EVs were obtained by NanoSight tracking analysis. (d) Correlation between total exosomal protein concentration and MPO-DNA complexes obtained from plasma of patients with calculous cholecystitis (e–f) CD63, TSG101, HSP70 as markers of EVs and CXCL16 were detected by western blotting. Levels of EVs markers and CXCL16 were compared with the normal group. (g) The expression of CXCR6 in gallbladder tissue was evaluated by immunohistochemical staining. *, **and ***denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the normal group, respectively.

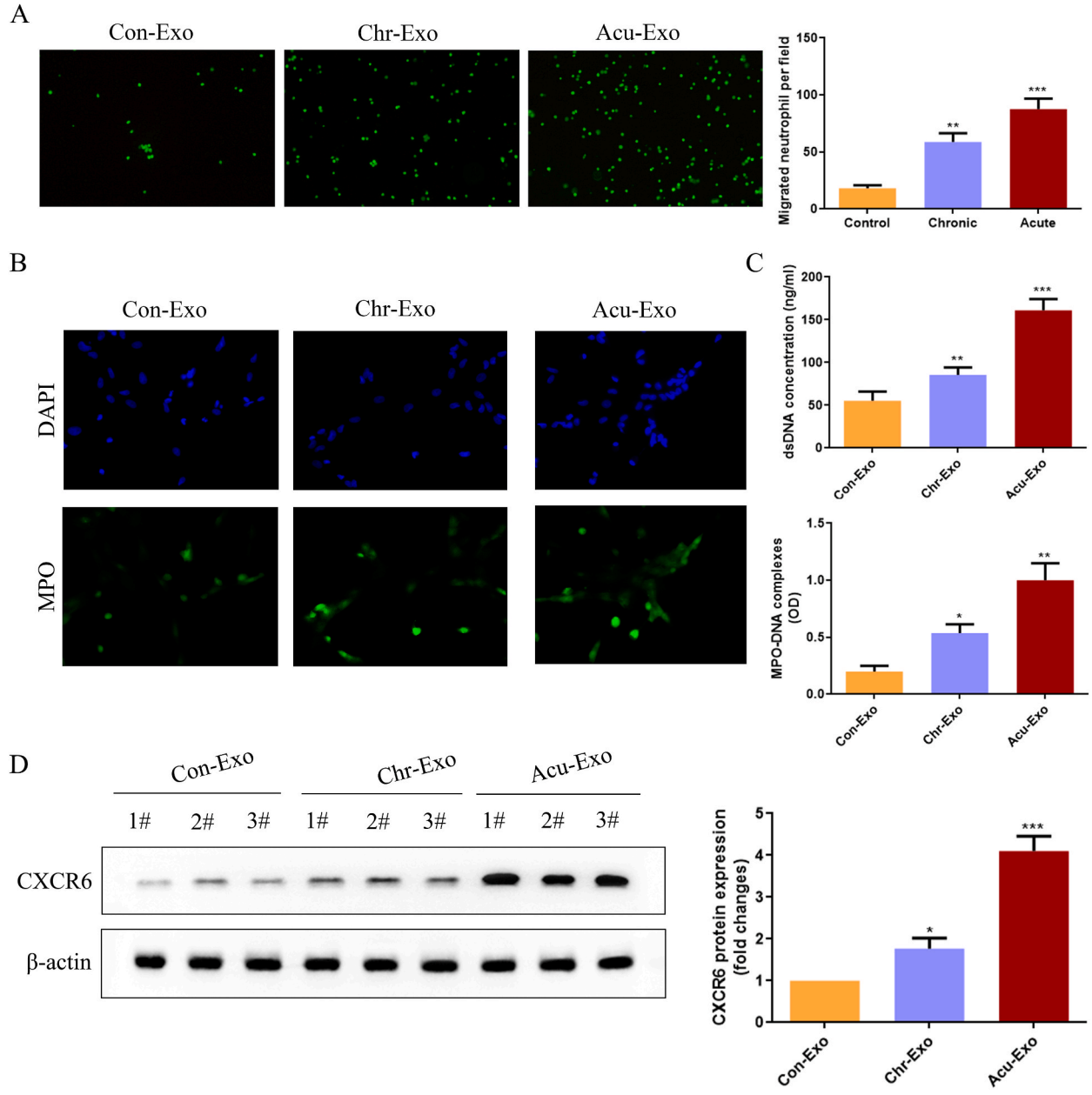

Fig. 4. Exosomes derived from plasma of patients with calculous cholecystitis promote neutrophil migration and NETs induction. Neutrophils (polymorphonuclear neutrophil, PMN) were co-cultured with exosomes obtained from healthy volunteers or patients with chronic and acute cholecystitis and stained with calcein-AM (a). (b) NETs were identified by MPO staining observed by confocalmicroscopy (green). (c) NETs in the supernatant of cultured PMNs were quantifited by dsDNA and MPO-DNA using PicoGreen fluorescent dye and MPO-DNA-ELISA, respectively. (d) CXCR6 was detected by western blotting, and β-actin was used as a control. *, **and ***denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the normal group, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

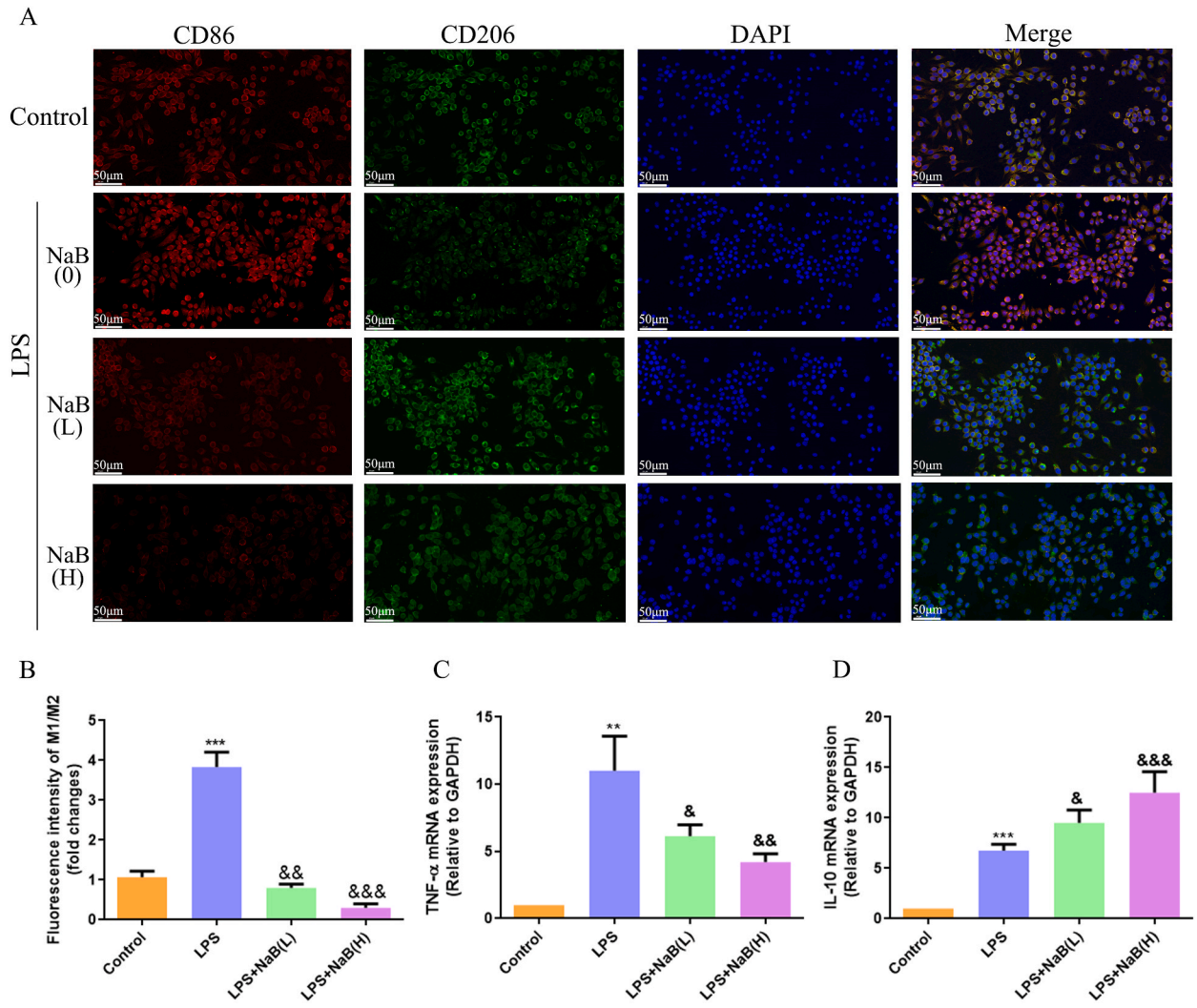

Fig. 5. Sodium butyrate reduced M1 macrophages polarization stimulated by LPS in RAW264.7 cells. (a) Immunofluorescent staining of CD86 and CD206 in LPS with or without Sodium butyrate (NaB) induced RAW264.7 mouse macrophages, Scale bar: 20 μm. (b) Quantitative analysis of fluorescence intensity for CD86 and CD206 in RAW264.7 cells. (c–d) qRT-PCR results for TNF-α and IL-10 mRNA levels after LPS stimulation with or without Sodium butyrate treatment. ** and *** denote $P < 0.01$ and $P < 0.001$ compared to the control group, $^{\&}$, $^{\&\&}$and$^{\&\&\&}$denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the NaB (0) group, respectively.

calculous cholecystitis, especially acute cholecystitis.

Macrophages modulated neutrophil trafficking and activation in inflammatory conditions [13]. Fewer studies have shown the role of macrophages polarization in calculous cholecystitis. Therefore, the activation of macrophages in the gallbladder tissue from patients with calculous cholecystitis was detected. Consistently with the previous study [32], our study showed that M2 accompanied with M1 macrophages, especially M1 macrophages were significantly increased in acute cholecystitis, which was further confirmed by the upregulated expression of M1-macrophages related inflammation factors as TNF-α [33] and IL-1β [34] in acute cholecystitis. Moreover, MPO-DNA complex, an indicator of NETs formation [35,36], was positively correlated with the expression of TNF-α or IL-1β in calculous cholecystitis.

Activated macrophages produced more inflammatory cytokines and chemoattractants. CXCL16, as a neutrophil chemoattractant [16], was significantly increased in patients with gallstone in Chinese population, and also involved in inflammatory responses [14,37]. We detected the expression of CXCL16 and its receptor CXCR6 in gallbladder tissue of patients with calculous cholecystitis, results showed that CXCL16 and CXCR6 expression was increased in acute calculous cholecystitis compared with chronic ones, especially CXCL16 expression. Macrophages mediate innate immune responses due to their releasing many inflammatory cytokines. Exosomes derived from macrophages were reported to induce NETs formation [38]. CXCL16 was found in exosomes and communicates between cells in this way [17]. We then detected CXCL16 in exosomes from the plasma of patients with calculous cholecystitis. The total concentration of proteins including exosome markers as CD63, TSG101 and HSP70 along with CXCL16 were all elevated pathological

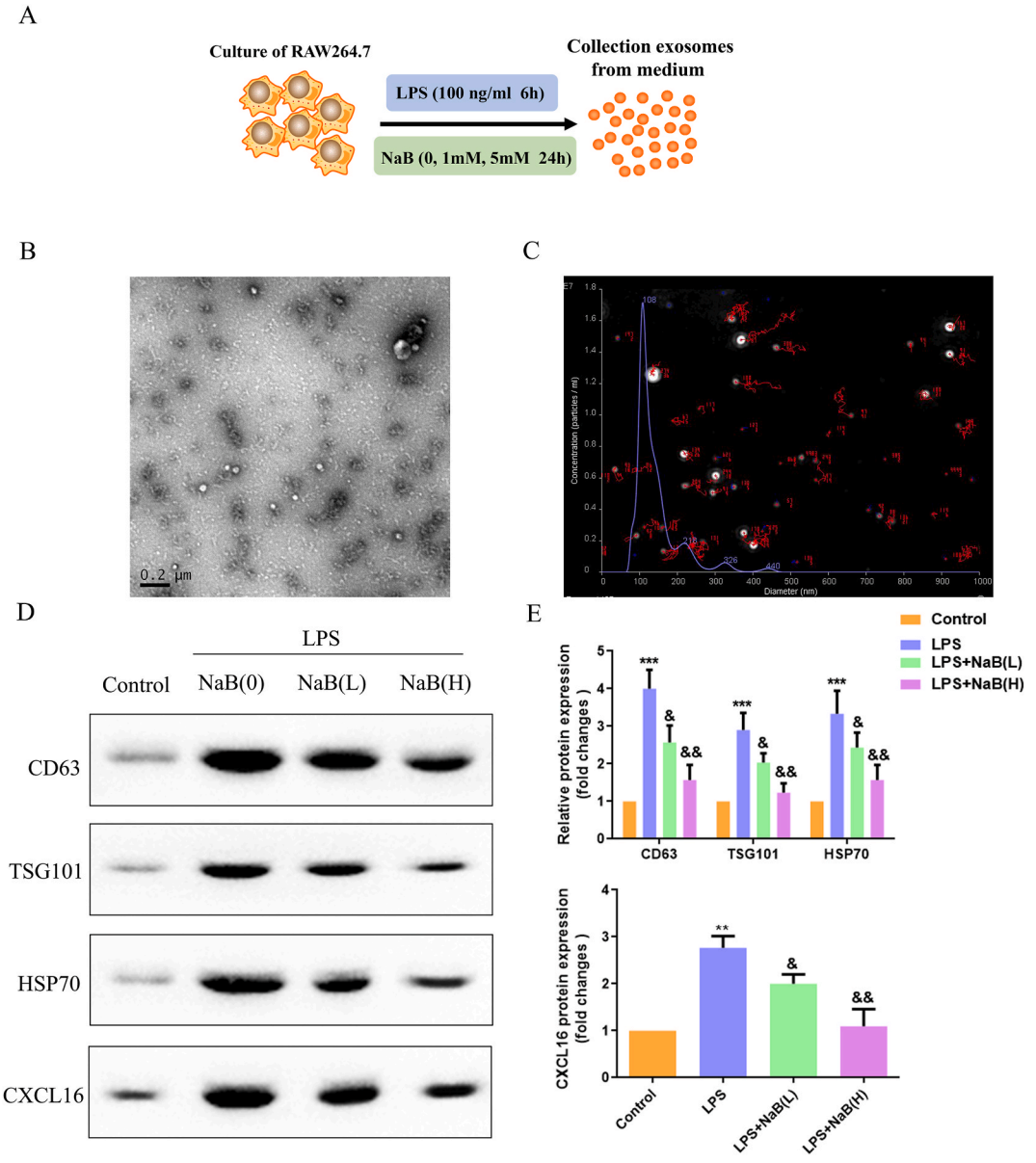

Fig. 6. Sodium butyrate reduced exosomal CXCL16 secreted by LPS-induced RAW264.7 cells. (a) Schematic of the protocol for exosome collection. (b) EVs obtained from medium were observed by TEM. Scale bar 200 nm. (c) The distribution of EVs were obtained by NanoSight tracking analysis.(d–e) Westernblot analysis (d) of exosomal proteins (CD63, TSG101, HSP70 and CXCL16) collected from the same volume RAW264.7 cells culture supernatant and quantitative analysis (e). **and***denote $P < 0.01$ and $P < 0.001$ compared to the control group, $^{\&}$and $^{\&\&}$ denote $P < 0.05$ and $P < 0.01$ compared to the NaB (0) group, respectively.

inflammation type-dependently in calculous cholecystitis patients. What is particularly striking is that exosomes derived from plasma of patients induced neutrophils migration and NETs formation as well as CXCR6 expression. However, knockdown of CXCL16 would be more convincing and might be considered in our further study. In addition, there are many other important chemokines that may contribute to gallstones, which would be explored in our further studies.

As one of the SCFAs, Butyrate acid is fermented from non-digestible carbohydrates by gut microbiota [36,39], which could alleviate cholesterol gallstones by regulating bile acid metabolism [40]. Butyrate acid was reported to regulate macrophages polarization by promoting M2 macrophages [41,42]. The role of Sodium butyrate in inflammation-mediated macrophages polarization *in vitro* was explored in our study. It was found that Sodium butyrate dose-dependently decreased M1 macrophages and increased M2 macrophages, as well as downregulated TNF-α and upregulated IL-10 expression in RAW264.7 cells under LPS stimulation. Investigative results of Sodium butyrate in exosomes from RAW264.7 cells induced by LPS indicated a similarly effect on the protein concentration of CD63, TSG101, HSP70 and CXCL16 and macrophages polarization. The effect of Sodium butyrate on neutrophils migration and

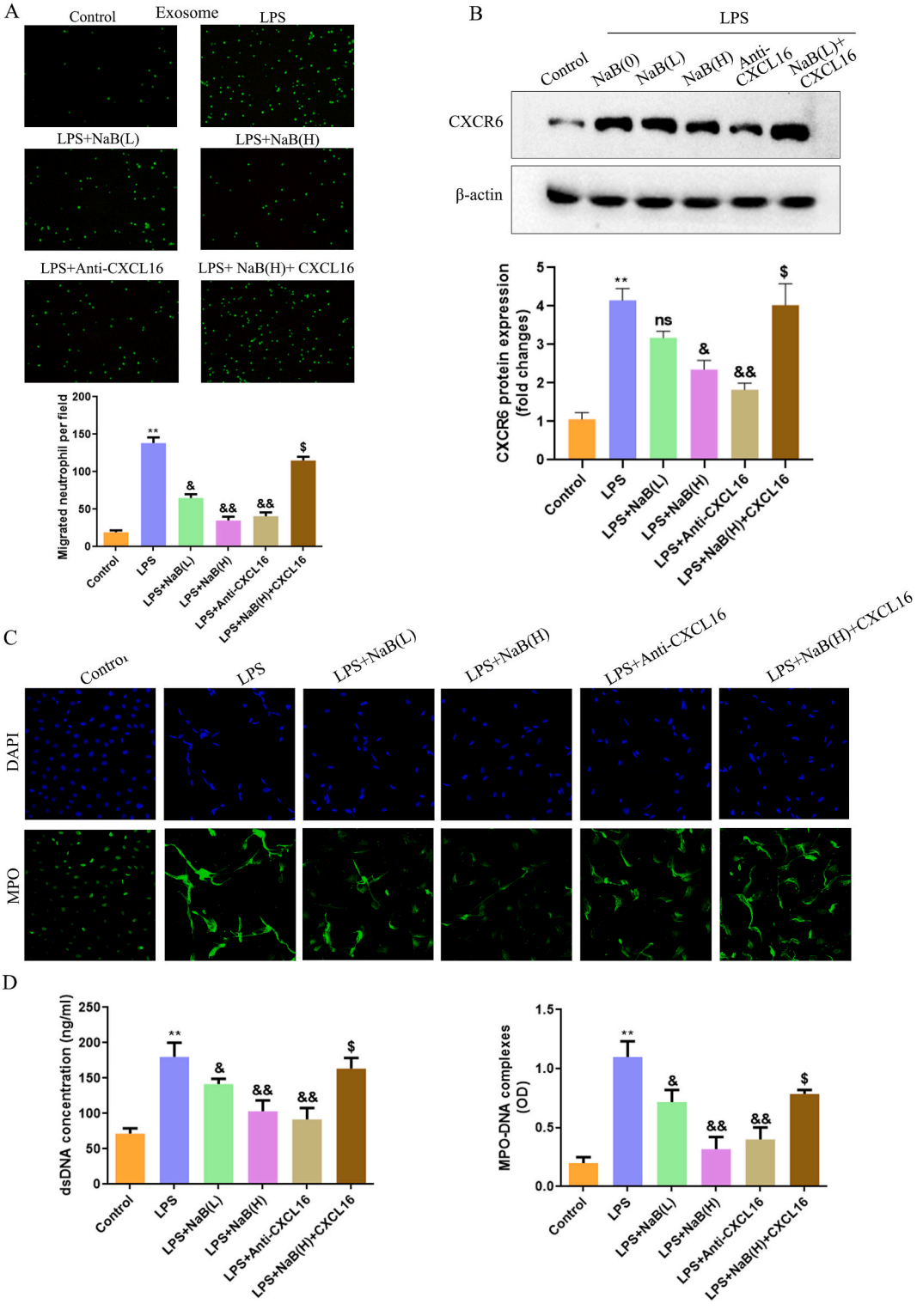

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Fig. 7. Sodium butyrate reduced neutrophil migration and NETs formation by inhibiting exosomal CXCL16 secretion. (a) Representative fluorescence images of neutrophil migration assay. Neutrophils were stained with calcein-AM and co-cultured with RAW264.7 cells exosomes in the presence of LPS, Sodium butyrate or CXCL16 antagonist/recombinant CXCL16, quantitative assessment of neutrophil migration across a permeable transwell chamber. Data represent mean ± SD (n = 5), ***p < 0.001 compared to control group, $^{\&\&}$and$^{\&\&\&}$denote p < 0.01 and p < 0.001 compared to LPS group. (c) CXCR6 protein levels were detected by Western blot. (d–e) Neutrophilic extracellular traps (NETs) were identified by MPO staining observed by confocal microscopy (green). Quantification of dsDNA and circulating NET structures in the supernatant of cultured PMNs using PicoGreen fluorescent dye and MPO-DNA-ELISA, respectively.**$P < 0.01$ compared to control group, $^{\&}$ and $^{\&\&}$ denote $P < 0.05$ and $P < 0.01$ compared to LPS group. $^{\$}$ denote $P < 0.05$ compared to LPS + NaB(H) group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

NETs formation co-cultured with LPS-induced RAW264.7 cells or their released exosomes was further explored accompanied with a CXCL16 antagonist or recombinant CXCL16. Sodium butyrate and CXCL16 antagonist were equivalent to suppressing neutrophils migration and NETs formation, while recombinant CXCL16 attenuated the inhibitory effect of sodium butyrate in the present study.

5. Conclusions

In conclusion, sodium butyrate may inhibit neutrophils migration and NETs formation to alleviate calculous cholecystitis through reduced exosomal CXCL16 secretion from macrophage and macrophage polarization. Although it would be necessary for us to conduct further study in more samples of human gallbladder tissue from calculous cholecystitis and normal gallbladder tissue as well as an animal model of calculous cholecystitis i*n vivo*, our findings may provide a link between exosomes and neutrophils to serve as a potential therapeutic interventionin calculous cholecystitis.

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Data availability statement

Data will be made available on request.

Ethical statement

All the experiments were performed in accordance with the Declaration of Helsinki. The present study was approved by the ethics committee of The Second Affiliated Hospital of Baotou Medical College (Approval # LW-012#) and written informed consent was obtained from all subjects.

CRediT authorship contribution statement

Hongsuo Chen: Writing – original draft, Methodology, Data curation. **Jing Wang:** Writing – original draft, Methodology, Investigation, Data curation. **Qingyu Ji:** Writing – review & editing, Project administration, Funding acquisition. **Zhenyu Jiang:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25189.

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