


ORIGINAL ARTICLE

ROR γ t inhibitor SR1001 alleviates acute pancreatitis by suppressing pancreatic IL-17-producing Th17 and $\gamma\delta$ -T cells in mice with ceruletide-induced pancreatitis

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Abstract

The management of acute pancreatitis (AP) remains a challenge to clinicians worldwide for limited effective interventions. Retinoid orphan receptor gamma t (ROR γ t) is a therapeutic target for several diseases; however, it is unclear whether inhibiting ROR γ t can ameliorate AP. The relative expression of ROR γ t, IL-17 and IL-23 in the peripheral blood mononuclear cells of AP patients was measured by RT-PCR. An AP mouse model was induced by ceruletide, and SR1001 was injected before ceruletide administration. ROR γ t+ cells, T helper 17 cells (Th17), regulatory T cells (Tregs) and $\gamma\delta$ T cells were assessed in the pancreas and spleen by flow cytometry. Higher ROR γ t expression in patients indicated the potential role of ROR γ t in AP progression. Analyses of the IL-17/IL-23 axis confirmed its role. SR1001 significantly alleviated AP histologically in the mouse model. Serum levels of amylase, IL-6, TNF α , IL-17 and IL-23 decreased upon SR1001 treatment. SR1001 selectively decreased the number of ROR γ t+, Th17, Tregs and $\gamma\delta$ T cells in the pancreas but not the spleen. Collectively, these results showed that SR1001 exerted therapeutic effects on AP by suppressing IL-17-secreting Th17 and $\gamma\delta$ T cells in the pancreas. Thus, SR1001 may be a promising drug for the treatment of AP in the clinic.

KEYWORDS

acute pancreatitis, IL-17, ROR γ t, SR1001

Abbreviations: AP, acute pancreatitis; PBMCs, peripheral blood mononuclear cells; ROR γ t, retinoic acid-related orphan receptor gamma t.

Jianfa Wang and Yayun Xu contributed equally to this work.

1 | INTRODUCTION AND BACKGROUND

Acute pancreatitis (AP) is a common acute gastrointestinal disease that afflicts nearly 34/100000 individuals globally every year, especially middle-aged and older adults.

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The elevated incidence and substantial sequelae of AP, such as post-pancreatitis diabetes mellitus and exocrine pancreatic dysfunction, impose a huge economic burden.¹ However, therapeutic agents are limited to octreotide and preventative antibiotics.² Thus, to improve the prognosis of AP, the development of novel effective drugs is promptly needed.

Studies have attempted to elucidate the molecular mechanisms underlying AP. The crucial role of the activated immune system, initiated by pancreatic injury, in amplifying the inflammatory response and worsening tissue damage in AP has garnered increasing attention.³ Recent studies have shown T helper cell 1 (Th1)/Th17 differentiation in the pancreatic tissue of a ceruletide-induced mouse model of AP⁴ and improvement of an L-arginine-induced AP model by oxymatrine via the reduction of Th1/Th17 cells secreting pro-inflammatory cytokines.⁵ However, it remains unclear if the selective inhibition of IL-17-secreting T cells exerts protective effects on AP.

Retinoid orphan receptor gamma t (ROR γ t) is a master transcription factor for Th17-producing interleukin 17 (IL-17) cells.⁶ ROR γ t inhibitors exert therapeutic efficacy in autoimmune diseases by inhibiting the differentiation of naïve T cells to Th17 cells.^{7,8} In addition, enhanced ROR γ t expression in Th17 cells exacerbates experimental autoimmune encephalomyelitis.⁹ ROR γ t is generally recognized as a promising pharmacological target in some autoimmune diseases characterized by the high production of IL-17 from Th17 cells. Rats with L-arginine-induced AP exhibit overexpression of ROR γ t protein⁵; however, whether ROR γ t is upregulated in AP patients and its role in the pathogenesis and progression of AP remains unclear.

In these contexts, a series of novel compounds targeting ROR γ t have been continuously synthesized, and their potential feasibility in treating Th17-mediated diseases has been assessed.¹⁰ SR1001, which specifically targets ROR α / γ t, functions as a prominent Th17 inhibitor, and its therapeutic efficacy has been widely evaluated in many autoimmune disorders such as multiple sclerosis¹¹ and pathological retinal angiogenesis.¹² Whether SR1001 contributes to the amelioration of AP by inhibiting IL-17-secreting T cells is unknown.

Here, we determined the expression of ROR γ t and IL-17 in the peripheral blood mononuclear cells (PBMCs) of AP patients and established a ceruletide-induced mouse model of AP pretreated with SR1001. Our findings demonstrated the therapeutic efficacy of SR1001 in AP, providing some evidence for the potential promising clinical application of SR1001 in patients with this condition.

2 | MATERIALS AND METHODS

2.1 | Human samples

According to the 2012 Revised Atlanta Classification criteria of typical abdominal pain, at least triply elevated serum amylase and/or lipase, and significant imaging findings (abdominal computed tomography [CT] or magnetic resonance imaging [MRI]),¹³ patients diagnosed with AP and confirmed by three surgeons separately were recruited in this study. Blood samples were collected from 17 healthy controls (HCs) and 22 AP patients within 24 h after admission to our ward. Demographic and clinical characteristics including the results of routine blood, liver function, renal function, C-reactive protein (CRP) and amylase tests were recorded (Table 1). Serum was extracted after coagulation and stored at -80°C until use. All procedures were approved by the Ethics Committee of Minhang Hospital, Fudan University (2021-Approval-035-01K, Shanghai, China), and written informed consent was obtained from the volunteers. This study was conducted in accordance with the *Basic & Clinical Pharmacology & Toxicology Policy for Experimental and Clinical Studies*.¹⁴

2.2 | PBMC isolation

PBMCs were isolated from fresh anticoagulant blood within 6 h according to the manufacturer's protocols. Briefly, blood samples were diluted with sterile phosphate-buffered saline (PBS), followed by the gentle addition of Lymphocyte Separation Medium (Biosharp Life Sciences, Beijing, China). After centrifugation, the PBMC layer was extracted and washed twice, resuspended in PBS, snap-frozen with liquid nitrogen and stored at -80°C until use.

2.3 | Reagents

Ceruletide and SR1001 were purchased from Abmole Bioscience Inc. (Houston, TX, USA). A Mouse Th17 Staining Kit and Mouse Treg Staining Kit were purchased from MultiScience (Hangzhou, China), together with human and mouse IL-6, IL-10, tumour necrosis factor alpha (TNF- α), IL-17 and IL-23 enzyme-linked immunoassay (ELISA) kits. FITC Mouse Anti-Mouse CD45.1, PE Hamster Anti-Mouse $\gamma\delta$ T-cell receptor, APC Hamster Anti-Mouse CD3e and BV421 Mouse Anti-Mouse ROR γ t Clone Q31-378 (RUO) were purchased from BD Pharmingen™ (San Jose, CA, USA). An RNA Purification Kit was purchased from ES

TABLE 1 Demographic and clinical information of AP patients and healthy controls

	Healthy control	AP patient	P-value
Cases	17	22	
Age (years)	51.59 ± 17.71	56.86 ± 14.38	0.9036
Sex			
Male (%)	8 (47.06)	13 (59.09)	0.7626
Female (%)	9 (52.94)	9 (40.91)	0.6429
Weight (kg)	64.59 ± 9.69	71.36 ± 9.30	0.4135
Height (m)	1.67 ± 0.08	1.71 ± 0.08	0.7395
BMI (kg/m ²)	23.20 ± 2.78	24.44 ± 2.76	0.5328
WBC count ([^] 10 ⁹ /L)	6.47 ± 1.66	12.69 ± 4.39	0.4862
Neutrophil count ([^] 10 ⁹ /L)	3.66 ± 1.33	10.50 ± 4.64	0.3263
Neutrophil %	55.71 ± 7.86	80.75 ± 9.76	0.8536
Lymphocyte count ([^] 10 ⁹ /L)	2.07 ± 0.61	1.39 ± 0.60	0.4568
Lymphocyte %	32.61 ± 6.89	12.24 ± 6.55	0.0024
Monocyte count ([^] 10 ⁹ /L)	0.65 ± 0.35	0.76 ± 0.54	0.7815
Monocyte %	9.27 ± 2.62	5.57 ± 2.02	0.1632
ALT (U/L)	42.18 ± 29.16	196.73 ± 254.64	0.1841
AST (U/L)	28.47 ± 12.47	171.41 ± 275.21	0.1168
ALT/AST	1.38 ± 0.46	1.39 ± 0.93	0.5416
Amylase (U/L)	60.29 ± 16.32	1239.27 ± 1053.44	0.001
CRP (mg/ml)	2.97 ± 6.75	85.22 ± 87.41	0.001
Creatinine (U/L)	65.12 ± 15.13	73.05 ± 21.81	0.3247
BUN (U/L)	4.67 ± 1.02	5.67 ± 2.56	0.7163
Total	39		

Notes: Data are shown as the mean ± standard deviation. The chi-squared test and Fisher's exact test were used to compare the differences between two groups of each item.

Science (Shanghai, China), PrimeScript™ RT Master Mix was purchased from TaKaRa Bio Inc. (Beijing, China), and PowerUp™ SYBR™ Green Master Mix was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.4 | Mice

Eight-week-old male C57BL/6 mice were used in our study. All specific pathogen-free mice were purchased from JieSiJie Laboratory Animals Co., Ltd. (Shanghai, China), maintained under ambient temperature (24°C) and humidity and kept at a 12-h light/dark cycle. All mice had access to standard rodent chow (Shore Biotechnology Co., Ltd., Shanghai, China) and sterile water ad libitum. All animal experiments were approved by the Ethics Committee of Minhang Hospital, Fudan University (2021-Approval-035-01K, Shanghai, China).

2.5 | Mouse model of AP

After acclimation for 1 week, 20 mice were equally divided into four groups: control group received dimethyl sulfoxide (DMSO), AP model group was intraperitoneally administered 50 µg/kg ceruletide every hour for 7 consecutive hours, and SR1001 25 mg/kg and SR1001 50 mg/kg groups received 25 and 50 mg/kg SR1001 for 2 days by intraperitoneal injection twice daily, which started 1 day before AP model construction, respectively. Mice were anaesthetized and killed at 24 h after the last injection of ceruletide, and then serum and other tissues were collected. All animal experiments were repeated three times independently.

2.6 | Histological analyses

Haematoxylin and eosin (H&E) staining was conducted to evaluate the severity of AP, and mouse pancreatic

tissues were processed as previously described.¹⁵ Multiple sections of the same slide were assessed by two pathologists in a blinded manner, and AP severity was scored according to the following criteria: oedema, inflammatory cell infiltration, haemorrhage and fat necrosis and acinar necrosis; each item was scored 0–3, as previously described.¹⁶

2.7 | Measurement of serum alanine aminotransferase and creatinine

Serum levels of alanine aminotransferase (ALT) and creatinine in all mice were measured with the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The absorbance of ALT and creatinine was measured at 510 and 546 nm, respectively, in a plate reader (Cytation 5; Shanghai BioGene Biotech Co., Ltd., Shanghai, China).

2.8 | Flow cytometry

The pancreas and spleen were harvested from the mice, and a single-cell suspension was prepared by incubating with collagenase IV and trypsin solution. After washing twice and resuspending in 10% foetal bovine serum to reach a concentration of 1×10^5 /ml, cells were stained with CD3e-FITC, CD4-PerCP-Cy5.5 and IL-17A-PE for Th17 (CAT#KTH217, LOT#A00613) and Mouse Anti-Mouse CD45.1-FITC, $\gamma\delta$ T-Cell Receptor-PE (331210, A014586) and CD3e-APC for $\gamma\delta$ T cells. CD4-FITC and CD25-APC antibodies were used to measure the regulatory T cells (Tregs), after which the cells were fixed, permeabilized and stained with Foxp3 + -PE antibody (KTR201, A01115). ROR γ t was stained with ROR γ t + -BV421 antibody (25-6981-82, RO73620). Finally, 1×10^5 cells were measured using the Beckman Coulter Cyan ADP Analyser (Beckman Coulter Inc., Sykesville,

MD, USA) and analysed by FlowJo software v.10 (TreeStar Inc., Ashland, OR, USA).¹⁷

2.9 | ELISA

According to the manufacturer's instructions, the concentrations of pro-inflammatory and anti-inflammatory cytokines including IL-6 (CAT#EK106, LOT#A10610535 for mouse cytokine, EK206, A20610435 for human), IL-10 (EK110, A11001144, EK210, A21000714), TNF- α (EK182, A18201042, EK217, A21710141), IL-17 (EK117, A11700641, EK223, A22310354) and IL-23 (EK123, A12301234, EK282, A28210345) in all mouse and human blood samples and serum amylase (ab102523; Abcam) in mice were measured with the corresponding ELISA kits.

2.10 | Real-time PCR

RNA in PBMCs was isolated using the RNA Purification Kit, and 500 ng RNA was reverse-transcribed into cDNA with PrimeScriptTM RT Master Mix, after which SYBRTM Green Master Mix was added to the RT-PCR reaction. The relative expression of IL-17, IL-23 and ROR γ t was calculated by $\Delta\Delta$ CT, and β -actin was chosen as the reference gene. The primer sequences of each target gene are shown in Table 2.

2.11 | Statistical analyses

One-way analysis of variance was used to compare data among groups and the Mann–Whitney *U* test was used for data comparisons between two groups. Data are expressed as the mean \pm standard error of the mean. GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was applied for all statistical analyses. *P* < 0.05 was considered statistically significant, and *P* < 0.01 was considered highly significant.

TABLE 2 Primer sequences for RT-qPCR in this study

Gene name	Primer sequence	NCBI accession number
<i>IL-17</i>	F: 5'-TACAACCGATCCACCTCACC-3' R: 5'-AGTGGTCTTCCAGGTTTGA-3'	NG_033021.1
<i>IL-23</i>	F: 5'-CAGGTATGAAGTAGGGCGTG-3' R: 5'-GGGACTGAGGCTTGAATCT-3'	NC_000012.12
<i>RORγt</i>	F: 5'-GTGGGGACAAGTCGTCTGG-3' R: 5'-AGTGCTGGCATCGGTTTCG-3'	NM_001001523.2
<i>β-Actin</i>	F: 5'-GAGACCTTCAACACCCAGC-3' R: 5'-ATGTCACGCACGATTCCCC-3'	NM_001083538.3

3 | RESULTS

3.1 | ROR γ t is upregulated and IL-17 is overexpressed in AP patients

In this study, 39 participants were recruited including 17 HCs and 22 patients with AP. The demographic and clinical characteristics of the AP patients and HCs including age; sex; body mass index; white blood cell count and classification; and serum levels of ALT, aspartate transaminase (AST), amylase, CRP, creatinine and blood urea nitrogen (BUN) were also recorded. As indicated in Table 1, AP patients exhibited significantly elevated CRP and amylase levels. PBMCs and serum were separated from the blood samples. RT-PCR was conducted to detect the relative expression of ROR γ t and IL-17, and ELISA was used to measure the IL-17 level. As shown in Figure 1A, ROR γ t was upregulated by approximately 4.8-fold in AP patients compared to HCs. Highly expressed IL-17 at the transcriptional and translational levels (Figure 1B,C) was detected as well. These results demonstrated the overexpression of ROR γ t and IL-17 in AP patients.

3.2 | SR1001 effectively attenuates AP in a ceruletide-induced mouse model of AP

To investigate whether AP can be alleviated by an ROR γ t inhibitor, a mouse model of AP model was constructed by consecutive intraperitoneal injection of ceruletide,¹⁵ and SR1001 (25 and 50 mg/kg), a traditional ROR γ t inhibitor, was administered before AP induction. The experimental design is shown in Figure 2A. Histological analysis of the pancreas by H&E staining (Figure 2B) showed that acinar cell oedema and inflammatory cell infiltration were significantly improved in SR1001-treated pancreatic tissues compared with the AP group. The pathological score (Figure 2C) showed alleviated pancreatic inflammation upon SR1001 treatment. The serum levels of amylase, ALT and creatinine were increased by more than sixfold in AP mice compared to control mice, whereas the level decreased by at least one-half in SR1001-treated mice

(Figure 2D–F). These results demonstrated that ROR γ t inhibition by SR1001 had marked therapeutic efficacy in mice with ceruletide-induced AP.

IL-6 is an early detector and biomarker for evaluating the severity of AP.¹⁸ We found that the serum level of IL-6 was greatly increased in the AP group and largely decreased in the SR1001-treated group (Figure 2G), indicating the substantial alleviation of AP. As evidenced by the reduced pro-inflammatory cytokine TNF- α (Figure 2H) and elevated anti-inflammatory cytokine IL-10 (Figure 2I), there was great amelioration of the inflammatory response following SR1001 treatment.

3.3 | SR1001 selectively inhibits ROR γ t in the pancreas rather than spleen of mice with ceruletide-induced AP

Because SR1001 is a traditional ROR γ t inhibitor with high affinity, it specifically suppresses the transcription of IL-17 and reduces the differentiation of Th17.¹¹ Overexpression of ROR γ t in sepsis is associated with pancreatic injury in a mouse model,¹⁹ L-arginine-induced AP in a rat model⁵ and elevated serum IL-17 in AP patients.¹⁸ It remains unknown whether ROR γ t is upregulated in a ceruletide-induced AP mouse model. In this study, the percentage of ROR γ t+ cells in the pancreas of ceruletide-induced AP mice was analysed by flow cytometry. As shown in Figure 3A,B, the proportion of pancreatic ROR γ t+ was increased by approximately threefold in AP mice compared to control mice, while SR1001 significantly reduced the elevated ROR γ t+ cells in SR1001-treated mice. To investigate whether SR1001 functions systemically, splenic ROR γ t+ cells were detected as well. Figure 3C,D shows that the percentage of ROR γ t+ cells was comparable among the three groups. ROR γ t plays a fundamental role in the transcription of IL-17; therefore, we investigated whether the level of IL-17 was affected by SR1001 administration. As shown in Figure 3E, serum IL-17 was markedly elevated by 18-fold in mice with ceruletide-induced AP compared to the controls and was greatly declined in the 25 and 50 mg/kg SR1001-treated groups. Furthermore, IL-23 was also measured and found to be altered, similar to IL-17

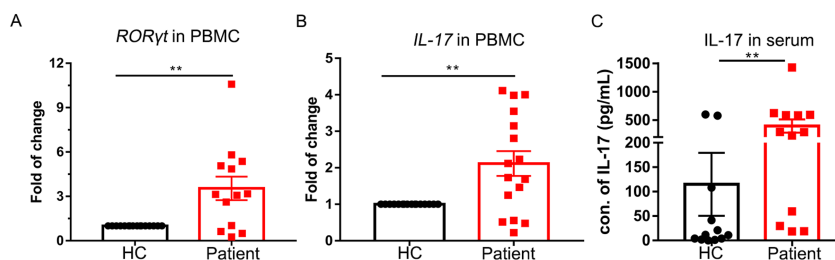


FIGURE 1 ROR γ t is highly expressed in the PBMCs of AP patients. (A,B) elevated expression of ROR γ t (A) and IL-17 (B) in the PBMCs of AP patients was detected by RT-PCR. (C) Serum levels of IL-17 in AP patients. Data are presented as the mean \pm SEM; $n = 8$ –12. ** $P < 0.001$, *** $P < 0.005$

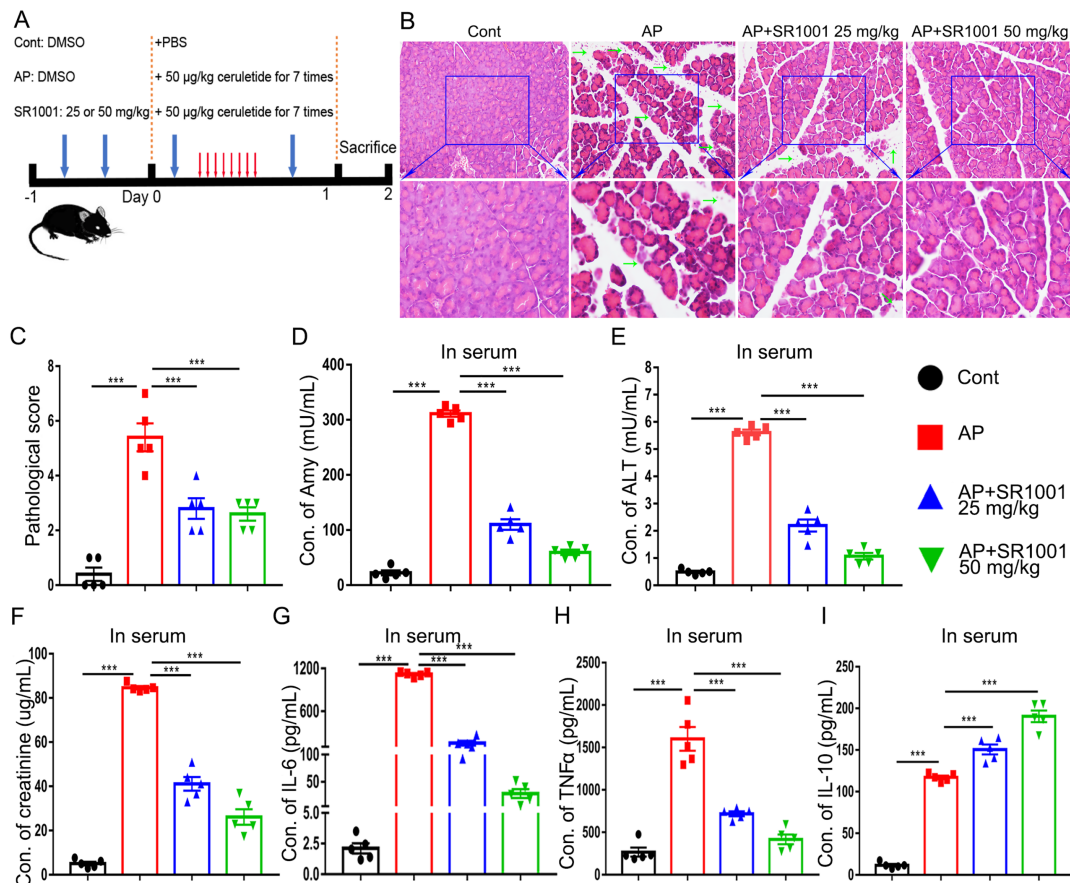


FIGURE 2 Pharmacological therapeutic effects of SR1001 on a mouse model of ceruletide-induced AP. (A) Representative images of H&E-stained sections of the pancreas in control and mice with AP. Magnification of upper panels: 20 \times and lower panels: 40 \times . The green arrows show typical acinar oedema, vacuolization and inflammatory infiltration of acinar cells in pancreatic tissues. (B) Quantified pathological score of pancreatic injury according to the above criteria. (C–E) quantitation of serum amylase (C), ALT (D) and creatinine (E) in different groups. (F–H) SR1001 attenuated the pro-inflammatory response in a mouse model of ceruletide-induced AP. ELISA was applied to determine the levels of pro-inflammatory cytokines IL-6 (F) and TNF- α (G) and anti-inflammatory cytokine IL-10 (H) in the serum of mice with ceruletide-induced AP and control mice. Data are expressed as the mean \pm SEM; $n = 5$. *** $P < 0.005$. All experiments were repeated three times independently

(Figure 3F). These findings suggest that SR1001 targets pancreatic ROR γ t+ cells, possibly through suppressing the IL-17/IL-23 immune pathway, and this effect was independent of immune cells in the spleen.

3.4 | Inhibition of ROR γ t by SR1001 significantly reduces Th17 differentiation in the pancreas of mice with AP

Mounting evidence has shown that IL-17 is a distinctive cytokine generated by Th17 cells, which in turn works with IL-23 to stimulate Th17.²⁰ During AP progression, IL-17 + Th17 cells increase at all time points after ceruletide induction.⁴ Similar results were obtained in our study by flow cytometry. The percentage of pancreatic CD4+/IL-17 + Th17 cells was increased by nearly

twofold in mice with ceruletide-induced AP; however, it declined after SR1001 injection (Figure 4A,B). Tregs are vital for maintaining immune homeostasis. We detected the significant reduction of CD25+/Foxp3 + Tregs in the pancreas of mice with AP, which decreased further in the SR1001-treated group (Figure 4C,D). Figure 4I shows that the pancreatic Th17/Treg ratio in AP mice was increased by more than sixfold compared to the control, but was significantly decreased in the SR1001-treated group, indicating that the immune imbalance in AP mice was significantly reversed upon SR1001 treatment. Splenic Th17 and Tregs were also measured, and no differences in their levels were found (Figure 4E–H,J). Together, these results demonstrated that targeted pancreatic ROR γ t inhibition by SR1001 suppressed Th17 and Treg differentiation, thereby reversing the immune imbalance in AP.

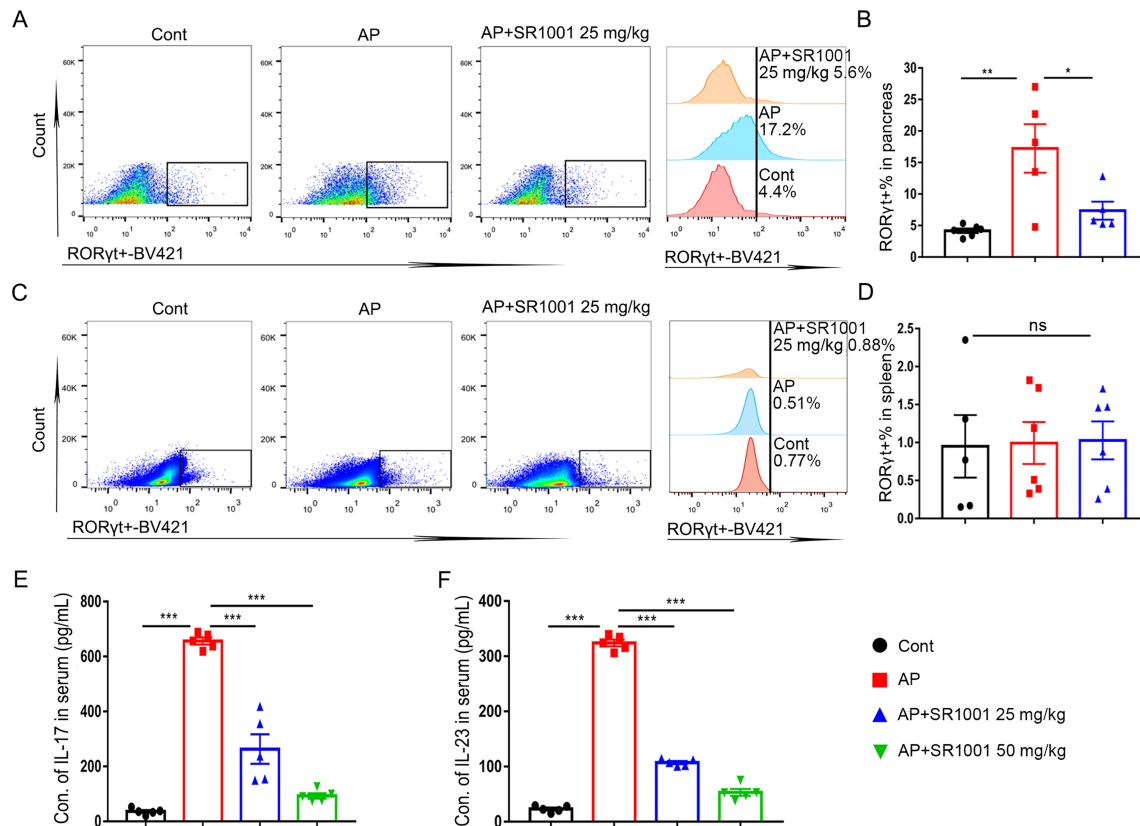


FIGURE 3 SR1001 targets ROR γ t in the pancreas rather than spleen of mice with ceruletide-induced AP. Flow cytometry analyses of ROR γ t in the pancreas and spleen of mice with ceruletide-induced AP and control mice. (A) Representative images of ROR γ t in the pancreas of mice with ceruletide-induced AP (left) and a fluorescence histogram of ROR γ t+ cells (right). (B) Quantitative percentage of ROR γ t+ cells in the pancreas. (C) Representative images of ROR γ t in the spleen of mice with ceruletide-induced AP (left) and fluorescence histogram of ROR γ t+ cells (right). (D) Quantitative percentage of ROR γ t+ cells in the spleen. (E,F) quantitation of serum levels of IL-17 (E) and IL-23 (F) by ELISA in mice with ceruletide-induced AP and control mice. Data are presented as the mean \pm SEM; $n = 5$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. ns, not significant

3.5 | SR1001 significantly decreases pancreatic $\gamma\delta$ T cells in the mouse model of AP

$\gamma\delta$ T cells are considered an important early first line of immune defence of acute infection and initial IL-17 producer.²¹ ROR γ t antagonism has obvious inhibitory effects on IL-17-secreting $\gamma\delta$ T cells (also named T $\gamma\delta$ 17) in patients with spondyloarthritis.¹⁷ Therefore, to evaluate the function of $\gamma\delta$ T cells in AP progression, we measured the percentage of $\gamma\delta$ T cells in the pancreas and spleen. The flow cytometry results revealed that pancreatic CD3e +/ $\gamma\delta$ -TCR + $\gamma\delta$ T cells increased from 0.875% in control mice to 5.112% in mice with ceruletide-induced AP but decreased to 1.884% in SR1001-treated mice (Figure 5A, B). However, similar results were not observed in the spleen (Figure 5C,D). These findings suggest the crucial role of T $\gamma\delta$ 17 in promoting the development of AP, while reducing T $\gamma\delta$ 17 by the ROR γ t inhibitor SR1001 may contribute to a good prognosis of AP.

3.6 | IL-17/IL-23 immune axis is activated in AP patients

Given that levels of IL-17 and IL-23 were increased in mice with AP and significantly decreased upon SR1001 treatment (Figure 3D,E), we investigated whether the IL-17/IL-23 immune axis is activated in AP patients. ELISA results showed that pro-inflammatory cytokines IL-6 and TNF- α were increased by about 200-fold (from 0.39 ± 0.15 to 82.45 ± 54.16 pg/ml) and 4-fold (from 334.23 ± 149.55 to 1227.03 ± 250.67 pg/ml), respectively, and the anti-inflammatory cytokine IL-10 was increased by 18-fold in AP patients. Furthermore, the RT-PCR assay confirmed activation of the IL-17/IL-23 immune pathway in AP patients, as indicated by the upregulated expression of IL-17 and IL-23 and corresponding protein concentrations in serum (Figures 1B,C and 6D,E). These results demonstrated that the IL-17/IL-23 immune axis was considerably activated in AP patients.

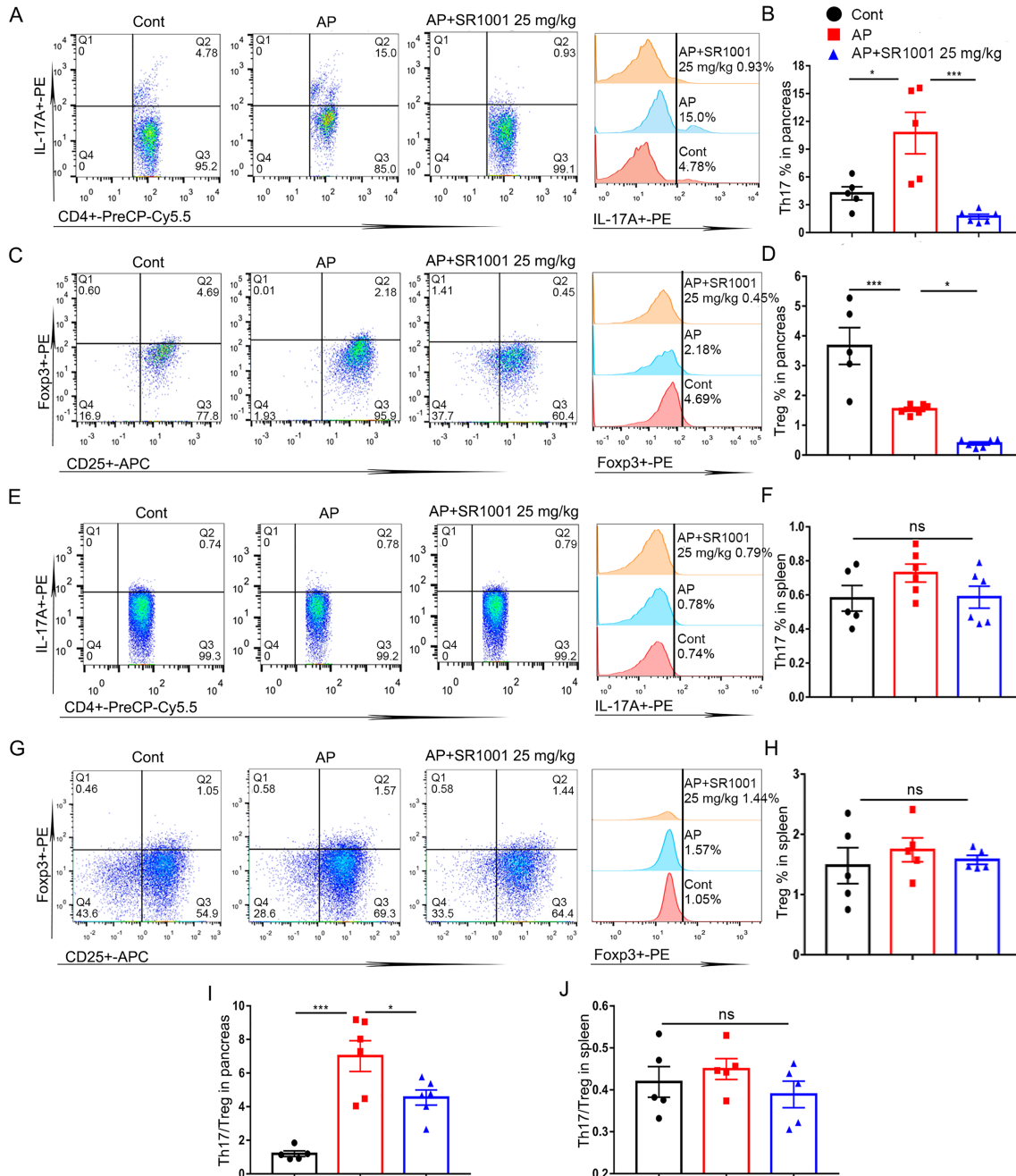


FIGURE 4 SR1001 selectively inhibits Th17 differentiation in pancreatic rather than splenic tissues. Flow cytometry was conducted to determine the proportion of CD4+/IL-17A + Th17 cells in the pancreas (A,B) and spleen (C,D) of mice with AP. (A) Representative images of cytometry results (left) profiling pancreatic CD4+/IL-17A + Th17, fluorescence histogram of Th17 cells (right) and (B) quantified analyses of Th17 cells in pancreatic tissues. (C) Representative images of cytometry results (left) profiling splenic Th17, fluorescence histogram of Th17 (right) and (D) quantitative analyses of Th17 in spleen. The proportion of CD25+/Foxp3 + Treg cells in the pancreas (E,F) and spleen (G,H) of mice with AP was assessed by flow cytometry. (E) Representative images of cytometry results (left) showing the percentage of Tregs, fluorescence histogram of Tregs (right) and (F) quantitative analyses of Treg in pancreatic tissues. (G) Representative images of cytometry results (left), fluorescence histogram of Tregs (right) and (H) quantitative analyses of Tregs in the spleen. (I,J) the ratio of Th17 to Tregs in the pancreas (I) and spleen (J) of mice with ceruletide-induced AP. Data are expressed as the mean \pm SEM; $n = 5$. * $P < 0.05$, *** $P < 0.005$. ns, not significant

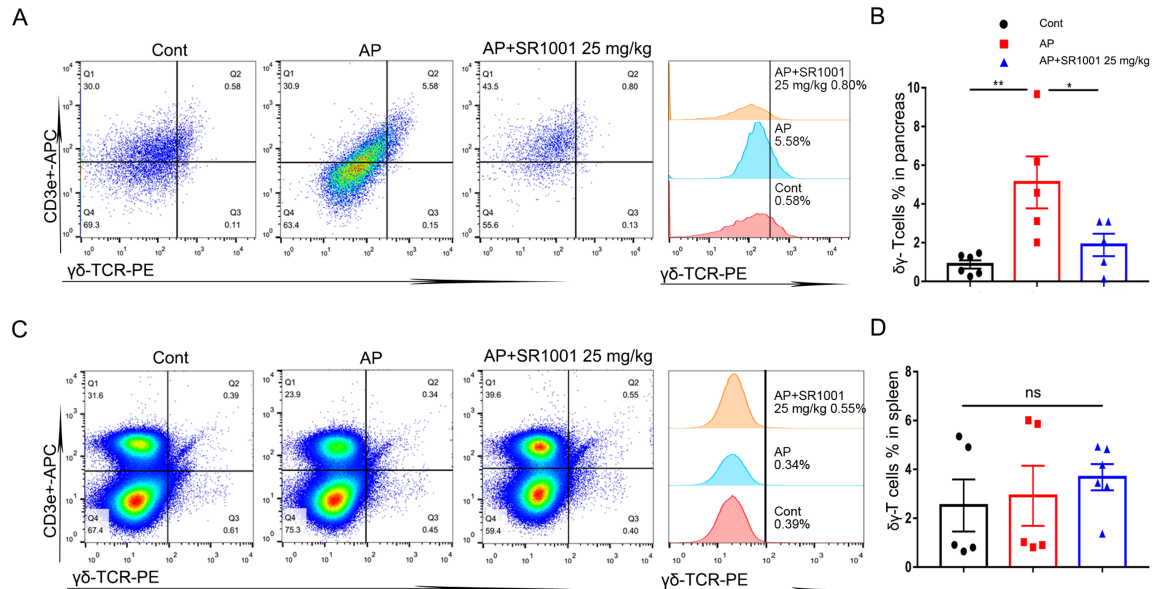
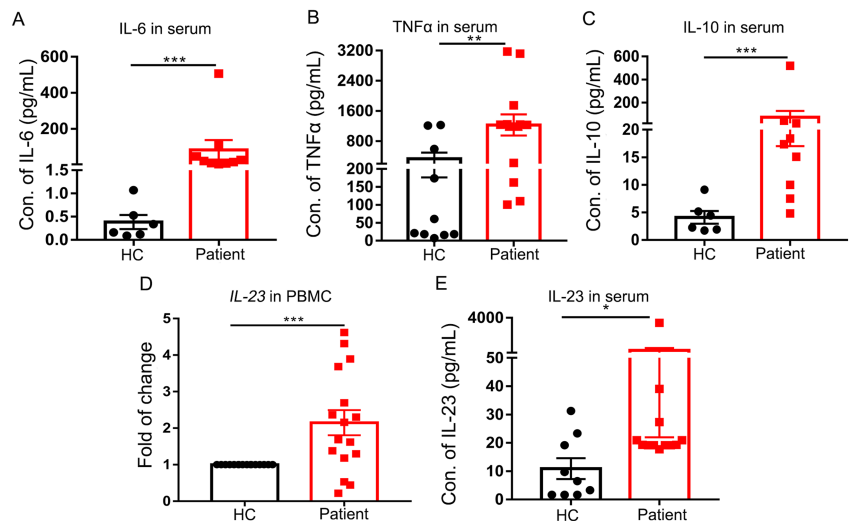


FIGURE 5 ROR γ t inhibitor SR1001 targets pancreatic CD3e⁺/TCR- $\gamma\delta$ + $\gamma\delta$ T cells in mice with ceruletide-induced AP. (A) Proportion of pancreatic CD3e⁺/TCR- $\gamma\delta$ + $\gamma\delta$ T cells analysed by flow cytometry (left) and fluorescence histogram of $\gamma\delta$ -T cells (right) are shown. (B) Quantitation of pancreatic $\gamma\delta$ T cells. (C) Proportion of splenic CD3e⁺/TCR- $\gamma\delta$ + $\gamma\delta$ T cells by flow cytometry (left), fluorescence histogram of $\gamma\delta$ T cells (right) and (D) quantitative analyses of splenic $\gamma\delta$ T cells are shown. Data are expressed as the mean \pm SEM; $n = 5$. * $P < 0.05$, ** $P < 0.001$. ns, not significant

FIGURE 6 IL-17/IL-23 immune pathway is activated in AP patients. (A–C) The level of pro-inflammatory cytokines IL-6 (A), TNF- α (B) and anti-inflammatory cytokine IL-10 (C) in the serum of AP patients was measured by ELISA. (D) Quantified relative expression of IL-23 in the PBMCs of AP patients. (E) IL-23 level in the serum of AP patients. Data are presented as the mean \pm SEM; $n = 5$ –12. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.005$



4 | DISCUSSION

Here, we report ROR γ t is highly expressed in the PBMCs of AP patients, making it a potential therapeutic target for AP. ROR γ t was discovered as a novel transcriptional factor for IL-17, which plays a key role in maintaining Th17 differentiation.²² Th17, characterized by IL-17 secretion, is a newly discovered subset differentiated from naïve T cells, which normally contributes to anti-inflammatory immune homeostasis.^{22,23} However, its pathogenicity in

eliciting various autoimmune or inflammatory diseases has been widely reported.²⁴ Similar to Th17, IL-17-producing $\gamma\delta$ T cells are another main source of innate IL-17 in the early stage of infectious or autoimmune diseases, which are also regulated by the expression of ROR γ t.²⁵ AP is a local and systemic inflammatory disease, in which complex immunological reactions occur.³ In this study, we found that Th17 and $\gamma\delta$ T cells were significantly increased in the pancreas of an AP mouse model, accompanied by the elevated secretion of IL-17. These

results indicate the possible function of IL-17-producing Th17 cells and $\gamma\delta$ T cells in the pathogenesis of AP.

AP, mainly caused by gallstones, alcohol abuse or hypertriglyceridaemia, is becoming one of the most common emergent GI diseases. Nearly 80% of AP patients exhibit a mild course, and the remaining 20% progress to severe disease, in which systemic inflammatory response syndrome or fatal complications might occur.^{26,27} Thanks to the updated 2012 Revised Atlanta Classification for AP diagnosis and grading¹³ and multiple Pancreatitis Activity Scoring System for severity prediction,²⁸ AP patients in the early stage can be identified and undergo initial therapies. Accordingly, the American Gastroenterological Association recommends initial management paradigms in the early stage of AP, such as goal-directed fluid management.²⁹ Despite these advancements in clinical guidelines and interventions, the mortality rate of AP remains 1.16/100000 person-years annually.¹ Therefore, to achieve the goal of alleviating acute inflammatory responses and improving outcomes of AP, more emphasis is suggested to be attached to effective drug development.

Since ROR γ t is specific for IL-17 and acts as an important mediator for type 17T cells, it has been universally recognized as an attractive target for many autoimmune diseases.⁶ ROR γ t protein level was proved to be associated with AP in animal model⁵; furthermore, our results detected ROR γ t upregulation in transcriptional level of AP patients and overexpression in translational level in pancreas of ceruletide-induced AP mice. A recent study underlines that ROR γ t inhibitors show great pharmacological potentials in ameliorating aberrant 'type 17 responses' in patients with spondyloarthritis¹⁷ and enhanced ROR γ t expression in Th17 cells would accelerate the progression of experimental autoimmune encephalomyelitis.⁹ Therefore, a series of small molecular ROR γ t inhibitors is constantly developed, and their therapeutical applications are continuously elucidated in several autoimmune disorders.¹⁰ SR1001 is a traditional ROR γ t inhibitor, and it could conspicuously alleviate multiple sclerosis by inhibiting Th17 differentiation and IL-17 secretion. Its inhibitory effects on oxygen-induced retinopathy and spontaneous subretinal neovascularization mouse model have been demonstrated.¹² Therefore, SR1001 was administered to ceruletide-induced AP mice in this study, and our results demonstrate a remarkable therapeutical efficacy on AP. Furthermore, pancreatic Th17 and $\gamma\delta$ -T cells decline upon SR1001 treatment in an AP mice model, which means that SR1001 exerts an inhibitory effect on Th17 and $\gamma\delta$ -T cells in pancreas. Upregulated ROR γ t and IL-17 in PBMC of AP patients provide evidence for the potential application of SR1001 in clinic.

Type 17 cells, comprising Th17, $\gamma\delta$ -T, natural killer T and innate lymphoid cells, bear the capability of IL-17 production and function as sentinels when tissue injury or acute infection occurs.³⁰ Increased proportion of Th17 in pancreas, together with reduced Tregs, has been demonstrated in Xu's study,⁴ which is in accordance with our results; ulteriorly, we found a significant augment of pancreatic $\gamma\delta$ -T cells as well. Intriguingly, upon administration of SR1001, pancreatic Th17 and $\gamma\delta$ -T cells simultaneously decreased and reduced Th17/Treg ratio help maintain immune tolerance. Th17 differentiation is mainly dependent on IL-17 expression. The transcription of IL-17 is induced by ROR γ t, which then triggers early-stage Th17 differentiation.²² During the AP progression, over-secreting IL-17 contributes to the imbalance of Th17 homeostasis. However, SR1001 administration can reverse the excessive expression of Th17 by inhibiting ROR γ t transcription and reverse IL-17 secretion in the pancreas. Even though similar findings are not presented in spleen of mice with AP, it still means that SR1001 functions in local pancreas rather than peripheral immune organs in the early stage of AP. These negative results from the spleen may be attributed to the gaps between ceruletide-induced AP mouse model and pancreatitis in the clinic. Th17 cells bear pathogenic and non-pathogenic capacities. IL-6-STAT3 pathway, a prerequisite for the expression of ROR γ t, jointly with IL-17-IL-23 axis and IL-1 β functions as inducing factor for pathogenic Th17.^{20,31} In our study, IL-6, IL-17 and IL-23 are collectively increased notably in AP patients and ceruletide-induced AP model, which hints inducible pathogenic Th17 in pancreas. Tregs plays a fundamental role in regulating immune balance between Th1/Th2 differentiation and immune suppression, and Tregs presents absent during AP.⁴ Here, Th17/Tregs ratio was reversed, yet Tregs decreased upon SR1001 treatment. We speculated that this result was caused by SR1001 administration because of the complexity of Tregs.³² Effects of SR1001 on Tregs and the complicated interrelationship between ROR γ t and Tregs require more investigations.

5 | CONCLUSION

We first demonstrated that inhibiting ROR γ t by SR1001 exerted significant therapeutic effects on ceruletide-induced AP mice, which was dependent on the selective inhibition of pancreatic IL-17-producing Th17 and $\gamma\delta$ T cells. The overexpression of ROR γ t was observed in the PBMCs of AP patients. The IL-17/IL-23 immune pathway was activated during AP with SR1001 treatment, leading to downregulation of this axis. These findings indicate

that SR1001 may have therapeutic efficacy in the early stage of AP.

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CONFLICT OF INTEREST

These authors declare that they have no conflicts of interest.

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