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# GV-971 prevents severe acute pancreatitis by remodeling the microbiota-metabolicimmune axis

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Despite recent advances, severe acute pancreatitis (SAP) remains a lethal inflammation with limited treatment options. Here, we provide compelling evidence of GV-971 (sodium oligomannate), an anti-Alzheimer's medication, as being a protective agent in various male mouse SAP models. Microbiome sequencing, along with intestinal microbiota transplantation and mass cytometry technology, unveil that GV-971 reshapes the gut microbiota, increasing *Faecalibacterium* populations and modulating both peripheral and intestinal immune systems. A metabolomics analysis of cecal contents from GV-971-treated SAP mice further identifies short-chain fatty acids, including propionate and butyrate, as key metabolites in inhibiting macrophage M1 polarization and subsequent lethal inflammation by blocking the MAPK pathway. These findings suggest GV-971 as a promising therapeutic for SAP by targeting the microbiota metabolic immune axis.

Acute pancreatitis is a medical condition characterized by inflammation of the pancreas, elevated levels of serum lipase and amylase, and the presence of acute abdominal pain<sup>1,2</sup>. This condition often leads to necrosis of pancreatic acinar cells and can result in multiple organ dysfunction<sup>2</sup>. While the majority of acute pancreatitis cases are selflimiting and resolve within a week, -20% of patients develop a more severe form known as severe acute pancreatitis (SAP)<sup>3,4</sup>. SAP is marked by tissue necrosis, organ failure, and mortality rates of up to 40%<sup>5-8</sup>. Current clinical treatments for SAP, including medical intervention and surgical procedures, have limited efficacy. This underscores the urgent need for the development of pharmaceutical interventions.

The pathogenesis of SAP involves a sequence of events, including acinar cell death, infiltration of innate immune cells, and the release of inflammatory mediators<sup>9-13</sup>. In the early stages, damaged acinar cells release damage-associated molecular patterns (DAMPs), triggering

local sterile inflammation<sup>14-17</sup>. Subsequently, macrophages and neutrophils are recruited to the site of injury, enhancing the production of cytokines and contributing to systemic inflammation<sup>17-19</sup>. Intestinal dysfunction and alterations in gut microbiota further exacerbate the inflammatory response, advance SAP progression, and contribute to the development of systemic complications<sup>20</sup>. Conversely, the utilization of probiotics and prebiotics is a vital strategy for microbial management aimed at improving the health of the host organism and attenuating immune responses in human diseases<sup>21-24</sup>. However, the available clinical drugs for modulating gut microbiota remain limited.

GV-971, also known as sodium oligomannate, is an oligosaccharide derived from marine brown algae extract. The China National Medical Products Administration has approved GV-971 for enhancing cognitive function in individuals with mild-to-moderate Alzheimer's disease. In this disease, GV-971 exhibits a wide range of

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In this study, we provide the evidence that GV-971 effectively prevents mortality in multiple experimental SAP mouse models. Through comprehensive omics, animal experiments, and molecular analyses, we reveal that GV-971's anti-inflammatory effects in SAP are associated with the microbiota metabolic immune axis. Specifically, GV-971 promotes the production of propionate and butyrate through remodeling the microbiota, such as *Faecalibacterium*, which inhibit macrophage M1 polarization and neutrophil infiltration by blocking the mitogen-activated protein kinase (MAPK) pathway. These findings highlight a promising application of GV-971 in treating lethal SAP.

#### Results

#### GV-971 protects against experimental SAP

To induce SAP in mice, a commonly used method involves administering caerulein plus lipopolysaccharide (LPS) stimulation. First, we aimed to assess the potential of GV-971 in reducing the severity of experimental SAP induced by caerulein/LPS in mice. Before SAP induction (Fig. 1a), we orally administered GV-971 to mice in three different dose groups (refer to the dosage used in our previous study<sup>25</sup>): low dose, middle-dose, and high dose for 1 week. SAP pathology involves the production of inflammatory cytokines, such as IL-8 (a neutrophil chemokine) and IL-6 (predominantly produced by macrophages)<sup>29</sup>. ELISA results indicate that GV-971 effectively inhibited the levels of these cytokines in both serum and pancreatic tissues of SAP mice (Fig. 1b, d).

Furthermore, morphological examinations of the pancreas confirmed the protective effect of GV-971, as it reduced acinar cell death, leukocyte infiltration, interstitial edema, and collagen content (as determined by Sirius staining) (Fig. 1c, d). Additionally, GV-971 reduced serological markers of SAP, specifically serum lipase and amylase activity (Fig. 1e). SAP often leads to complications such as acute kidney and lung injury, which significantly contribute to morbidity and mortality in critically ill septic patients<sup>30</sup>. GV-971 also demonstrated protective effects on kidney function, as indicated by reduced kidney damage and by serum creatinine levels in SAP model mice (Supplementary Fig. 1a–c). Similarly, GV-971 mitigated pulmonary damage in SAP model mice (Supplementary Fig. 1d, e).

Moreover, GV-971 exhibited protective effects against L-arginine–induced SAP in mice (Supplementary Fig. 2a). This protection was associated with decreased lipase and amylase activity in serum, reduced acinar cell death, decreased leukocyte infiltration, interstitial edema, and collagen content in the pancreas, as well as lower levels of IL-6 in the serum of SAP mice (Supplementary Fig. 2b–e). Notably, the oral administration of GV-971 at the highest dose did not affect food intake or body weight in mice (Supplementary Fig. 2f).

Collectively, these animal findings demonstrate that GV-971 administration effectively alleviates experimental SAP, mitigating associated systemic inflammatory reactions in mice while exhibiting low toxicity.

#### GV-971 suppresses innate immune cell infiltration during SAP

The dysregulated infiltration of innate immune cells, particularly macrophages and neutrophils, plays a crucial role in the development of SAP<sup>2</sup>. To investigate this, we measured the presence of neutrophils (using myeloperoxidase [MPO] staining) and macrophages (using

CD68 staining) in pancreatic tissue. Notably, the induction of SAP resulted in a significant increase in the infiltration of both macrophages and neutrophils within the pancreas (Fig. 1f, g and Supplementary 2g, h).

However, the administration of GV-971 resulted in a remarkable reduction in the infiltration of both macrophages and neutrophils (Fig. 1f, g and Supplementary 2g, h). Macrophages can differentiate into two distinct phenotypes, known as M1 and M2 macrophages, which have different effects on inflammation responses<sup>31-34</sup>. Immuno-histochemistry assays revealed that GV-971 treatment facilitated the transition of pro-inflammatory M1 macrophages (indicated by CD86 expression) toward anti-inflammatory M2 macrophages (indicated by CD206 expression; Fig. 1f, g and Supplementary Fig. 2g, h). These findings suggest that GV-971 has the potential to suppress the infiltration of innate immune cells in the pancreas during experimental SAP.

#### GV-971 remodels gut microbiota during SAP

Next, we examined the regulatory effects of high doses of GV-971 on the gut microbiota using Illumina MiSeq sequencing, specifically analyzing the V3-V4 variable region of bacterial 16S rRNA in cecal contents. Following stringent quality filtering, we identified a total of 4157 amplicon sequence variants (ASVs) from 24 samples, using a 100% sequence similarity threshold. Among these, 2419 ASVs were found in the control group, 1353 in the SAP group, and 1455 in the high dose + SAP group. The control group had 1999 unique ASVs, the SAP group had 644 unique ASVs, and the high dose + SAP group had 689 unique ASVs. Additionally, we discovered 116 ASVs present in the high dose + SAP group but not in the SAP group, while ASVs were present in the control group (Fig. 2a and Supplementary Data 1). Principal coordinate analysis based on weighted UniFrac distance revealed distinct clustering patterns in the microbiota composition among the control, SAP, and high dose + SAP groups (Fig. 2b and Supplementary Data 2). These patterns suggest significant alterations in gut microbiota composition induced by GV-971.

We also conducted inter-group comparisons of bacteria at the phylum and genus levels. At the phylum level, *Firmicutes* was the dominant phylum in the control group, followed by *Proteobacteria* and *Bacteroidetes*. In contrast, the SAP group showed an increased abundance of *Proteobacteria* as the predominant phylum, with a depletion of *Firmicutes* (Supplementary Fig. 3a and Supplementary Data 3). After 1 week of GV-971 pretreatment, high doses inhibited the reduction of *Firmicutes* and the increase of *Proteobacteria* in SAP mice, although these trends were not statistically significant (Supplementary Fig. 3b). Notably, GV-971 inhibited the increase of *Cyanobacteria* (p < 0.05) (Supplementary Fig. 3b). These findings suggest that the combination of caerulein and LPS treatment can shift the dominant taxa from *Firmicutes* to *Proteobacteria* while increasing *Cyanobacteria*, and that these changes tend to be reversed by GV-971.

At the genus level, GV-971 treatment induced alterations in the abundance of Melissococcus, Pseudomonas, Sphingomonas, Ralstonia, Faecalibacterium, and unidentified Streptophyta compared to the SAP group (Fig. 2c). To assess the relationship between changes in gut microbiota and pancreatic function indicators, we conducted correlation analyses between differential pancreatic function parameters and differential genera. We found that Faecalibacterium was negatively correlated with serum IL-6 level, while Melissococcus, Pseudomonas, Sphingomonas, Ralstonia, and unidentified\_Streptophyta exhibited positive correlations with serum IL-6 level (Fig. 2d and Supplementary Data 4). Correlation analysis of the differential bacteria revealed interactions between the probiotic Faecalibacterium and the five other classes of bacteria (Fig. 2e and Supplementary Data 5). Together, these data suggest that GV-971 prophylaxis can modulate gut microbial richness and community structure in SAP, notably increasing the levels of probiotic Faecalibacterium.





staining, Sirius staining, and IL-6 immunohistochemical staining (**c**), while bar plots illustrate pathological scores and associated quantification (**d**) in different groups (n = 8 samples). **e** Bar plots present the quantification of serum lipase and amylase in different groups (n = 8 samples). **f**, **g** Representative images show pancreatic immunohistochemical staining of MPO, CD68, CD86, and CD206 (**f**), and bar plots demonstrate associated quantification (**g**) in different groups (n = 8 samples). Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA with Dunnett's test for statistical significance. Scale bar, 100 µm. Source data are provided as a Source Data file. NS normal saline, LPS lipopolysaccharide, IL-8 interleukin-8, H&E hematoxylin-eosin, MPO myeloperoxidase, CD68 cluster of differentiation 68.



**Fig. 2** | **GV-971 modifies gut microbial richness and community structure in mice during SAP. a** Cecal contents were collected from various groups, including control mice (CON, n = 8 samples), mice with SAP (SAP, n = 8 samples), and SAP mice pre-treated with a high dose of GV-971 (HG + SAP, n = 8 samples). Analysis was conducted using the Illumina MiSeq platform. The Venn diagram and its corresponding bar plot are based on ASV levels in different groups. **b** Principal coordinate analysis based on ASV level in different groups (n = 8 samples). **c** Bar plots illustrating differential bacterial richness at the genus level in different groups (n = 8 samples). **d** A two-tailed Spearman

correlation heatmap showing the differential genera and their relationships with pancreatic function indicators between the SAP group and HG + SAP group. Positive correlations are denoted in red, while negative correlations are in blue. **e** The symbiotic network illustrating relationships among the differential genera between the SAP group and HG + SAP group. Data are presented as mean  $\pm$  SD. Statistical comparisons were conducted using one-way ANOVA with Dunnett's test for statistical significance. Source data are provided as a Source Data file. PCoA principal coordinate analysis, ASV amplicon sequence variant, Cor correlation coefficient.

## GV-971-affected intestinal microbiota transplantation prevents experimental SAP

To assess the potential impact of gut microbiota influenced by GV-971 in mitigating the severity of SAP, we conducted a series of experiments in mice. First, the mice underwent a 1-week course of antibiotic treatment to deplete their gut microbiota. Following this, they received intestinal microbiota transplantation (IMT) from corresponding donor groups for another week before caerulein/LPS was used to induce SAP in them (Fig. 3a).

The severity of SAP in mice pre-treated with GV-971-affected IMT was notably reduced in comparison to the control group. This reduction was evident through decreased serum IL-8 and pancreatic IL-6 levels (Fig. 3b–d). Histopathological examination and Sirius Red staining of the pancreas further affirmed the protective effect, characterized by reduced acinar cell death,

diminished leukocyte infiltration, decreased interstitial edema, and decreased collagen content (Fig. 3c, d). Additionally, we observed a decrease in MPO, CD68, and CD86, coupled with an increase in CD206 expression in the pancreas (Fig. 3e, f).

Moreover, mice receiving GV-971-affected IMT exhibited reduced serum lipase and amylase activity (Fig. 3g). Renal CD68 levels and tissue damage in SAP model mice were also attenuated by GV-971affected IMT (Supplementary Fig. 4a, b). GV-971-affected IMT also demonstrated protective effects on liver function, as indicated by serum ALT levels in SAP model mice (Supplementary Fig. 4c). Similarly, pulmonary damage and MPO levels were reduced in SAP model mice receiving GV-971-affected IMT (Supplementary Fig. 4d, e). Furthermore, GV-971-affected IMT led to an increase in the length of ileal villi and occludin levels in SAP model mice, indicating improved intestinal integrity (Supplementary Fig. 4f, g).



**Fig. 3** | **Effects of intestinal microbiota transplantation modulated by GV-971 on SAP severity in mice. a** Mice were pre-treated with antibiotics (ABX) in their drinking water for 7 days. Subsequently, they were pre-treated with or without cecal contents from the HG + SAP group for 7 days. Afterward, mice were induced with SAP by injecting caerulein (50 µg/kg/hour) for 11 times and LPS (10 mg/kg) once. Tissue samples were collected 24 hours later from the following groups: ABX + IMT (SAP) + SAP mice (n = 8 samples) and ABX + IMT (HG + SAP) + SAP mice (n = 8 samples), and these samples were analyzed using various techniques. Thirdparty elements were modified from the Scidraw under a CC BY 4.0 license. **b** Bar plots illustrating the quantification of serum cytokines IL-8 and IL-6 in different groups (n = 8 samples). **c**, **d** Representative images displaying pancreatic H&E staining, Sirius staining, and IL-6 immunohistochemical staining (**c**), along with bar plots showing pathological scores and associated quantification (**d**) in different groups (n = 8 samples). **e**, **f** Representative images show pancreatic immunohistochemical staining of MPO, CD68, CD86, and CD206 (**e**), and bar plots demonstrate associated quantification (**f**) in different groups (n = 8 samples). **g** Bar plots presenting the quantification of serum lipase and amylase in different groups (n = 8 samples). Data are presented as mean  $\pm$  SD. Statistical comparisons were performed using two-tailed unpaired Student's *t* test for statistical significance. Scale bar, 100 µm. Source data are provided as a Source Data file. ABX antibiotics, IMT intestinal microbiota transplantation, LPS lipopolysaccharide, IL-8 interleukin-8, H&E hematoxylin-eosin, MPO myeloperoxidase, CD68 cluster of differentiation 68.

## The depletion of gut microbiota diminishes GV-971-mediated protection against SAP

Next, we investigated whether the protective effects of GV-971 against SAP could be reversed by depleting gut microbiota using antibiotics (ABX). After a 1-week ABX treatment to deplete gut microbiota, mice were pre-treated with or without GV-971 for an additional week before induction of caerulein/LPS-induced SAP (Supplementary Fig. 5a). The depletion of gut microbiota by ABX abolished GV-971's ability to reduce serum lipase and amylase activity in SAP mice (Supplementary Fig. 5b). Furthermore, GV-971 did not mitigate acinar cell death, reduce leukocyte and macrophage infiltration, alleviate interstitial edema, or decrease collagen content in the pancreas of SAP mice following ABX treatment (Supplementary Fig. 5c–f). These results highlight that GV-971's efficacy in preventing SAP depends on the presence of gut microbiota.

#### GV-971 regulates microbiota metabolites during SAP

To elucidate the beneficial mechanisms of GV-971, we conducted targeted metabolic profiling of microbiota-associated metabolites within the cecal contents of the control, SAP, and high dose + SAP groups. A comprehensive analysis identified 172 metabolites, encompassing organic acids, fatty acids, bile acids, amino acids, phenylpropionate, benzene derivatives, indices, benzoic acids, short-chain fatty acids (SCFAs), carbohydrates, and other compounds (Fig. 4a and Supplementary Data 6). Principal component analysis demonstrated distinct clustering of metabolite compositions among the three groups, indicating that GV-971 induces alterations in metabolite composition (Fig. 4b and Supplementary Data 7).

We further examined the taxonomic similarity of microbiota metabolites at the metabolite class level to assess the overall structure of microbiota-related metabolites in different groups. GV-971 was found to increase the content of SCFAs, carbohydrates, and phenyl-propanoic acids while reducing the content of amino acids and fatty acids within the cecal environment of SAP mice (Fig. 4c, d, Supplementary Fig. 6a, and Supplementary Data 8). Given that the average relative abundance of SCFAs ranked highest among the five differential metabolites, SCFAs were identified as the primary differential metabolites and were speculated to play a central role in the preventive action of high doses against SAP. Consequently, our metabolite analysis would primarily focus on SCFAs.

Subsequently, we screened the metabolites within the three groups and identified 135 differential metabolites, including 5 types of SCFAs, based on predefined criteria for potential differential metabolites (Fig. 4e and Supplementary Data 9). Volcano plot analysis revealed that intestinal acetic acid and butvrate were diminished in the SAP group when compared to the control group (Fig. 4f and Supplementary Data 10). However, in the high dose + SAP group, intestinal acetic acid, propionate, and butyrate were elevated in comparison to the SAP group (Fig. 4g, Supplementary Fig. 6b, and Supplementary Data 11). To elucidate potential metabolic pathways through which GV-971 exerts its protective effects in SAP, we conducted pathway enrichment analysis. It revealed that the propanoate metabolism and butyrate metabolism pathways were significantly influenced (Fig. 4h and Supplementary Data 12). Notably, acetic acid, propionic acid, and butyric acid displayed negative associations with the levels of pancreatic inflammation markers (Fig. 4i and Supplementary Data 13).

#### Propionate and butyrate protect against experimental SAP

To investigate the direct effects of metabolites identified through metabolomics screening, namely acetate, propionate, and butyrate, we conducted experiments to validate their potential beneficial impact on SAP. This involved a two-step approach: first, mice underwent 1 week of gut microbiota depletion using antibiotics, followed by 1 week of administration of sodium acetate, sodium propionate, and sodium butyrate, and finally, SAP modeling induced by caerulein/ LPS (Fig. 5a).

Our results demonstrated that sodium propionate or sodium butyrate significantly reduced the levels of IL-6 and IL-8 in both serum and pancreatic tissues of SAP mice, indicating their beneficial effects. However, sodium acetate did not show a similar positive effect (Fig. 5b–d). Further examination of the pancreas confirmed the protective effect of sodium propionate and sodium butyrate. This was evidenced by reduced acinar cell death, diminished leukocyte infiltration, decreased interstitial edema, lower levels of MPO and CD68, reduced collagen content, and a shift towards CD206 expression with a concurrent decrease in CD86 (Fig. 5e, f). Additionally, sodium propionate and sodium butyrate reduced serum lipase activity, and sodium propionate reduced serum amylase activity (Fig. 5g). Moreover, compared to the model group without pretreatment, sodium propionate pretreatment reduced pancreatic weight in SAP mice (Fig. 5h).

Remarkably, sodium propionate and sodium butyrate also exhibited protective effects in other organs. They reduced kidney damage and CD68 levels, mitigated lung fibrosis, lowered MPO levels, and increased ileal villus length and occludin levels in SAP model mice (Supplementary Fig. 7a–f). These collective findings demonstrate that supplementation with sodium propionate and sodium butyrate can effectively alleviate SAP and related systemic inflammatory responses.

## GV-971 mitigates SAP-induced alterations in peripheral and intestinal immune systems

The gut microbiome and its metabolites are intricately linked to the functions of peripheral immune cells and the intestinal immune microenvironment. We evaluated the impact of GV-971 on the peripheral immune system through high-dimensional analysis using mass cytometry. We collected peripheral blood cells and labeled them with 22 antibodies targeting immune markers. Employing high-dimensional viSNE analysis, we visualized the data in two dimensions, generating viSNE maps to represent the expression of 10 markers on each cell (Fig. 6a). This facilitated the identification of 11 distinct cell populations based on their marker expression (Fig. 6b, c).

The analysis of peripheral blood immune cells unveiled significant alterations induced by SAP. Specifically, SAP induction led to a substantial increase in granulocytes and monocytes, accompanied by a decrease in B cells, dendritic cells, and all subsets of T cells (Fig. 6d, e and Supplementary Fig. 8a). Notably, pretreatment with high doses mitigated the SAP-induced increase in granulocytes and reversed the SAP-induced decrease in B- and T-cell subsets. Additionally, GV-971 pretreatment exhibited a trend towards increased percentages of macrophages (Fig. 6e).

To delve deeper into the analysis of T-cell subsets, we employed viSNE analysis using 8 markers to identify 11 distinct subsets (Fig. 6f, g and Supplementary Fig. 8b). The SAP model displayed a decrease in regulatory T cells, while high-dose administration increased their percentages (Fig. 6h). Moreover, high-dose pretreatment increased the percentages of resting non-Th1/17 CD44<sup>+</sup>CD4 T cells and Ly6C<sup>+/-</sup>CD44<sup>+</sup>CD8 T cells following SAP induction. However, it had no effect on the SAP-induced decrease in Th1, Th17, non-Th1/17 CD44<sup>-</sup>CD4 T-cell and Ly6C<sup>+/-</sup>CD44<sup>-</sup>CD8 T-cell subsets (Fig. 6h and Supplementary Fig. 8c).

We also explored the impact of GV-971 on the intestinal immune microenvironment using mass cytometry analysis with the viSNE algorithm (Supplementary Fig. 9a-c). In the SAP model, we observed an increase in monocytes and granulocytes, while other cell types, including T-cell subsets, resting CD11b<sup>+</sup> cells, and dendritic cells, decreased in proportion (Supplementary Fig. 9d, e). GV-971 pretreatment further elevated monocytes in the SAP model, along with a significant increase in double-negative T cells (Supplementary Fig. 9d). Neither SAP induction nor GV-971 treatment induced significant changes in macrophage proportions in the intestinal tissue (Supplementary Fig. 9f). However, GV-971 administration increased the percentage of M2 macrophages in the SAP model (Supplementary Fig. 9g). Furthermore, the SAP model displayed compromised intestinal integrity, characterized by reduced villus length and occludin levels in the intestinal tissue. GV-971 treatment effectively reversed these detrimental effects, restoring both villus length and occludin levels (Supplementary Fig. 9h).

Taken together, these findings confirm the extensive role of GV-971-mediated gut microbiome remodeling in shaping both peripheral and intestinal immune systems during SAP.



**Fig. 4** | **Effects of acetate, propionate, and butyrate on anti-SAP effect of GV-971. a** Cecal contents were collected from different groups, including control mice (CON, n = 8 samples), mice with SAP (SAP, n = 8 samples), and SAP mice pre-treated with a high dose of GV-971 (HG + SAP, n = 8 samples), and analyzed using UPLC-MS/ MS. A category statistics pie chart showing the composition ratio of the identified metabolites and the number of metabolites in each category in different groups. **b** Results of a partial least squares discriminant analysis model based on the metabolic profiles of cecal content in different groups (n = 8 samples). **c** A percentage chart illustrating the relative abundance of metabolite classes in different groups. **d** A bar plot displaying the relative abundance of SCFAs in different groups (n = 8 samples). **e** A heatmap showing potential differential metabolites in different

groups (n = 8 samples). **f**, **g** Volcano plots depicting 172 metabolites between the CON group and SAP group (**f**), and between the SAP group and HG + SAP group (**g**). **h** Pathway analysis based on the mmu set using differential metabolites between the SAP group and HG + SAP group. **i** A two-tailed Spearman correlation heatmap demonstrating the correlation between differential metabolites and differential pancreatic function parameters between the SAP group and HG + SAP group, with positive correlations in red and negative correlations in blue. Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA with Dunnett's test for statistical significance. Source data are provided as a Source Data file. SCFAs short-chain fatty acids, Cor correlation coefficient.

## GV-971-mediated macrophage polarization is required for the prevention of SAP

Having demonstrated that GV-971 administration leads to macrophage M2 polarization in an anti-inflammatory state, our next step was to determine whether depleting macrophages using clodronate liposomes could reverse the protective effects of GV-971 in SAP. Mice were pre-treated with or without GV-971 for 7 days, and macrophages were

depleted through intraperitoneal (IP) injections of clodronate liposomes or control PBS liposomes on the sixth day, followed by caerulein/LPS-induced SAP (Fig. 7a). When macrophages were depleted, GV-971 was unable to reduce lipase and amylase activity in the serum of SAP mice (Fig. 7b). GV-971 was also unable to decrease acinar cell mortality, lower leukocyte infiltration, reduce macrophage infiltration, alleviate interstitial edema, and diminish collagen content in the



pancreas of mice with SAP (Fig. 7c-f). This supports the notion that GV-971 relies on macrophages to prevent SAP.

To gain further insights into the molecular mechanisms underlying GV-971-mediated macrophage M2 polarization, we focused on the role of propionate and butyrate in macrophage polarization. Initially, we assessed their impact on  $H_2O_2$ -induced cell death, which models caerulein-induced death of pancreatic acinar cells in vivo<sup>30</sup>. Notably, sodium propionate and sodium butyrate did not directly inhibit cell death in mouse pancreatic acinar cell line MPC-83 (Supplementary Fig. 10a), suggesting that they do not interfere with DAMP release from acinar cells to affect macrophage polarization. Additionally, sodium propionate and sodium butyrate had no significant effects on RAW 264.7 macrophage cell viability in the absence or presence of LPS (Supplementary Fig. 10b–d). However, sodium propionate and sodium butyrate did reduce macrophage polarization induced by LPS, as evidenced by a decreased proportion of M1 macrophages (CD86<sup>+</sup>), an increased proportion of M2 macrophages (CD206<sup>+</sup>), and reduced secretion of IL-6, TNF- $\alpha$ , and IL-8 in the cell culture medium (Fig. 8a–f).

Then, RNA sequencing (RNA-seq) was used to assess the impact of propionate and butyrate on the transcriptome. Principal component analysis revealed distinct clustering of RNA compositions across the

Fig. 5   Reduction of SAP severity by propionate and butyrate. a Mice were pre-
treated with antibiotics (ABX) in their drinking water for 7 days. They were then pre-
treated with or without sodium acetate (SA, 400 mg/kg/day), sodium propionate
(SP, 400 mg/kg/day), and sodium butyrate (SB, 400 mg/kg/day) for 7 days. Finally,
SAP was induced by injecting caerulein (50 µg/kg/hour) for 11 times and LPS
(10~mg/kg) once. Tissue samples were collected 24 hours later from the following
groups: ABX + SAP mice ( $n = 11$ samples), ABX + SA + SAP mice ( $n = 8$ samples),
ABX + SP + SAP mice (n = 8 samples), and ABX + SB + SAP mice (n = 8 samples), and
these samples were analyzed using various techniques. Third-party elements were
modified from the Scidraw under a CC BY 4.0 license. <b>b</b> Bar plots illustrating the
quantification of serum cytokines IL-8 and IL-6 in different groups (ABX + SAP,
n = 11 samples; ABX + SA + SAP, $n = 7$ samples; ABX + SP + SAP, $n = 8$ samples;
ABX + SB + SAP, $n = 8$ samples). <b>c</b> , <b>d</b> Representative images displaying pancreatic
H&E staining, Sirius staining, and IL-6 immunohistochemical staining ( $\mathbf{c}$ ), along with
bar plots showing pathological scores and associated quantification $(\mathbf{d})$ in different

four experimental groups, indicating that propionate and butyrate induce significant alterations in RNA profiles (Supplementary Fig. 11a). Volcano plot analysis identified 1641 upregulated genes and 1504 downregulated genes in the LPS group compared to the CON group, 2239 upregulated genes and 1057 downregulated genes in the SP + LPS group compared to the LPS group, and 1964 upregulated genes and 666 downregulated genes in the SB + LPS group compared to the LPS group (Supplementary Fig. 11b and Supplementary Data 14). Kyoto Encyclopedia of Genes and Genomes enrichment analysis highlighted that propionate and butyrate primarily influenced MAPK signaling pathway, with a significant number of genes involved (Supplementary Fig. 11c). Given that macrophage M1 polarization is mediated through the MAPK pathway in response to LPS<sup>35</sup>, propionate and butyrate likely exert their effects through modulation of the MAPK pathway. At the molecular level, western blot analysis revealed that sodium propionate and sodium butyrate effectively inhibited the phosphorylation of JNK, ERK1/2, and p38 in the MAPK signaling pathway of macrophages (Fig. 8g, h). The inhibitory effects of sodium propionate and sodium butvrate on macrophage polarization were prevented by the p38 MAPK agonist U46619, a known antagonist of their anti-inflammatory actions (Fig. 8i-1)<sup>36</sup>.

#### Discussion

In this study, we have demonstrated an application for GV-971 in protecting against lethal pancreatic inflammation in two distinct SAP mouse models. The mechanism of action for GV-971 involves the promotion of intestinal probiotics (such as *Faecalibacterium*), resulting in subsequent anti-inflammatory microbiota metabolites (such as propionate and butyrate) that inhibit macrophage M1 polarization (Fig. 9). These findings not only enhance our understanding of the signals within the microbiota metabolic immune axis but also highlight the potential of microbiome therapeutic-related drugs in treating lethal inflammatory diseases, such as SAP.

Gut microbiota dysbiosis plays a pivotal role in the development of SAP, contributing to the activation of both innate and adaptive immunity, as well as impairing intestinal barrier function<sup>37,38</sup>. Prebiotics, which are selectively digested by gut microbiota and influence the composition of the intestinal flora by promoting biodiversity and beneficial bacteria, have been considered as potential treatments for SAP<sup>22,24,39-44</sup>. For example, pretreatment with COS, a type of oligosaccharide found in prebiotics, has been shown to attenuate experimental SAP. Similarly, GV-971, also an oligosaccharide, possesses anti-inflammatory properties attributed to its impact on gut bacteria and their metabolic products. A previous study has demonstrated that GV-971 can restore disrupted gut microbiota, reduce phenylalanine and isoleucine concentrations in blood and feces, and mitigate neuroinflammation linked to Th1 responses in the brain<sup>25</sup>. Our study suggests that GV-971 plays a distinct role in remodeling the gut microbiota and metabolism in the groups (ABX + SAP, n = 11 samples; ABX + SA + SAP, n = 8 samples; ABX + SP + SAP, n = 8 samples; ABX + SB + SAP, n = 8 samples). **e**, **f** Representative images show pancreatic immunohistochemical staining of MPO, CD68, CD86, and CD206 (**e**), and bar plots demonstrate associated quantification (**f**) in different groups (ABX + SAP, n = 11 samples; ABX + SA + SAP, n = 8 samples; ABX + SP + SAP, n = 8 samples; ABX + SB + SAP, n = 8 samples; ABX + SP + SAP, n = 8 samples; ABX + SB + SAP, n = 8 samples). **g** Bar plots presenting the quantification of serum lipase and amylase in different groups (ABX + SAP, n = 11 samples; ABX + SP + SAP, n = 8 samples). **g** Bar plots presenting the quantification of serum lipase and amylase in different groups (ABX + SAP, n = 11 samples; ABX + SP + SAP, n = 8 samples). **h** A bar plot indicating pancreatic weight differences in different groups (ABX + SAP, n = 11 samples; ABX + SA + SAP, n = 8 samples; ABX + SP + SAP

SAP model, implying that GV-971 may exhibit varying effects on gut homeostasis under different disease conditions.

Proteobacteria, implicated in SAP pathogenesis due to its correlation with inflammation<sup>42</sup>, was found to be increased significantly (64.83 times more than in control mice), while Firmicutes decreased significantly (to 19.2% of that in control mice) in SAP mice. GV-971 pretreatment showed trends of reversing these changes but not statistically significantly, while it significantly reduced Cyanobacteria in SAP mice, suggesting GV-971's selective role in regulating intestinal microbiota. Faecalibacterium, a beneficial bacterium in Firmicutes, is known to reduce intestinal inflammation and improve intestinal permeability<sup>43</sup>. GV-971 pretreatment increased Faecalibacterium, which correlated with reduced inflammation in the intestine and pancreas. The gut microbiota's role in metabolic functions is increasingly recognized, and the introduction of specific probiotics like Faecalibacterium into the intestine can produce SCFAs, including butyrate and propionate<sup>44-47</sup>. Our study emphasizes the importance of SCFAs, as they rank highest in average relative abundance among the differential metabolites, suggesting their potential significance in GV-971's SAP prevention. However, our results diverge from previous studies<sup>25</sup>. possibly due to variations in animal models used. Further experiments confirmed that GV-971-mediated IMT reduced acute inflammation in SAP mice, while depleting gut microbiota with ABX reversed GV-971's protective effects in SAP. Overall, GV-971 affected the gut microbiota, alleviating the acute inflammatory phenotype in SAP mice, with Faecalibacterium potentially playing a key role. However, future research needs to investigate how GV-971 regulates the gut microbiota, specifically Faecalibacterium. Whether Faecalibacterium directly uptakes and utilizes GV-971 remains to be studied.

Butyrate, a specific SCFA, is of great interest due to its consistent association with the gut microbiota and its role in SAP<sup>2</sup>. Firmicutes, including genera such as Faecacterium, and Bifidobacterium, are primary contributors to butyrate production<sup>48,49</sup>. In SAP-afflicted mice, there is a decrease in intestinal butyrate levels. Oral or IP administration of butyrate shows promise in reducing SAP-related mortality<sup>42</sup>. Butyrate helps maintain the intestinal barrier's integrity, inhibits the inflammatory marker IL-6, and effectively mitigates caerulein-induced acute pancreatitis and associated intestinal damage<sup>2,50,51</sup>. Propionate can also have some anti-inflammatory effects<sup>52</sup>. However, the effect of propionate during SAP is unknown. In the context of GV-971 treatment, our metabolite analysis showed significant increases in acetate (p = 0.007), propionate (p = 0.001), and butyrate (p = 0.003), all SCFAs. Subsequent experiments confirm that propionate and butyrate reduce the acute inflammatory phenotype in SAP-afflicted mice. These results suggest that propionate and butyrate produced by Faecalibacterium mediate GV-971's therapeutic effects on SAP.

Furthermore, in our search for downstream targets of propionate and butyrate, we observed that GV-971 promotes macrophage M2



polarization and inhibits neutrophil infiltration. Furthermore, propionate and butyrate do not directly act on pancreatic acinar cells. Instead, they have a direct inhibitory effect on neutrophils, suppressing their chemotaxis<sup>53</sup>. Our study also confirmed that propionate and butyrate can effectively suppress macrophage M1 polarization. Macrophage M1 polarization is regulated by various signaling pathways, including the MAPK and NF-κB pathways<sup>35,54,55</sup>. Our RNA-seq and western blot analysis results suggest that propionate and butyrate can inhibit macrophage M1 polarization via the MAPK signaling pathway.

While the anti-inflammatory effects of SCFAs are wellestablished<sup>56</sup>, our research uncovers GV-971's role in modulating gut microbiota to enhance SCFA production for SAP protection. Our study suggests GV-971, approved for Alzheimer's disease, as a potential treatment for SAP. SCFAs themselves could also serve as therapeutic Fig. 6 | GV-971 improves SAP-induced dysregulation in peripheral blood.

**a** Peripheral blood samples were collected from different groups, including control mice (CON, n = 8 samples), mice with SAP (SAP, n = 7 samples), and SAP mice pretreated with a high dose of GV-971 (HG + SAP, n = 8 samples), and were analyzed using mass cytometry. The viSNE maps are colored by the normalized expression of indicated surface markers in peripheral blood (PB) CD45<sup>+</sup> cells. **b** A heatmap displaying the normalized expression of 10 indicated markers in 10 PB cell populations. **c** These viSNE maps are colored by 11 main cell populations in PB after clustering. **d**, **e** Bar plots showing the frequencies of the indicated populations in PB CD45<sup>+</sup> cells of mice in different groups (CON, n = 8 samples; SAP, n = 7 samples;

agents in SAP. However, the lack of overlap in function between GV-971 and SCFAs remains unclear and warrants further investigation.

While patients in the acute phase of SAP traditionally undergo extended fasting periods, clinical practice continues to incorporate oral medications such as the purgative agent magnesium sulfate<sup>57</sup>. Clinical studies highlight GV-971's efficacy in attenuating inflammation in Alzheimer's disease, suggesting its potential as a valuable adjunct therapy alongside conventional treatments for SAP patients. Our animal studies have demonstrated significant protective effects against SAP development; however, further research and clinical trials are necessary to fully elucidate its utility and integration into SAP treatment regimens. Given the broad role of gut microbiota in various inflammatory diseases, GV-971 is anticipated to have wide-ranging applications in treating various inflammatory conditions beyond Alzheimer's disease and SAP, as demonstrated in this study.

In summary, we have unraveled the mechanism underlying GV-971's protective effect in SAP through a comprehensive analysis of microbial, metabolic, and immune cell profiles. Our investigation highlights the promising therapeutic potential of targeting the microbiota metabolic immune axis for the treatment of SAP. These findings signify an approach that could hold significant promise in SAP therapy.

### Methods

#### Animals

Male C57BL/6 mice aged 6–8 weeks were procured from Charles River (Beijing, China). These mice were housed in a specific pathogen-free animal facility maintained at a temperature of  $23 \pm 2^{\circ}$ C with relative humidity ranging from 44% to 52% and subjected to a 12-hour light-dark cycle. The mice were provided unrestricted access to both food (Regular Chow Diets; 05073, Guangdong Medical Laboratory Animal Center) and water throughout the study. To ensure unbiased group allocation, the animals were randomly assigned to different experimental groups based on their body weight. Each cage accommodated no more than 5 mice. All animal experiments have been approved by the Animal Ethics Committee of Guangzhou Medical University (Approval number: GY2022-212).

For the caerulein and LPS-induced SAP model, the mice received IP injections of caerulein (50 µg/kg; D&B, Shanghai, China) in normal saline (NS) every hour for 10 hours. Immediately following the last caerulein injection, a single injection of LPS (10 mg/kg; Sigma-Aldrich, Saint Louis, MO, USA) was administered. Control mice received NS only, as this represents the standard SAP model<sup>37,38</sup>. Prior to the injections of caerulein and LPS, the mice were orally gavaged with GV-971 at low (100 mg/kg/day, LG), middle (200 mg/kg/day, MG), and high (400 mg/kg/day, HG) doses (GreenValley, Shanghai, China) dissolved in water for 7 days. On the eighth day, caerulein and LPS were administered to the mice. For murine sample collections 24 hours after the first caerulein injection, euthanasia was performed through gaseous anesthesia with isoflurane, followed by cervical dislocation, and the euthanasia protocol complied with the Guideline for the Care and Use of Laboratory Animals by the Animal Ethics Committee of Guangzhou Medical University. Samples that included the pancreas, peripheral HG + SAP, n = 8 samples). **f** A heatmap displaying the normalized expression of eight indicated markers in 11 PB T-cell populations. **g** These viSNE maps are colored by 11 T-cell subpopulations in all PB T cells after clustering. **h** Bar plots showing the frequencies of the indicated T-cell subpopulations in PB CD45<sup>+</sup> cells of mice in different groups (CON, n = 8 samples; SAP, n = 7 samples; HG + SAP, n = 8 samples). Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA with Dunnett's test for statistical significance. Source data are provided as a Source Data file. DNT double-negative T cell, TCR T-cell receptor, Gran granulocyte, Mono monocytes, DC dendritic cell, r- rest of, Treg regulatory T cell, Th1 T helper 1 cell.

blood, lungs, kidneys, cecum, and ileum were collected for enzyme and cytokine assays, histopathology, microbiome analysis, metabolomics, and mass cytometry analysis.

In the case of L-arginine–induced SAP, the mice received 2 hourly IP injections of L-arginine hydrochloride (4 g/kg; Macklin, Shanghai, China), while control mice were given NS<sup>58,59</sup>. Similar to the previous model, prior to L-arginine injections, the mice received oral gavages of the middle dose and high dose dissolved in water for 7 days. On the eighth day, L-arginine was administered, and euthanasia was performed at 72 hours after the first L-arginine injection. Samples, including the pancreas and peripheral blood, were collected for enzyme assays, cytokine assays, and histopathological examination.

#### 16S rDNA profiling of gut microbiota in mice

At the end of the experiment, cecal contents were as eptically collected from the mice, flash-frozen in liquid nitrogen, and subsequently stored at -80 °C. These samples, carefully packaged with dry ice, were dispatched to the laboratory of Metabo-Profile Biotechnology Co., Ltd. (Shanghai, China) for analysis.

Total DNA extraction from the cecal content samples was carried out using a QIAamp Rapid DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommended protocol. Following DNA concentration and integrity assessments, the 16S rRNA V3-V4 variable region (338 F: 5'-ACTCCTACGGGAGGCAGCAG-3': 806 R: 5'-ACTCC-TACGGGAGGCAGCAG-3') underwent PCR amplification to generate the amplicon sequencing library<sup>60</sup>. Subsequently, the qualified library underwent paired-end sequencing on an Illumina MiSeq platform, in accordance with the manufacturer's instructions. Microbiome bioinformatics was performed with QIIME 2 2019.4 with slight modifications according to the official tutorials (https://docs.qiime2.org/2019.4/ tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin. Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin. Non-singleton ASVs were aligned with mafft and used to construct a phylogeny with fasttree2.

#### Intestinal microbiota transplantation

For the purpose of IMT, we obtained cecal contents from terminal mice under sterile conditions. These contents were then resuspended in pre-chilled PBS at a concentration of 100 mg/mL and filtered through a sterile  $70 \,\mu\text{m}$  filter. Before storage at  $-80 \,^{\circ}\text{C}$ , we added glycerol (20%) as a preservative.

To normalize the gut microbiota, we dissolved ampicillin (0.25 mg/mL; MB1507, Meilunbio, Dalian, China), metronidazole (0.25 mg/mL; MB2200, Meilunbio), neomycin (0.25 mg/mL; MB1716, Meilunbio), and vancomycin (0.125 mg/mL; MB1260, Meilunbio) in high-pressure water to create an ABX mixture, as previously described<sup>61</sup>. The mice received ABX in their drinking water for a period of 1 week, followed by oral gavage of a bacterial suspension ( $10 \mu L/g$ ) at 1-day intervals for an additional week.

Subsequently, on the fifteenth day, SAP was induced in the mice through the administration of caerulein (50  $\mu$ g/kg/hour) 11 times and a single dose of LPS (10 mg/kg). Euthanasia was performed, and relevant samples were collected for testing 24 hours after SAP induction.



**Fig. 7** | **GV-971 relies on macrophages for the prevention of SAP. a** Mice were pretreated with or without HG for 7 days. On the sixth day, macrophages were depleted through intraperitoneal injections of PL (PBS liposomes) or CL (clodronate liposomes). Subsequently, mice were induced with SAP by injecting caerulein (50 µg/ kg/hour) for 11 times and LPS (10 mg/kg) once on the eighth day. After 24 hours, tissues were collected from mice in four groups (SAP, n = 8 samples; HG + SAP, n = 11 samples; CL + SAP, n = 7 samples; HG + CL + SAP, n = 7 samples) and analyzed using various techniques. Third-party elements were modified from the Scidraw under a CC BY 4.0 license. **b** Bar plots showing the quantification of serum lipase and amylase in different groups (SAP, n = 8 samples; HG + SAP, n = 11 samples; CL + SAP, n = 7 samples; HG + CL + SAP, n = 7 samples). **c**, **d** Representative images displaying pancreatic H&E staining, Sirius staining, and IL-6 immunohistochemical staining (c), along with bar plots showing pathological scores and associated quantification (d) in different groups (SAP, n = 8 samples; HG + SAP, n = 11 samples; CL + SAP, n = 7 samples; HG + CL + SAP, n = 7 samples). e, f Representative images show pancreatic immunohistochemical staining of MPO, CD68, and CD206 (e), and bar plots demonstrate associated quantification (f) in different groups (SAP, n = 8 samples; HG + SAP, n = 11 samples; CL + SAP, n = 7 samples; HG + SAP, n = 11 samples; CL + SAP, n = 7 samples; HG + CL + SAP, n = 7 samples). Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA with Dunnett's test for statistical significance. Scale bar, 100 µm. Source data are provided as a Source Data file. LPS lipopoly-saccharide, H&E hematoxylin-eosin, IL-6 interleukin-6, MPO myeloperoxidase, CD68 cluster of differentiation 68.



#### Gut microbiota depletion

After a 1-week period of ABX treatment to deplete the gut microbiota, mice were subsequently orally administered high-dose GV-971 for an additional week.

On the 15 day, SAP was induced in the mice through injections of caerulein (50  $\mu$ g/kg/hour) for 11 times and LPS (10 mg/kg) once. Euthanasia was performed, and relevant samples were collected from

mice in the two groups 24 hours after SAP induction and subjected to the analysis of serum lipase and amylase and immunohistochemical staining.

#### MicrobioMET

At the end of the experiment, we collected samples of cecal contents under sterile conditions, which were promptly frozen in liquid **Fig. 8** | **Propionate and butyrate inhibit MAPK signaling pathway in macrophages. a**–**d** RAW 264.7 cells were pre-treated with or without sodium propionate (SP) at concentrations of 1.6 mM, 3.2 mM, and 6.4 mM, and sodium butyrate (SB) at concentrations of 0.2 mM, 0.4 mM, and 0.8 mM for 24 hours. Subsequently, the corresponding metabolites were combined with 1 µg/mL LPS and used to treat the cells for an additional 6 hours. The RAW 264.7 cells were then collected for western blotting and immunofluorescent staining, and the supernatants were collected for enzyme-linked immunosorbent assay (ELISA). Representative images showing immunofluorescence staining of CD86<sup>+</sup> cells and CD206<sup>+</sup> cells in RAW 264.7 cells (**a**, **c**), with bar plots displaying their quantitative analysis (**b**, **d**) in different groups (*n* = 3 independent experiments). **e**, **f** Bar plots showing the levels of IL-6, TNF- $\alpha$ , and IL-8 in the supernatant of RAW 264.7 medium determined by ELISA in different groups (*n* = 3 independent experiments). **g**, **h** Representative images showing the

relative levels of p-JNK, p-ERK1/2, p-p38, and p-p65 in RAW 264.7 cells determined by western blotting in different groups (n = 3 independent experiments). i–I RAW 264.7 cells were pre-treated with SP (6.4 mM), SB (0.8 mM), and U46619 (10  $\mu$ M, a p38 MAPK agonist) for 24 hours, and then were combined with 1  $\mu$ g/mL LPS and used to treat the cells for another 6 hours. Representative images show immunofluorescence staining of CD86<sup>+</sup> cells and CD206<sup>+</sup> cells in RAW 264.7 cells (i, k), with bar plots displaying their quantitative analysis (j, l) in different groups (n = 3independent experiments). Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA with Dunnett's test for statistical significance. Scale bar, 50  $\mu$ m. Source data are provided as a Source Data file. LPS lipopolysaccharide, CD86 cluster of differentiation 86, DAPI 4',6-diamidino-2phenylindole, IL-6 Interleukin-6, TNF- $\alpha$  tumor necrosis factor- $\alpha$ , p- phospho-, GAPDH glyceraldehyde-3-phosphate dehydrogenase.



**Fig. 9** | **Schematic diagram of the protective effects of GV-971 on SAP.** In response to caerulein and LPS injections, a reduction in intestinal *Faecalibacterium* led to decreased levels of the essential metabolites propionate and butyrate. These injections further triggered an inflammatory cascade, characterized by heightened macrophage polarization and increased neutrophil migration into the pancreas. This cascade culminated in severe acute pancreatic inflammation and a concurrent systemic inflammatory response. The administration of GV-971 via an oral route acted as a therapeutic intervention. It effectively restored the balance of gut

microbiota by promoting the growth of intestinal *Faecalibacterium*. As a result, levels of propionate and butyrate were increased. This metabolic shift played a pivotal role in dampening macrophage polarization and reducing neutrophil migration within the pancreas. Consequently, pancreatic inflammation was resolved, ultimately leading to the amelioration of the systemic inflammatory response. Third-party elements were modified from the Scidraw under a CC BY 4.0 license. LPS lipopolysaccharide, p-MAPK phospho-mitogen-activated protein kinase.

nitrogen and stored at -80 °C. These cecal content samples, carefully packaged with dry ice, were then dispatched to the laboratory of Metabo-Profile Biotechnology Co., Ltd. (Shanghai, China) for subsequent analysis.

A metabolomics analysis, as previously reported, was conducted on these cecal content samples<sup>62</sup>. First, the samples underwent homogenization and centrifugation, and the resulting supernatant was pooled. We employed an MPS2 multifunctional robot (Gerstel, Demilheim, NRW, Germany) for automated sample extraction and separation. Quantitative analysis of microbial metabolites was carried out using UPLC-MS/MS (Acquity UPLC, Xevo TQ-S, USA).

201 standard substances from different chemical categories were prepared as previously reported<sup>62</sup>. Retention solutions were meticulously prepared for each compound of microbial metabolites. These solutions were based on methanol, ultrapure water, or sodium hydroxide solution. To ensure data quality and compensate for potential matrix effects, internal standards were incorporated. Subsequently, the raw data files generated by UPLC-MS/MS were processed using TMBQ software (Shanghai, China). This software facilitated the integration, calibration, and quantification of each metabolite.

For comprehensive analysis, various statistical techniques were employed, including principal component analysis, PLS-DA, univariate analysis, and pathway analysis. These analyses were performed using a self-developed iMAP platform (v1.0, Metabo-Profile, Shanghai, China).

#### Treatment of the metabolites

To prepare the metabolite solution, we dissolved sodium acetate (SA; 241245, Sigma-Aldrich, Saint Louis, MO, USA), sodium propionate (SP; P1880, Sigma-Aldrich), and sodium butyrate (SB; C26319, Alfa Aesar, Waltham, MA, USA) in water. After a 1-week period of ABX treatment, mice were subsequently orally administered sodium acetate, sodium propionate, and sodium butyrate at a dose of 400 mg/kg/day for an additional week.

On the 15 day, SAP was induced in the mice through 11 hourly injections of caerulein ( $50 \mu g/kg/hour$ ) and a single dose of LPS (10 mg/kg). Euthanasia was performed and relevant samples were collected for analysis at 24 hours after SAP induction.

#### Macrophage depletion

Mice were pre-treated with high-dose GV-971 for 7 days. On the sixth day, macrophages were either depleted by intraperitoneal injections of clodronate liposomes (CL, CP-005-005, Liposoma, Holland) or received PBS liposomes (PL, CP-005-005, Liposoma). On the eighth day, SAP was induced in the mice through 11 hourly injections of caerulein ( $50 \mu g/kg/hour$ ) and a single dose of LPS (10 mg/kg). Euthanasia was performed, and relevant samples were collected from mice in the four groups 24 hours after SAP induction and subjected to the analysis of serum lipase and amylase and immunohistochemical staining.

#### Determination of serum biochemical indexes and cytokines

After peripheral blood collection, the blood was centrifuged at room temperature at  $2348 \times g$  for 20 minutes, and the resulting supernatants were collected for analysis. The activities of serum amylase (C016-1) and lipase (A054-2) were determined using commercial kits from Jiancheng Biotech (Nanjing, China) following the manufacturer's protocol. Serum creatinine (K131) and alanine aminotransferase (ALT, K114) levels were measured using commercial kits from KHB (Shanghai, China). Concentrations of serum cytokines IL-6 (EMC004), IL-8 (EMC104), and TNF- $\alpha$  (EMC102a) were determined in accordance with instructions provided in a commercial kit from Neobioscience (Shenzhen, China).

#### Histopathological analysis and immunohistochemistry

Fresh pancreas, kidney, lung, and ileum tissues were initially fixed overnight in 4% paraformaldehyde. They were subsequently rinsed in tap water for 2 hours, followed by dehydration in an ethanol gradient. Afterward, the tissues were embedded in paraffin and cut into 5-µm sections. These sections underwent dewaxing in xylene, rehydration through an ethanol series, and staining with H&E and Sirius red dyes. Pancreatic damage was assessed by a pathologist in a blinded manner according to previously described criteria<sup>63</sup>. Briefly, the evaluation of pancreatic pathology included four categories: edema, inflammatory cell infiltration, necrosis, and vacuolization. In the immunohistochemical staining, various antibodies, including anti-MPO, anti-CD68, anti-CD86, anti-CD206, and anti-IL-6, were employed to assess mouse pancreatic neutrophil, total macrophages, M1 macrophages, M2 macrophage infiltration, as well as lung neutrophil and renal total macrophage infiltration. After blocking endogenous peroxidase activity with 3% H<sub>2</sub>O<sub>2</sub> for 8 minutes, the cells were incubated overnight with diluted anti-MPO antibody (1:500; ab9535, Abcam, Cambridge, England), anti-CD68 antibody (1:500; GB113109, Servicebio, Wuhan, China), anti-CD86 antibody (1:500; 13395-1-AP, Proteintech, Wuhan, China), anti-CD206 antibody (1:500; GB113497, Servicebio), and anti-IL-6 antibody (1:500; GB11117, Servicebio). Subsequently, each section was incubated with an HRPlabeled secondary antibody, developed with a 3,3-diaminobenzidine solution, and counterstained with hematoxylin, followed by dehydration. All slides were observed and captured under a microscope (Leica CS2, Wetzlar, Germany), and at least six fields for each slide

#### Cell suspension preparation

Peripheral blood was collected from the heart following the administration of pentobarbital sodium. Intestinal tissues were collected from the cecum, and the cell suspension was prepared following established protocols<sup>64</sup>.

All cell suspensions were filtered through a 70  $\mu$ m cell strainer and subsequently fixed with Fix I buffer (Fluidigm, South San Francisco, CA, USA) for 10 minutes at room temperature. After washing, a red blood cell lysis buffer was used to remove erythrocytes. Cell pellets were then resuspended in cell staining buffer supplemented with 10% dimethyl sulfoxide and stored at -80 °C.

#### Mass cytometry analysis

In total,  $0.5 \times 10^6$  peripheral white blood cells and intestinal cells from mice were individually barcoded using a Cell-ID 20-Plex Pd Barcoding Kit (Standard BioTools) for 30 minutes at room temperature. After thorough washing, 20 samples, each with a distinct barcode, were pooled together, while the remaining barcoded samples were pooled separately. To reduce nonspecific antibody binding, we added antimouse CD16/32 antibody (FcR III/II, Biolegend, CA, USA). These cells were subsequently incubated with a mixture of metal isotope-conjugated antibodies (see Table S1) for 30 minutes at room temperature. Following another wash, the cells were stained with antimouse Foxp3 and anti-mouse RORgt antibodies using a Nuclear Antigen Staining Buffer Set (Standard BioTools) for 45 minutes at room temperature.

To visualize the cell nucleus, Cell-ID Intercalator-Ir (Fluidigm) was applied and incubated overnight at 4 °C. After further washing with staining buffer and double-distilled water, the cells were resuspended in ultrapure water supplemented with 10% EQ Four Element Calibration Beads (Fluidigm). Subsequently, these samples were analyzed using a Helios mass cytometer (Fluidigm).

Mass cytometry data underwent randomization, normalization, and de-barcoding using CyTOF 6.7 software (Fluidigm). These data were uploaded to Cytobank.cn for further analysis. Prior to highdimensional analysis utilizing the viSNE algorithm, all cell debris and doublets were excluded.

#### Cell culture and treatment

Mouse pancreatic acinar cell line (MPC-83) was obtained from Huofei Biology (HF-0087, Wuhan, China). A mouse macrophage cell line (RAW 264.7) was procured from the cell bank of the Chinese Academy of Sciences (TCM13, Shanghai, China). They were cultured in Dulbecco's modified Eagle's medium (Corning, NY, USA) supplemented with 10% fetal bovine serum (BI, Kibbutz Beit Haemek, Israel). The cell cultures were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> and at 95% humidity. All cells used in this study were confirmed as mycoplasma-free.

MPC-83 cells were treated with or without a concentration gradient of sodium propionate and sodium butyrate for 24 hours. Subsequently, the cells were exposed to a concentration gradient of hydrogen peroxide for an additional 2 hours. Cell viability of MPC-83 cells was assessed using a Cell Counting Kit-8 (CCK8; CK04, DOJINDO, Kyushu, Japan).

For metabolite treatment, prior to experimentation, sodium propionate, and sodium butyrate were pre-incubated with RAW 264.7 cells for 24 hours. Subsequently, the corresponding metabolites were administered in combination with 1  $\mu$ g/mL LPS for 6 hours. Following this treatment, RAW 264.7 cells were collected for CCK8 assay, immunofluorescence staining, RNA-Seq, and western blotting, while the supernatants were collected for enzyme-linked immunosorbent assay.

In rescue experiments, sodium propionate at a concentration of 6.4 mM and sodium butyrate at 0.8 mM were individually preincubated with 10  $\mu$ M U46619 (a p38 MAPK agonist; B6890, APEx-BIO, Houston, TX, USA) for 24 hours. These were followed by combined treatment with 1  $\mu$ g/mL LPS for 6 hours. Subsequently, RAW 264.7 cells were collected for immunofluorescence staining.

#### Cell viability assay

Cell viability was analyzed with CCK8. Cells were seeded at  $1 \times 10^4$  cells per well into 96-well plates and incubated with the indicated drugs. Subsequently, 100 µL of fresh medium was added to cells containing 10 µL of CCK8 solutions and incubated for 2 h in an incubator of 5% CO<sub>2</sub> at 37 °C. Absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, Mass, USA).

#### Immunofluorescence staining

The RAW 264.7 cells were gently rinsed with sterile PBS and subsequently fixed in 4% PFA for 20 minutes at room temperature. Afterward, the cells were treated with 0.1% Triton X-100 for 15 minutes at room temperature and then blocked with 5% (w/v) BSA for 1 hour at room temperature. The cells were incubated with anti-CD86 primary antibody (1:200; 13395-1-AP, Proteintech) and anti-CD206 primary antibody (1:500; GB113497, Servicebio), respectively. Following overnight incubation, the cells were treated with fluorescent material-conjugated secondary antibodies for 1 hour at room temperature, followed by DAPI staining. Images were captured using an Axio Observer5 microscope (ZEISS, Oberkochen, Germany) and subsequently analyzed using ImageJ software.

#### **RNA** sequencing

Total RNA was extracted with AG RNAex Pro Reagent (AG21102, Accurate Biology, Hunan, China) as per the manufacturer's instruction<sup>65</sup>. RNA purity and concentration were measured using a Nanophotometer Pearl (IMPLEN, CA, USA). The preparation of RNA library and transcriptome sequencing was conducted by Novogene Co., LTD (Beijing, China). Genes with adjusted p value  $\leq$  0.05 and  $|\log_2(FoldChange)| \geq 1$  were considered as differentially expressed.

#### Western blotting

The RAW 264.7 cells were lysed using a lysis buffer (P0013, Beyotime, Shanghai, China) containing phenylmethyl sulfonyl fluoride (ST506, Beyotime). Protein concentration was determined using a BCA protein assay (P0011, Beyotime). After ensuring equal protein amounts, the total protein was separated on an SDS-PAGE gel (10%–12%). Subsequently, the protein bands were transferred onto a PVDF membrane using the wet tank transfer method.

Following sealing with 10% skim milk, the PVDF membrane was washed with TBST. The protein bands were probed with the primary antibodies overnight at 4°C, including JNK (1:1000; 9252, CST, Boston, MA, USA), p-JNK (1:1000; 9255, CST), ERK1/2 (1:1000; 4695, CST), p-ERK1/2 (1:1000; 4370, CST), p38 (1:1000; 8690, CST), p-p38 (1:1000; 4511, CST), p-p65 (1:1000; 3033, CST), p65 (1:1000; ab16502, Abcam), and GAPDH (1:5000; AP0063, Bioworld, MN, USA).

After further washing with TBST, the protein bands were incubated with HRP-conjugated secondary antibodies and developed using an ECL buffer (sc-2048, Santa Cruz Biotechnology, CA, USA).

#### Statistical analysis

The data were subjected to statistical analysis using GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA). Before statistical analysis, data were subjected to the Kolmogorov–Smirnov test to determine distribution. Variance similarity was tested using an F test for two groups and Bartlett's test for multiple groups. Two groups were compared using the two-tailed *t* test for parametric data or the Mann–Whitney *U* test for non-parametric data. Multiple groups were

compared using one-way ANOVA with Tukey's or Dunnett's test for parametric data or using the Kruskal–Wallis test with Dunn's correction for non-parametric data. Food intake, body weight, and cell viability curves were analyzed using two-way ANOVA with Sidak's, Tukey's, or Bonferroni's test for multiple comparisons. Two-tailed Spearman's correlation analysis was used to examine the relationships between clinical parameters and gut microbiota or metabolites. Statistical significance was defined as a *p* value of <0.05 (*p* < 0.05). All data are presented as mean ± SD.

#### **Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

#### **Data availability**

Microbiomics data sets were deposited at Mendeley Data under the DOI 10.17632/v8tx42gchw.1 (https://data.mendeley.com/datasets/ v8tx42gchw/1). Targeted metabolomics data sets were deposited at Mendeley Data under the DOI 10.17632/4kb39tg8gj.1 (https://data. mendeley.com/datasets/4kb39tg8gj/1). RNA-Seq data sets were deposited into the NCBI GEO under the accession number GSE271034. Other information is available in the supplementary information and otherwise available upon request. Source data are provided with this paper.

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### **Author contributions**

J.L., D.T., and Jinheng W. designed the experiments. Xi C., Xin C., D.Y., N.Z., W.F., F.G., Jiangtuan W., M.W., and Jinheng W. performed the

experiments. Xi C., Jinheng W., and D.T. analyzed the data and wrote the paper. Xin C., X.L., and M.G. assisted in data interpretation and edited the manuscript. J.L. supervised the whole study and edited the manuscript.

### **Competing interests**

The authors declare no competing interests.

### **Additional information**

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