Special issue article

Innate Immunity

Innate Immunity
2020, Vol. 26(1) 48–61

DOI: 10.1177/1753425919881502
journals.sagepub.com/home/ini
SAGE

Combinatory antibiotic treatment protects against experimental acute pancreatitis by suppressing gut bacterial translocation to pancreas and inhibiting NLRP3 inflammasome pathway

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Abstract

Gut bacterial translocation following impaired gut barrier is a critical determinant of initiating and aggravating acute pancreatitis (AP). Antibiotic combination (ABX; vancomycin, neomycin and polymyxin b) is capable of reducing gut bacteria, but its efficacy in AP prevention and the underlying mechanism have not been investigated yet. AP was induced in BALB/c mice by caerulein (CAE) hyperstimulation. We found that ABX supplementation attenuated the severity of AP as evidenced by reduced pancreatic oedema and myeloperoxidase activity. The protective effect was also confirmed by improved histological morphology of the pancreas and decreased pro-inflammatory markers (IL-1β, TNF-α, MCP-1) in pancreas. ABX administration inhibits the activation of colonic TLR4/NLRP3 inflammasome pathway. Subsequently, down-regulated NLRP3 resulted in decreased colonic pro-inflammation (IL-1β, IL-6, MCP-1) and enhanced gut physical barrier as evidenced by up-regulation of tight junction proteins including occludin, claudin-1 and ZO-1, as well as improved histological morphology of the colon. Together, combinatory ABX therapy inhibited the translocation of gut bacteria to pancreas and its amplification effects on pancreatic inflammation by inhibiting the pancreatic NLRP3 pathway, and inhibiting intestinal-pancreatic inflammatory responses. The current study provides the basis for potential clinical application of ABX in AP.

Keywords

Gut bacterial translocation, antibiotic, gut barrier, acute pancreatitis, Toll-like receptor 4

Date Received: 31 December 2018; accepted: 9 September 2019

Introduction

Acute pancreatitis (AP) is a pancreatic inflammatory disorder caused by gallstones, alcohol misuse, and other risk factors (such as genetic factors and drugs).[1] During the past decades, the worldwide incidence of AP has grown.[1] The direct cause of AP is abnormal activation of proenzymes[2] and uncontrolled diffuse inflammation in the pancreas and adjacent organs,[3] but the mechanism of AP remains unclear. Complications of severe AP, such as multiple organ failure and sepsis, enormously increase the mortality,[4] so it is critically important to elucidate the aggravated pathogenesis of mild AP turning to severe AP.

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Gut bacteria have been increasingly recognized for their role in the regulation of local and distant inflammatory responses.[5] Severe illness enables gut bacteria to enter the extraintestinal site (such as blood, pancreas and other organs);[6] this phenomenon is referred to as 'bacteria translocation'. Delayed intestinal transit time induced by AP changes the composition of the intestinal microflora, promotes intestinal bacterial overgrowth, and subsequently promotes intestinal bacterial translocation.[7,8] In addition, severe AP is characterized by impairment of the intestinal barrier, intestinal dysmotility, ischemia, hyperpermeability, bacterial translocation and increased endotoxin. These studies suggest that dysfunctional intestinal homeostasis in the early stage of AP may enhance intestinal bacterial translocation, subsequently exacerbating AP. Gut decontamination by prophylactic antibiotic treatment may protect against severe AP by inhibiting intestinal bacterial translocation.

Bacteria can activate the host innate immune system via PRR Toll-like receptor 4 (TLR4) and its downstream leucine-rich repeat containing molecules (NLRs).[9] TLR4 triggers the inflammatory responses by mediating organ dysfunction and bacterial translocation in severe AP.[10] Thus, reduction of intestinal bacterial translocation may alleviate the activation of TLR4 and NLRP3, thereby inhibiting the uncontrolled diffuse inflammation in pancreas and adjacent organs during AP.

During AP, gut bacteria translocation is usually caused by an impaired gut barrier, gut bacterial overgrowth, and increased inflammation.[11–13] In the later stage of AP, bacteria in the blood and other organs account for multiple organ failure, sepsis and other serious complications. However, how the escaped gut bacteria participate in amplifying inflammation in the blood, pancreas and adjacent organs remains unknown.

The current study aimed to elucidate the role of escaped gut bacteria in AP. To elucidate the effects and mechanisms, first, a broad-spectrum antibiotic combination (vancomycin, neomycin and polymyxin b – ABX) was used to reduce gut bacteria. Eight days later, caerulein was used to induce AP in mice. Pathology and inflammation-associated indexes were then examined. The effect of ABX treatment on gut bacteria translocation in relation to the progression of AP was investigated. This study will facilitate the understanding on the gut–pancreas immune environment and provide a novel strategy for intervening in AP.

Materials and methods

Animals

For this study, 8-week-old female BALB/c mice (Su Pu Si Biotechnology Co., Ltd. Suzhou, Jiangsu, China) were maintained in specific pathogen-free environment at the Animal Housing Unit of Jiangnan University (Wuxi, Jiangsu, China) under 23–25°C and 12-h light/dark cycle with unlimited access to food and water. All mice were allowed to acclimatize to laboratory conditions over the course of 1 week prior to the experiments. All experimental procedures in this study were approved by the Institutional Animal Ethics Committee of Jiangnan University (JN.No20170711-20171020-84) and carried out in compliance with national and international guidelines for the Care and Use of Laboratory Animals.

Antibiotic treatment and AP induction

Vancomycin is one of the last antibiotics used to treat life-threatening infections caused by Gram-positive bacteria.[14–17] Polymyxin b is often used in combination with neomycin to deplete intestinal Gram-negative bacteria, and the combination has been used to treat Gram-negative infections approved by the FDA.[18–20] In this study, the purpose of the triple antibiotic regimen is to decontaminate intestinal Gram-positive and Gram-negative bacteria.

Female BALB/c mice (20 ± 2g) were randomly assigned to three groups ($n = 7$): (1) the control (CON) mice were fed with autoclaved water; (2) the caerulein-treated (CAE) mice were fed with autoclaved water; (3) the antibiotic-treated mice were fed with autoclaved water with antibiotic combination (ABX, 0.5 .mg·ml^{-1} vancomycin; 1 mg·ml^{-1} neomycin; 0.3 mg·ml^{-1} polymyxin b, all in Sangon, China) for 8 d. Then the CON-mice received hourly intraperitoneal injections with saline for 10 h, and the CAE-mice and ABX + CAE-mice received hourly intraperitoneal injections with caerulein for 10 h.

Tissues sampling

Mice were euthanized and sacrificed with pentobarbitone sodium (100 mg/kg) 1 h after the last caerulein injection. For serum analysis, blood samples were centrifuged (3000 *g*, 15 min), and supernatant was collected and stored at –80°C. Tissues (pancreas and colon) were excised, fixed in 4% paraformaldehyde or snap frozen in liquid nitrogen and stored at –80°C for later analysis. The colon was collected, cut along the axis of the intestine and washed three times with phosphate-buffered saline, and then stored at –80°C until used for subsequent western blotting/PCR analysis. For pancreatic and colonic cytokine assays, tissues (pancreas and colon) were homogenized with phosphate-buffered saline, and centrifuged (3000 *g*, 15 min). Supernatant was then collected and stored at –80°C.

Pancreatic oedema measurement

A portion of freshly harvested pancreatic tissue was trimmed of fat and weighed. Pancreatic water content was evaluated by the ratio of initial mass (wet mass) of the pancreas to its mass after incubation at 80°C for 48 h (dry weight).[21]

Pancreatic myeloperoxidase (MPO) measurement

The MPO activity was measured in homogenized pancreas using MPO assay kit according to the manufacturer's protocol (Jian Cheng Bioengineering Institute, China) to determine the neutrophil infiltration in the pancreas.

Bacterial culture

On TSA plates, we cultured bacterial colonies following a standard method for pancreatic bacteria.[22] After euthanasia, mouse skin was sterilized with 70% ethanol before opening the abdomen. Freshly harvested pancreatic and pancreatic lymph node (PLNs) samples (0.1 g) were obtained aseptically in a safety vertical laminar flow hood, homogenized in with phosphate-buffered saline (1:9). The dilution (0.2 ml) was plated on tryptone soy agar plates, incubated at 37°C for 48–72 h (until CFUs were evident). Tryptone soy agar plate counts (CFUs) were examined to determine whether bacterial translocation had occurred as previously described.[22–25]

Gut bacterial translocation

To evaluate the effect of ABX on the gut bacterial translocation during AP, after 8 d of antibiotic treatment, mice were given *Lactobacillus plantarum* with GFP-labelled plasmids suspended in saline (1×10^{10} CFU/kg) by gavage 18 h before the mice were euthanized. The *L. planterum*-GFP were detected in PLNs and mesenteric lymph nodes (MLNs) by flow cytometry.

The *L. planterum*-GFP (http://www.paper.edu.cn/releasepaper/content/201902-90) were kindly provided by Dr Bingyong Mao (State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, China).

Preparation of single cell suspensions

PLNs and MLNs were harvested and ground with gentle MACS™ Dissociators (Miltenyi Biotec, Bergisch Gladbach, Germany) and filtered with 70 μm filter screen.[26–28] GFP^+ *L. plantarum* were analysed on an Invitrogen™ Attune™ NxT Flow Cytometer (Thermo Fisher Scientific, Massachusetts, USA).

Short chain fatty acid (SCFAs) analysis

Faecal acetate, propionate and butyrate were detected by gas chromatography coupled mass spectrometry (GC-MS) as previously described[29].

Histological examination

Freshly harvested pancreatic samples were fixed with 4% paraformaldehyde overnight, washed with ddH_2O, dehydrated with gradient ethanol solutions and embedded with paraffin. Prepared sections (5 μm) were stained with hematoxylin and eosin (H&E) using standardized protocols. Morphological changes of pancreas were examined under a DM2000 light microscope (Leica, Germany).

Pancreatic injury was evaluated based on oedema, inflammatory cell infiltration, haemorrhage and necrosis.[30] The histopathologic scores were in accordance with the pathological scoring system of pancreas regulated by Schmidt et (Table 1), and Schmidt scores of normal pancreas were 0–3.

DNA extraction

The stool samples were stored at –80°C until analysis. Stool sample DNA was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals, 6560-200, California, USA), following the manufacturer's instructions. In detail, 50 mg frozen stools were added to Lysing Matrix A tube, then 1.0 ml CLS-TC were added to Sample Tube. The mixture was homogenized in the FastPrep Instrument for 40 s at a speed setting of 6.0, then centrifuged at 14,000 *g* for 5–10 min to pellet debris. Next, the supernatant was transfer to a 2.0 ml microcentrifuge tube, an equal volume of Binding Matrix was added, and the samples were mixed and incubated with gentle agitation for 5 min at room temperature on a rotator. Then, the suspension was transferred to a SPIN™, filtered and centrifuged (14,000 *g*, 1 min) twice. Subsequently, the pellet was re-suspended gently with 500 μl prepared SEWS-M, and centrifuged (14,000 *g*, 1 min). The contents of Catch Tube were discarded and the Catch Tube was replaced, centrifuged (14,000 *g*, 1 min) without any addition of liquid. Catch Tubes were replaced with new, clean Catch Tubes, the Binding Matrix above the SPIN

Table 1. Schmidt score of pancreatic pathology.

Scores	Interstitial edema	Inflammatory infiltration	Parenchymal necrosis	Parenchymal haemorrhage
0	–	–	–	–
1	Mild	< 20	< 5%	1–2
2	Moderate	20–50	5–20%	3–5
3	Serious	> 50	> 20%	> 20%

filter was resuspended with 100 µl DNase/Pyrogen-Free Water to elute DNA, centrifuged at 14,000 *g* for 1 min to bring eluted DNA into the clean Catch Tube after incubating the tubes at 55°C for 5 min. DNA was now ready for downstream applications and was stored at –80°C until use.

RNA isolation, reverse transcription, and real-time quantitative polymerase chain reaction (RT-qPCR)

We detected the pancreatic and colonic cytokines by RT-qPCR in our study. Mice were euthanized and sacrificed with pentobarbitone sodium (100 mg/kg) 1 h after the last caerulein injection. Pancreas and colon tissues were collected and stored at –80°C until they were used for RNA extraction. Total RNA of pancreas and colon was homogenized in TRIzol (Life Technologies, MA, USA), quantitated by spectrophotometry (Thermo, USA) and subjected to reverse transcription using the Prime-Script RT reagent kit (TaKaRa Bio, Japan) following the manufacturer's instructions. SYBR® Green RT-PCR reagents (Yeasen, China) were used with a real-time PCR system (BIO RAD CFX Connect, CA, USA). Calculations were made based on the comparative cycle threshold method (2^{-DDCt}). Relative mRNA expression was normalized to the mRNA levels of β-actin (housekeeping control).[21,31–36] Detailed primer sequences are shown in Table 2.

Western blot analysis

Mice were euthanized and sacrificed with pentobarbitone sodium (100 mg/kg) 1 h after the last caerulein injection. Pancreas and colon tissues were collected and stored at –80°C until used for Western blot analysis. The tissues were homogenized in ice-cold RIPA lysis buffer (Beyotime, China) containing cocktail protease inhibitors (Beyotime, Shanghai, China), and centrifuged at 10,000 *g* for 15 min at 4°C; the supernatant was used for Western blot analysis at an equaled amount of protein (30 µg), and protein concentration was quantified using a BCA protein assay Kit (Beyotime, Shanghai, China). Equal amounts of total proteins were separated via SDS-PAGE, transferred onto polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer for 1 h at room temperature, washed with TBST, finally incubated overnight at 4°C with anti-cleaved-caspase-1 p20, anti-cleaved-IL-1β, anti-NLRP3, anti-TLR4 (CST, Beverly, MA, USA) and anti-GAPDH (Biogot, Nanjing, China). Incubation with fluorescently labelled secondary HRP-conjugated secondary antibodies (1:5000) was performed for 2 h at room temperature. Immunoreactivity was analysed using Western Lightening Plus enhanced chemiluminescence (PerkinElmer, MA, USA) according to the manufacturer's instructions. GAPDH was adopted as internal standard to control for unwanted sources of variation, and relative protein expression values were expressed as 'fold mean of the controls' by comparing with the corresponding control value,[32,37–40] and the control value was normalized to 1.0.

Statistic analysis

All data were expressed as mean ± SD. ANOVA was performed to determine the significance among three groups followed by the indicated post hoc test. Independent *t*-test was used for two independent groups. A *p*-value less than 0.05 was considered as a significant difference. All data were analysed using GraphPad Prism 5 software (version 5; GraphPad Software Inc, San Francisco, CA, USA).

Table 2 Specific primers for qPCR.

Target gene	Forward primer	Reverse primer
MCP-1	5′- GTGCTGACCCCAATAAGGAA -3′	5′- TGAGGTGGTTGTGGAAAAGA -3′
TNF-α	5′-AGGGTCTGGGCCATAGAACT-3′	5′-CCACCACGCTCTTCTGTCTAC-3′
IL-1β	5′-CTGAACTCAACTGTGAAATGC-3′	5′-TGATGTGCTGCTGCGAGA-3′
IL-6	5′-CTCTGCAAGAGACTTCCATCCAGT-3′	5′-GAAGTAGGGAAGGCCGTGG-3′
Oldn	5′-TTGAAAGTCCACCTCCTTACAGA-3′	5′-CCGGATAAAAAGAGTACGCTGG-3′
Cldn1	5′-GGGGACAACATCGTGACCG-3′	5′-AGGAGTCGAAGACTTTGCACT-3′
Tjp1	5′-GCCGCTAAGAGCACAGCAA-3′	5′-TCCCCACTCTGAAAATGAGGA-3′
Muc2	5′-CAAGGGCTCGGAACTCCAG-3′	5′-ATGCCCACCTCCTCAAAGAC-3′
Reg3g	5′-ATGCTTCCCCGTATAACCATCA-3′	5′-GGCCATATCTGCATCATACCAG-3′
Reg3b	5′-TACTGCCTTAGACCGTGCTTTCTG-3′	5′-GACATAGGGCAACTTCACCTCACA-3′
Defb1	5′-GCACAAGAAGGTCACACGGA-3′	5′-CTAAGGTTGCAGATGGGGTGT-3′
16SrDNA	5′-TCCTACGGGAGGCAGCAGT-3′	5′-GACTACCAGGGTATCTAATCCTGTT-3′
β-Actin	5′-GGCTGTATTCCCCTCCATCG-3′	5′-CCAGTTGGTAACAATGCCATGT-3′

Results

ABX supplementation alleviates the severity of caerulein-induced AP

As shown in Figure 1a, ABX + CAE-mice exhibited significantly lower body mass than the CAE-mice. ABX pretreated mice exhibited reduced pancreatic edema (Figure 1b) and MPO levels (Figure 1c) induced by caerulein injection. Morphological examination confirmed the protective effect of ABX on caerulein-induced AP mice evidenced by improved cellular morphology, pancreatic oedema, reduced inflammatory cell infiltration and acinar necrosis (Figure 1d). The pancreas pathology score of the CON group was 0, and there was no hyperemia, oedema or inflammatory cells infiltration, and there was less hyperemia, oedema and inflammatory in the CAE-ABX group than that in the CAE group (Figure 1d).

These findings demonstrate that the ABX may reduce the translocation of gut bacteria to the pancreas during caerulein-induced AP.

ABX supplementation inhibits pancreatic inflammation during caerulein-induced AP

Progression of AP is accompanied by increased production of pancreatic pro-inflammatory cytokine, which amplifies the condition and promotes systemic inflammatory responses.[41] To detect the effects of ABX on inflammatory responses during AP, we determined the mRNA levels of pro-inflammatory cytokines in the pancreas. The results showed that ABX significantly down-regulated the pancreatic pro-inflammation markers (IL-1β, TNF-α, MCP-1) compared with the caerulein-treated mice (Figure 2), suggesting that ABX administration effectively reduces pancreatic inflammatory tone.

ABX supplementation enhances the gut barrier during caerulein-induced AP

Impaired gut physical barrier with down-regulated tight junction protein expression is an early hallmark of severe AP.[42,43] To investigate the effects of ABX on

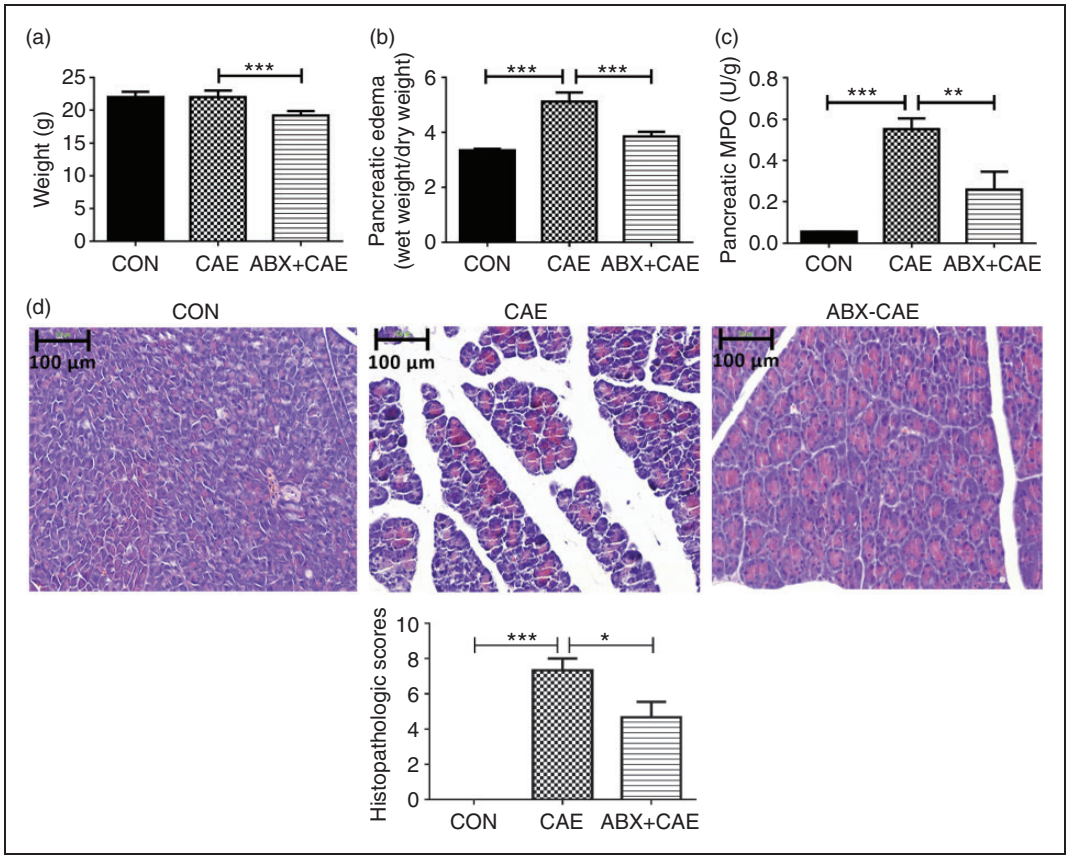

Figure 1. ABX supplementation alleviates the severity of caerulein-induced AP. Female BALB/c mice received normal water (CON and CAE group) or antibiotic-containing water (ABX + CAE group) for 8 days before AP induction by caerulein. Body mass (a), pancreatic oedema (b) and pancreatic MPO activity (c) were determined. (d) Representative photographs showed histomorphology of pancreatic tissues by H&E staining for the indicated groups (bar = 50 μm), and the pancreas pathology score. The results are shown as mean ± SD, $n \geq 3$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by one-way ANOVA followed by Dunnett's test.

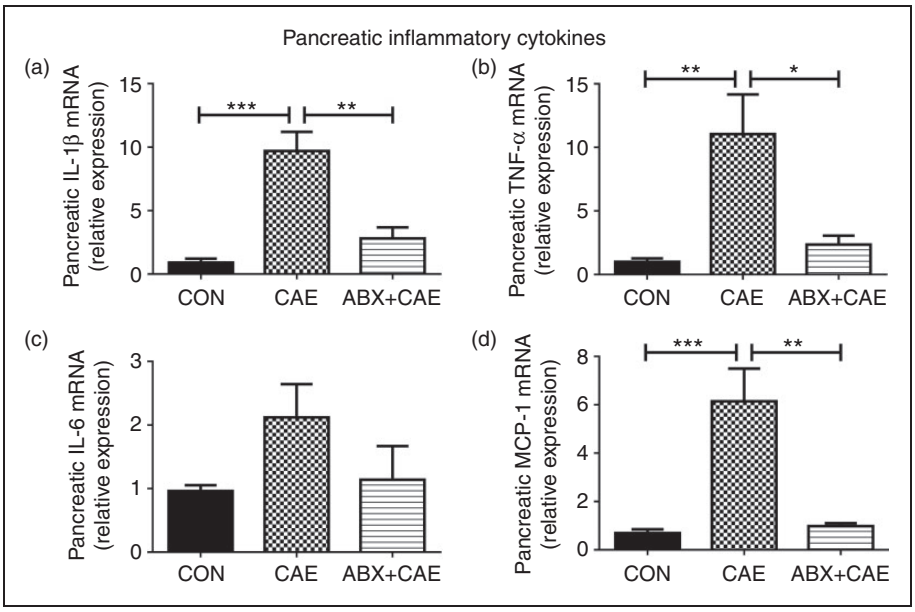

Figure 2. ABX supplementation inhibits pancreatic inflammations during caerulein-induced AP. Levels of pancreatic IL-1β (a), TNF-α (b), IL-6 (c) and MCP-1 (d) were determined by qPCR. The results are shown as mean ± SD, $n \geq 3$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by one-way ANOVA followed by Dunnett's test.

the gut barrier, we determined the expression of physical barrier-associated markers as well as chemical barrier-associated markers in colon.[44,45] As shown in Figure 3 and Supplementary Figure 1, both physical barrier-associated markers and chemical barrier-associated markers were greatly impaired by caerulein injection, while ABX protected against caerulein-induced down-regulation of physical barrier-associated markers (occludin, claudin-1, ZO-1) (Figure 3a–e) but not the mucus and the chemical barrier-associated markers (Supplementary Figure 1). Moreover, we found that ABX prevented the reduction of goblet cells and crypt length in the colon (Figure 3f), which was a sign of improved gut barrier dysfunction. These data demonstrate that ABX administration effectively restores the impaired gut physical barrier induced by AP.

ABX supplementation inhibits colonic inflammation during caerulein-induced AP

The integrity of the gut barrier is tightly coupled to the degree of severity in AP, and the intestine is the origin of systemic inflammation.[46] Having found clear effects on the gut integrity (Figure 3), we subsequently measured the colonic pro-inflammatory markers by qPCR to elucidate whether ABX lowered inflammatory tone in the colon. The ABX + CAE-treated mice had significant lower IL-1β, IL-6 and MCP-1 levels compared with the CAE-mice (Figure 4), suggesting the protective effect of ABX on AP could be potently induced by decreased inflammatory tone in colon.

ABX supplementation suppresses the translocation of gut bacteria to pancreas

Previous studies have suggested a possible interaction between altered intestinal bacteria and pancreas.[22,23] We next detected the bacteria inside the faeces and pancreas by nanodrop and 16S PCR analysis, and found that the ABX + CAE-mice exhibited significantly lower faecal (Figure 5a) and pancreatic bacterial DNA (Figure 5b) compared with the CAE-mice.

In addition, we detected the bacteria inside the pancreas and PLNs by CFU analysis. Interestingly, we found a significant increase of CFU in the pancreas and PLNs of CAE-mice compared with CON-mice (Figure 5c, 5d), suggesting the translocation of gut bacteria to pancreas during AP. ABX treatment effectively lowered AP-induced increase in CFU (Figure 5c, 5d).

To evaluate the effect of ABX on the gut bacterial translocation during AP, mice were given *L. planterum*-GFP suspended in saline (1×10^{10} CFU/kg) by gavage 18 h before the mice were euthanized. After the mice were sacrificed, the GFP^+ events were detected in PLNs and MLNs. We found that CAE-mice harboured more GFP^+ events in both PLNs (Figure 5e) and MLNs (Figure 5f) compared with the CON-mice, which was significantly inhibited by ABX treatment (Figure 5e, 5f).

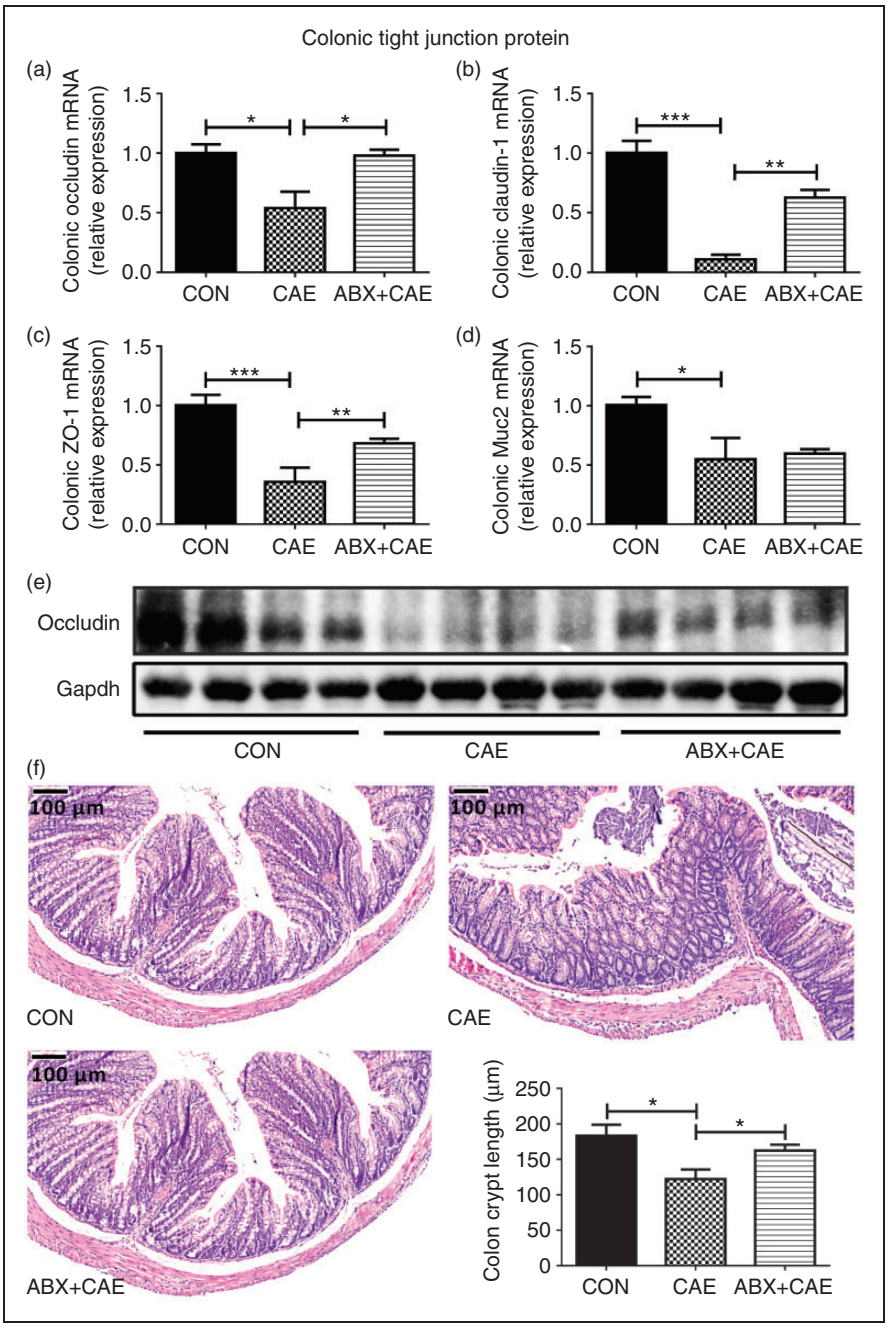

Figure 3. ABX supplementation enhances gut barrier function during caerulein-induced AP. Levels of colonic physical barrier markers occludin (a), claudin-1 (b), ZO-1 (c), Muc2 (d) were determined by qPCR. (e) Levels of colonic occludin were determined by Western blot. (f) Representative photographs showed histomorphology of colon by H&E staining for the indicated groups (bar = 100 μm). The results are shown as mean ± SD, $n \geq 3$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by one-way ANOVA followed by Dunnett's test.

In addition, GC-MS analyses revealed that ABX significantly reduced SCFA production (Figure 5g).

These data suggest that ABX administration attenuates intestinal bacterial content and inhibits their translocation to pancreas during caerulein-induced AP.

ABX supplementation down-regulates colonic TLR4 expression during caerulein-induced AP

We next examined pancreatic and colonic TLR4 levels by Western blotting. Interestingly, colonic TLR4 was significantly activated in the CAE-mice compared with the CON-mice, while ABX treatment significantly

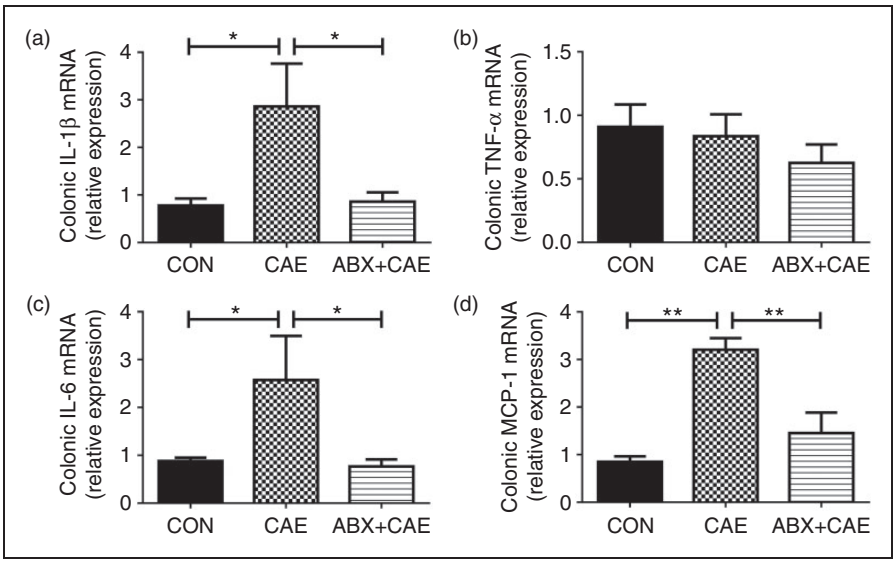

Figure 4. ABX supplementation inhibits colonic inflammation during caerulein-induced AP. Levels of colonic cytokines IL-1β (a), TNF-α (b), IL-6 (c) and chemokine MCP-1 (d) were determined by qPCR. The results are shown as mean ± SD, $n \geq 3$. *$P < 0.05$ and **$P < 0.01$ by one-way ANOVA followed by Dunnett's test.

reduced colonic TLR4 levels (Figure 6). However, there was no significant difference observed in pancreatic TLR4 levels among the groups. These findings demonstrate that the anti-AP mechanism of ABX may be associated with the inhibition of colonic TLR4.

ABX supplementation inhibits the activation of pancreatic and colonic NLRP3 inflammasome during caerulein-induced AP

Next, we investigated the signalling molecular pathways underlying the protective effects by ABX supplementation. Recent reports have suggested that NLRP3 inflammasome can be activated by bacteria during AP.[47,48] Thus we investigated whether the protective effect of ABX on AP was associated with the NLRP3 inflammasome pathway. We found that both pancreatic and colonic NLRP3 inflammasome pathways were significantly activated in caerulein-induced AP mice, while ABX supplementation effectively suppressed the activation of NLRP3 and its downstream signalling molecules (caspase-1 and cleaved 1β) (Figure 7). These data suggest that ABX supplementation inhibits the activation of pancreatic and colonic NLRP3 inflammasome pathway.

Discussion

The present study demonstrates that prophylactic ABX supplementation delays AP development by three graded actions: (1) reducing pancreatic inflammation and damage; (2) preventing intestinal barrier dysfunction and inflammation, and reducing gut bacterial translocation to the pancreas; (3) delaying the progression of AP into a systemic inflammatory response as the consequence. To our knowledge, this is the first study to reveal the positive effect of prophylactic ABX treatment on AP development.

Developmentally controlled lymphogenesis establishes a preferential trafficking route from the gut to the PLNs, where T cells can be activated by antigen drained from the gastrointestinal tract. Furthermore, intestinal stress also modifies the presentation of pancreatic self-antigens in PLNs.[11,49] Prophylactic broad-spectrum treatment prevents gut bacterial translocation to the PLNs in both streptozotocin-induced type 1 diabetes (T1D) and non-obese diabetic mice, subsequently protecting the mice from T1D.[23] With the progression of AP, pro-inflammatory cytokines including TNF-α and IL-1β are produced in the pancreas and reach the colon through the microcirculation.[21,43] These cytokines recruit more leukocytes and inflammatory mediators, ultimately contributing to intestinal barrier dysfunction and increased intestine permeability.[43] This promotes intestinal bacterial overgrowth and its translocation to the pancreas, subsequently resulting in a second round of inflammatory events in the pancreas and initiating excessive inflammatory responses and multi-organ dysfunctions.[50] In the present study, combinatory ABX therapy significantly inhibited gut bacterial translocation to the pancreas, finally alleviating intestinal-pancreatic inflammatory responses to prevent secondary excessive inflammatory responses.

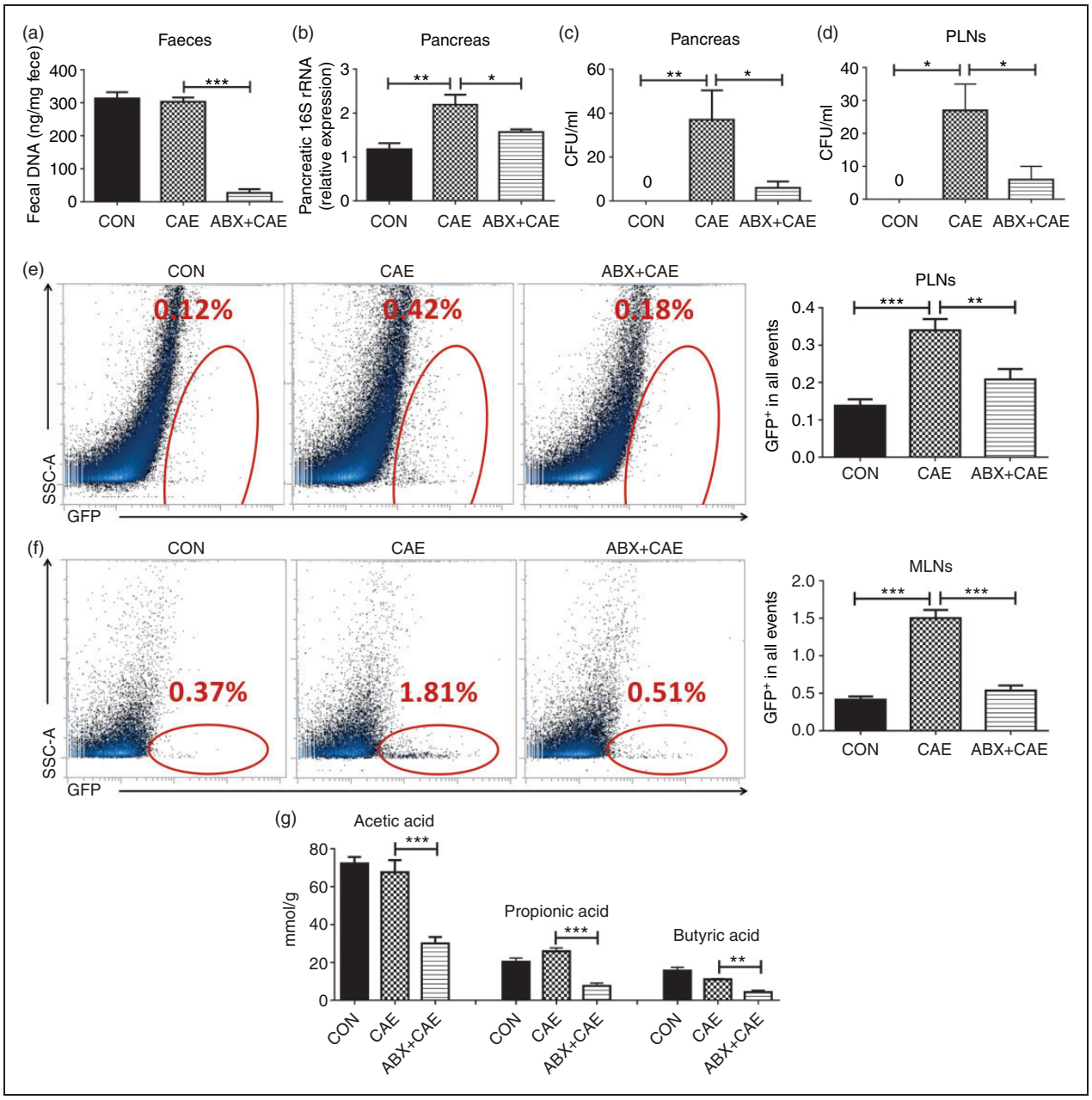

Figure 5. ABX supplementation suppresses the translocation of gut bacteria to pancreas. (a) Faecal bacterial DNA concentration. (b) Pancreatic bacterial DNA content. CFUs were counted on culture plates of pancreas (c) and PLNs (d). The GFP^+ events were detected in PLNs (e) and MLNs (f) by flow cytometry. (g) Faecal SCFAs concentration measured by GC-MS. The results are shown as mean $\pm$ SD, $n \geq 3$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by one-way ANOVA followed by Dunnett's test.

The innate immune system recognizes specific bacterial antigens through an extensive family of PRRs. The inflammasome complex is typically composed of three components: NLR, ASC and caspase-1, of which the NLRP3 inflammasome is the most investigated.[51] The combination of TLR4 (one of the PRRs, the receptor of LPS, which is a Gram-negative bacterial component) and LPS activates the downstream NLRP3. Once activated, NLRP3/ASC adaptor promotes the recruitment of pro-caspase-1 to generate enzymatically active caspase-1, which then converts pro-IL-1β into IL-1β (its mature active form).[52,53] IL-1β is an important mediator in systemic inflammation reaction syndrome and plays an important role in the early stages of AP.[12,54] However, there were no differences in pancreatic TLR4 levels among these three groups, probably due to relatively low abundance of bacteria in pancreas compared with that in colon. Consistent

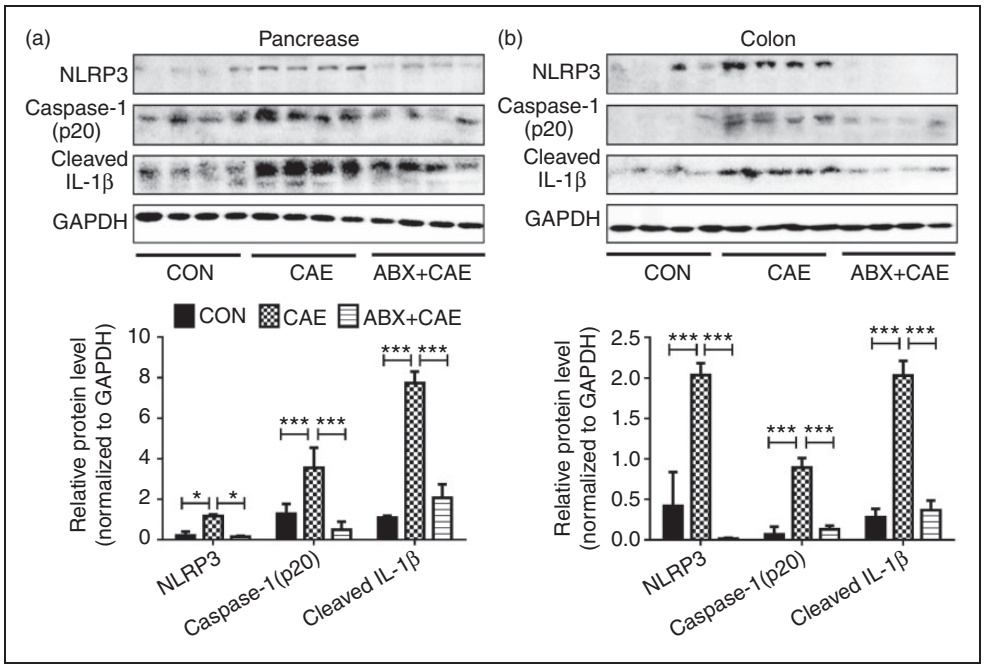

Figure 6. ABX supplementation down-regulates colonic TLR4 expression during caerulein-induced AP. Protein expression and quantitative analysis of TLR4 in pancreas (a) and colon (b) were examined by Western blot and analysed by Alphaview SA. The results are shown as mean ± SD, $n \geq 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$ by one-way ANOVA followed by Dunnett's test.

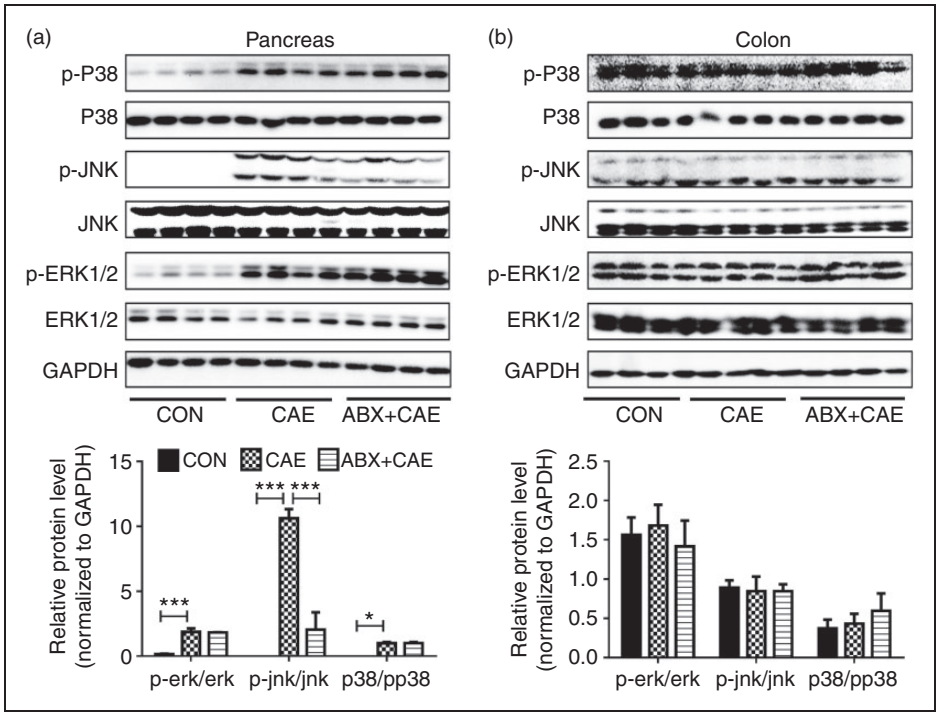

Figure 7. ABX supplementation inhibits pancreatic and colonic NLRP3 inflammasome activation during caerulein-induced AP. Protein expression and quantitative analysis of NLRP3, caspase-1 (p20), cleaved-IL-1β in pancreas (a) and colons (b) were examined by Western blot and analysed by Alphaview SA. The results are shown as mean ± SD, $n \geq 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by one-way ANOVA followed by Dunnett's test.

with previous studies, in colon, combinatory ABX therapy inhibits the activation of TLR4/NLRP3 pathway, subsequently resulting in decreased colonic inflammation and enhanced gut physical barrier. Enhanced gut physical barrier inhibited the translocation of gut bacteria to pancreas and pancreatic inflammation by inhibiting the pancreatic NLRP3 pathway, ultimately inhibiting the inflammatory response in pancreas.

The translocation of gut bacteria to pancreas is the underlying cause of exacerbating AP. Inhibition of gut bacterial translocation to pancreas improves the outcome of AP.[42,55] Antibiotics with high pancreatic tissue penetration significantly reduce pancreatic infection and mortality.[56,57] In addition, in human studies, both systemic antibiotic use and selective intestinal antibiotic intervention are beneficial for AP.[58,59] Consistently, in our results, 8 d of ABX pre-treatment significantly down-regulated total gut bacteria and their migration to pancreas, subsequently improving the outcome of AP. However, pre-treatment with broad-spectrum antibiotic meropenem for 2 d increases the mortality of SAP mice[60], which is probably because 2 d of meropenem treatment is insufficient to reduce gut bacteria and their translocation to pancreas. Instead it may lead to excessive proliferation of *Enterococcus faecium*.[60] These data suggest that different antibiotics or different intervention time may account for discrepant effects.

Our results demonstrate that ABX administration significantly improves the impaired gut barrier by enhancing the gut physical barrier-associated markers but not chemical barriers-associated markers. Gut microbiota modulates mucus composition and mucus thickness, and the mucus layer in germ-free mice, is relatively thinner than control mice.[61] In addition, the metabolites of gut microbiota (SCFAs, especially butyrate) also stimulate epithelial cells to produce chemical barrier-associated markers after sensing the luminal environment.[61,62] Moreover, previous findings show that butyrate up-regulates Muc 2 expression in a dose-dependent manner.[63–66] In our current study, ABX treatment contributed to drastically reduced gut bacteria and decreased SCFAs in the caerulein-induced AP mice, which might lead to a reduced stimulus signal and unraised chemical barrier-associated markers, including Muc 2.

In this study, we observed a significantly increase of TNF-α in pancreas but not colon in response to caerulein injection or ABX administration. Moreover, in colon, the other pro-inflammatory cytokines (IL-1β, IL-6 and MCP-1) of ABX-treated mice were significantly lower than in CAE-mice, probably because TNF-α can be rapidly cleared by liver[2,67] and disrupted by inactivating enzymes in the bloodstream (neutrophil elastase).[2,68,69] Therefore, serum and colonic TNF-α are difficult to detect in patients with AP, and TNF-α is not a good indicator of AP.[67] In addition, differential cytokine regulation in different tissues is also common in experimental AP.[70] Moreover, cytokine production differs based on their tissue-dependent cellular sources and the time point examined. In the current study,

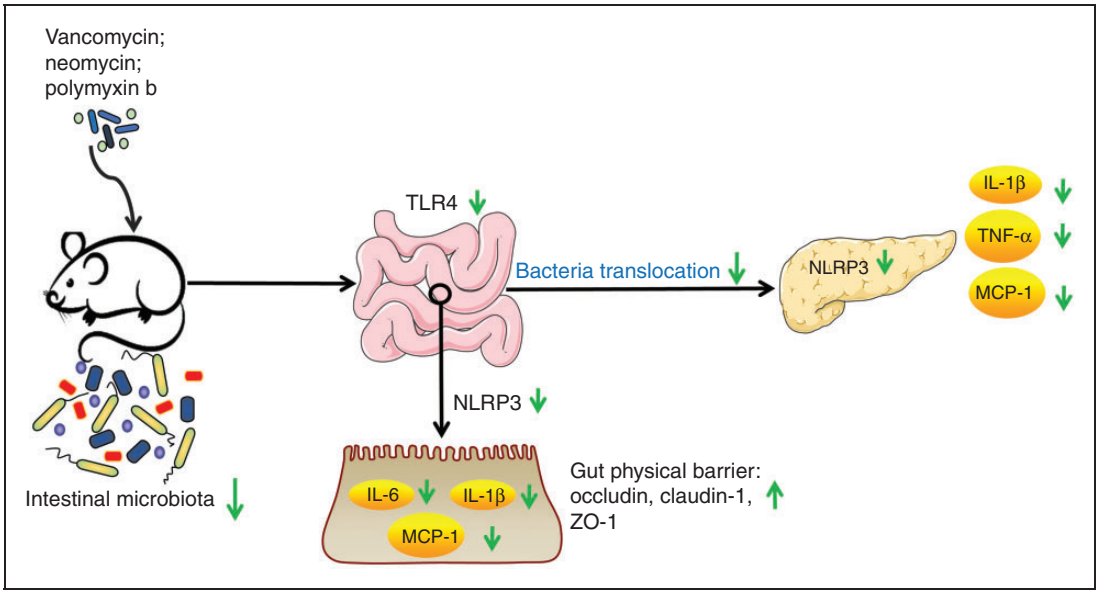

Figure 8. The ABX administration inhibits the activation of colonic TLR4/NLRP3 inflammasome pathway. Down-regulated NLRP3 resulted in decreased colonic inflammation (IL-1β, IL-6, MCP-1) and enhanced gut physical barrier, subsequently inhibiting the translocation of gut bacteria to pancreas and its amplification effects on pancreatic inflammation by inhibiting pancreatic NLRP3 pathway.

we measured a variety of cytokines to demonstrate their differential production in tissues and regulation by ABX treatment.

In summary, the study demonstrates that combinatory ABX administration protects against AP and delineates the underlying mechanism. The protective effects of ABX administration were achieved by attenuating pancreas inflammation, which was the result of decreased gut bacterial translocation to pancreas. Thus, our results suggest that ABX treatment targeting gut bacteria translocation may act as a novel strategy for prevention and treatment of AP and associated complication in clinical practice (Figure 8).

Acknowledgements

The authors thank Dr. Bingyong Mao (State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, China) for providing the *Lactobacillus plantarum* with green fluorescent protein-labelled plasmid.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: All authors participated in the discussion and commented on the paper.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The work was supported by funds from the National Natural Science Foundation of China (Grant Nos. 91642114, 81870439, 31570915 and 81573420), Jiangsu Province Recruitment Plan for High-level, Innovative and Entrepreneurial Talents, the Fundamental Research Funds for the Central Universities (Grant No. JUSRP11866), Jiangsu Province "Six Summit Talents" program (2019-YY-038), National first-class discipline program of Food Science and Technology (Grant No. JUFSTR20180103), Wuxi Social Development Funds for International Science & Technology Cooperation (Grant No. WX0303B010518180007PB), Chinese Postdoctoral Science Foundation (Grant No. 2018M642169), Jiangsu Postdoctoral Fund (Grant No. 2018K237C), Wuxi Medical Youth Talents Project of "Science and Technology Strengthening Health Project" (QNRC001) and Project of Public Health Research Center of Jiangnan University (JUPH201825).

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Supplemental material

Supplemental material for this article is available online.

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