ORIGINAL ARTICLE

Activity of acute pancreatitis is modified by secreted protein acidic and rich in cysteine ablation

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Abstract

Background: Acute pancreatitis (AP) is a frequent cause for hospitalization. However, molecular determinants that modulate severity of experimental pancreatitis are only partially understood.

Objective: To investigate the role of secreted protein acidic and rich in cysteine (SPARC) during cerulein-induced AP in mice.

Methods: AP was induced by repeated cerulein injections in SPARC knock-out mice (SPARC$^{-/-}$) and control littermates (SPARC$^{+/+}$). Secreted protein acidic and rich in cysteine expression and severity of AP were determined by histopathological scoring, immunohistochemistry, and biochemical assays. For functional analysis, primary murine acinar cell cultures with subsequent amylase release assays were employed. Proteome profiler assay and ELISA were conducted from pancreatic tissue lysates, and co-immunofluorescence was performed.

Results: Upon cerulein induction, SPARC expression was robustly induced in pancreatic stellate cells (PSCs) but not in acinar cells. Genetic SPARC ablation resulted in attenuated severity of AP with significantly reduced levels of pancreatic necrosis, apoptosis, immune cell infiltration, and reduced fibrosis upon chronic stimulation. However, the release of amylase upon cerulein stimulation in primary acinar cell culture from SPARC$^{+/+}$ and SPARC$^{-/-}$ was indistinguishable. Notably, immune cell derived C-C Motif Chemokine Ligand 2 (CCL2) was highly elevated in SPARC$^{+/+}$ pancreatic tissue potentially linking PSC derived SPARC with CCL2 induction in AP.

Conclusion: SPARC mediates the severity of AP. The potential link between SPARC and the CCL2 axis could open new avenues for tailored therapeutic interventions in AP patients and warrants further investigations.

KEYWORDS
acute pancreatitis, BM-40, CCL2, cerulein, osteonectin, SPARC

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INTRODUCTION

Acute pancreatitis (AP) is considered as one of the leading causes of hospital admissions in Gastroenterology and causes a relevant economic health burden.[1] Despite its overall low mortality of 2% AP is associated with high morbidity and mortality in complicated and severe cases.[2] The majority of patients experience mild disease and completely recover without long-lasting or permanent organ damages. However, 20% of patients suffer from moderate to severe AP with local and systemic complications, high morbidity and prolonged hospital stay.[3] In these cases mortality dramatically increases ranging between 36% and 50%.[4–6] Complications such as necrotic collections with potential superinfections, arrosion bleedings or thrombosis of the visceral vessels develop within weeks after disease onset. In the last decades intensive research efforts were conducted to anticipate the course of disease at the time of hospital admission and identify markers that are associated with the extent of local and systemic inflammation. However, sophisticated scoring systems such as the *Ranson score, Acute Physiology and Chronic Health Evaluation II score* or *Bedside Index for severity in acute pancreatitis* only yielded sensitivities around 70% and are often not feasible for clinical routine use.[7–9]

Secreted protein acidic and rich in cysteine (SPARC), also known as Osteonectin and Basement-membrane protein 40 is an evolutionarily highly conserved 43 kDa protein, which was initially isolated from calve bones.[10] It is secreted by endothelia, fibroblasts, pericytes, astrocytes, and macrophages and is involved in collagen mineralization, wound healing and organ fibrosis.[11] SPARC interacts with multiple extracellular matrix (ECM) proteins and modulates their function, thereby manipulating the composition of ECM.[12,13] Among others, it stimulates tumour-growth-factor-β (TGF-β) leading to an increased collagen I synthesis.[14] Furthermore, SPARC has binding sites for vascular-endothelial-growth-factor (VEGF) and platelet-derived-growth-factor (PDGF) inhibiting their proangiogenic pathways.[15]

Secreted protein acidic and rich in cysteine expression is associated with different inflammatory and oncogenic processes.[16–18] A recent study by Papapanagiotou et al. revealed a potential role of SPARC as an early biomarker for predicting severity of biliary AP. To this end, serum levels of SPARC were the only independent predictor for the clinical outcome of AP patients.[19] Furthermore, transcriptome profiling datasets collected from various compartments of the murine pancreas recently identified SPARC as candidate marker for activated PSCs during acute and chronic pancreatitis (CP).[20] However, the functional role of SPARC during experimental pancreatitis has not been investigated before. Here, we hypothesize that SPARC is causally involved in the inflammatory cascade of AP and the progression from recurrent AP (RAP) to CP and may determine the severity of acute experimental pancreatitis. To test this, we used a variety of in vitro and in vivo systems such as genetically engineered mouse models, acinar cell culture, and proteome profiler and identified SPARC as an important modulator of disease activity during early acute and CP.

Key summary

What is established knowledge on this subject:

- Cerulein-induced pancreatitis is a well-established model to investigate the pathophysiology of Acute pancreatitis (AP).
- Genetic ablation of C-C Motif Chemokine Ligand 2 (CCL2) attenuates experimental pancreatitis in mice.
- Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein with unknown functions in AP.

What are the new findings of this study:

- SPARC is induced upon cerulein induced pancreatitis in pancreatic stellate cells (PSCs).
- Genetic SPARC ablation attenuates cerulein induced pancreatitis in mice and reduces fibrosis upon chronic cerulein stimulation.
- SPARC expression in cerulein-induced pancreatitis is associated with significantly increased CCL2 levels in pancreatic tissue potentially establishing a SPARC-CCL2 axis that could be exploited for novel therapeutic avenues.

METHODS

Genetically engineered mouse model

Initially, SPARC$^{-/-}$ mice (B6; 129S-Sparctm1Hwe/J) and SPARC$^{+/+}$ (WT) mice (B6; 129S) were purchased from Jackson laboratory. Generations were bred as previously described.[21] Both mouse models were housed at a 12 h light, 12 h dark rhythm under pathogen-free conditions. All animal experiments were conducted following the protocols approved by the Institutional Animal Care and Use Committee at the University Medical Center Göttingen (15/1913).

Induction of pancreatitis

Cholecystokinin analogue cerulein was dissolved in NaCl at 20 µg/ml concentration and administrated at a dose of 0.1 µg/g body weight at an interval of 1 h (in total eight injections each day) for two consecutive days. Mice were sacrificed at different time points (8h, 24h, 7days and 21days) after the last treatment to harvest tissue samples as previously described[22] (Suppl. Figure 1). Prior to sacrificing, serum samples were collected via intracardial puncture in deep anaesthesia. The prolonged cerulein treatment regime was conducted over 4 weeks trice a week and 0.5 µg/g body weight was administrated.

Blood serum analysis

The clinical trial office at University Medical Center Goettingen analysed the serum samples for glucose, amylase and lipase levels with ARCHITECT cSystemsTM (Abbott Core Laboratory, USA). The Lipase assay (ref: 7D80-31), the amylase assay (Ref: 6K22-30) and the glucose assay (ref: 3L82-21/-41) were performed by using the manufacturer's instruction.

Cell lines

Murine acinar cells were isolated from freshly resected pancreata derived from $SPARC^{+/+}$ and $SPARC^{-/-}$ mice as previously described,[23] and maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, USA) with 10% Fetal Bovine Serum and 1% non-essential amino acids (Thermo Fisher, USA).

Amylase release assay

Freshly isolated pancreatic acinar cells from 4 to 8 weeks old $SPARC^{+/+}$ and $SPARC^{-/-}$ mice were subject to amylase release assay experiments. For each cerulein concentration (0 pmol/L, 10 pmol/L, 30 pmol/L, 100 pmol/L, 300 pmol/L, 1000 pmol/L) 1 ml of the isolated acinar cell suspension was added in doublets. Prior to plating the cells, 1 ml of freshly isolated cell suspensions were collected as 'pre-blank' reference. After plating the cells, 1 ml of the cell suspension was collected as a 'post-blank' reference. The 'pre-blank' and 'post-blank' samples were centrifuged for 4 min at 1200 rpm at 4°C. The supernatants were separated from the pellet and analysed for amylase levels to ensure that the time between plating all cells did not cause the release of amylase. The remaining pellet of acinar cells was mixed with 1 ml of protein lysis buffer and amylase levels were measured.

The 12 well-plates with cell treatment were incubated at 37°C for 30 min. Subsequently 1 ml of the cell suspension from each well was collected. Samples were centrifuged for 3 min at 1200 rpm at 4°C 200 µL of the supernatant were collected from each sample for the amylase level analysis. The amylase release by acinar cells within the incubation time was calculated and stated as ratio of the total amount of amylase in the pellet.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin for 24 h and transferred to 70% ethanol. Tissues were embedded in paraffin and 4 µm sections were processed for H&E staining, picrosirius staining, immunohistochemistry (IHC) and co-immunofluorescence (Co-IF) using standard protocol as previously stated.[18] The following antibodies were used: SPARC (R&D Systems, AF942, 1:100, Research Resource Identifiers (RRID): Antibody (AB)_2286625), α-SMA (Dako, Clone 1A4, 1:250, RRID: AB_2335694), CC3 (Cell signalling, 9664L, 1:100, RRID: AB_2335694) CCL2 (Invitrogen, #MA5-17040, Clone 2D8, 1:500, RRID: AB_2538512), CD45 (BD Biosciences, 550539, 1:100, RRID: AB_2174426).

Pictures were taken with 40x magnification for H&E and IHC staining and 20x magnification for Co-IF staining. Olympus DP27 and Olympus confocal FV1000 cameras were used for visualization.

Proteome profiler analysis

Mouse pancreatic tissues were homogenized and lysed in tissue lysis buffer (50 mM 2(4(2Hydroxyethyl)1piperazinyl)ethansulfonic acid (pH 7.5–7.9), 100 mM NaF, 150 mM NaCl, 10 mM $Na_4O_7P_2 \times 10$ H_2O, 1 mM EDTA, 10% Glycerine, 1% Triton X-100) supplemented with a cocktail of protease inhibitors (Complete, Roche Applied Science). 300 µg of protein from $SPARC^{+/+}$ and $SPARC^{-/-}$ mice tissue lysates were pooled together to perform proteome profiler analysis for 40 cytokines. The proteome profiler was applied following the manufacturer's protocol (R&D Systems, Inc. Minneapolis, USA.). Membranes were developed using chemiluminescence substrate Plus- Enhanced Chemoluminescence (ECL; PerkinElmer Inc., USA). The proteome bands were detected at the ChemiDoc™ X Ray Spectometer (XRS) + imaging system (Bio-Rad Laboratories GmbH, Germany) using Image Lab Software (version 5.2.1, RRID: Smart Connect Research [SCR] 014210).

Chemokine Chemokine Ligand 2 ELISA

The CCL2 amount in pancreas tissue lysates derived from $SPARC^{+/+}$ and $SPARC^{-/-}$ mice over 8 h with or without treatment were measured using a CCL2 Quantikine® ELISA (R&D Systems, Inc. Minneapolis, USA.) according to the manufacturer's instructions.

Western blot

Western blot analysis was performed as previously described.[24] The following primary antibodies were used: Hsp90 (Cell signaling, USA, #4875, RRID: AB_2233331) and SPARC (R&D Systems, USA #AF942, RRID: AB_2286625). Membranes were incubated with secondary HRP-antibodies (Jackson ImmunoResearch, USA) and developed using chemiluminescence substrate Plus-ECL (PerkinElmer Inc., USA) and protein bands were detected at the ChemiDoc™ XRS + imaging system (Bio-Rad Laboratories GmbH, Germany) using Image Lab Software (version 5.2.1, RRID: SCR 014210). Murine pancreatic fibroblasts were isolated and cultured as previously described.[24]

Methods of quantification

The Co-IF staining data were acquired by manual quantification of positive stained cells for CCL2 and CD45. The positive stained cells

were divided by the number of cell nuclei determined by Fiji/ImageJ (Fiji, RRID: SCR 002285). Histological changes, such as edema, immune cell infiltration, vacuolization, loss of parenchyma, acinar to ductal metaplasia and quantification of necrosis were scored manually on H&E stains based on modified histopathology scores[25,26] (Table 1). The quantifications were conducted in a non-biased, blinded manner.

Statistical analysis

All statistical analyses were performed using R and R-studio (version 4.0.3) with the following packages: openxlsx 4.2.3, tidyverse 1.3.0, ggpubr 0.4.0, car 3.0.1, rstatix 0.7.0. Before comparison of 2 groups, normal distribution and homogeneity of variance were checked with Shapiro-Wilk- and Levene test, respectively. If samples did not match t-test assumptions Wilcoxon test was performed, for example, for semiquantitative analysis of histological scores. P-values were adjusted with False discovery rate. Correlations were calculated by using Spearman test. P-values <0.05 were considered as statistically significant: < 0.05 *, <0.01 ** and <0.001 ***.

RESULTS

Genetic secreted protein acidic and rich in cysteine ablation attenuates severe acute phase of pancreatitis

To investigate whether SPARC expression has an impact on the severity of AP, pancreata were stained by H&E and a combined histopathology score, adapted from Freiburghaus et al. and Tito et al. was used.[25,26] Additionally to the well-established scoring system, we examined two more histological parameters: Acinar-to-ductal metaplasia and loss of parenchyma (Table 1). Histopathological examination of H&E staining 8h after cerulein treatment revealed signs of AP in both groups with morphological differences between SPARC^{+/+} and SPARC^{−/−} mice (Figure 1a,b). The results demonstrated increased severity of pancreatitis 8h after the last cerulein injection in SPARC^{+/+} compared to SPARC^{−/−} mice regarding the occurrence of immune cell infiltration, vacuolization, loss of parenchyma, and necrosis (Figure 1c,d,e,f). After the acute phase both groups do not differ significantly after 24h and showed complete resolution of histopathological abnormalities after 7–21 days. To confirm that the infiltrated cells are immune cell we further conducted CD45^{+}- staining 8h post cerulein treatment. Indeed, IHC-staining revealed a higher amount of CD45^{+} cells in SPARC^{+/+} compared to SPARC^{−/−} mice (Suppl. Figure 2). No significant differences between SPARC^{+/+} and SPARC^{−/−} mice were detected regarding acinar-to-ductal metaplasia and the expansion of edema (Suppl. Figure 3). Immunohistochemistry staining for cleaved caspase-3 (CC3) revealed significantly elevated levels of apoptosis in SPARC^{+/+} compared to SPARC^{−/−} mice further supporting the increased severity of AP in SPARC^{+/+} mice (Figure 2a-c).

TABLE 1 Histopathological Grading Scale for Acute pancreatitis (AP) adapted from Freiburghaus et al. and Tito et al.

Edema	
Score	Explanation
0	No edema
0,5	Focal expansion of interlobar septa
1	Diffuse expansion of interlobar septa
1,5	Same as 1 + focal expansion of interlobar septa
2	Same as 1 + diffuse expansion of interlobar septa
2,5	Same as 2 + focal expansion of interacinar septa
3	Same as 2 + diffuse expansion of interacinar septa
Necrosis	
Score	Explanation
0	No necrosis
0,5	Focal occurrence of necrotic cells
1	Diffuse occurrence of necrotic cells
1,5	Focal occurrence of areal necrosis <50% of cells
2	Diffuse occurrence of areal necrosis <50% of cells
2,5	Focal occurrence of areal necrosis >50% of cells
3	Diffuse occurrence of areal necrosis >50% of cells
Loss of parenchyma	
Score	Explanation
0	No destruction of acinus cells
0,5	Focal loss of acinus cells
1	Diffuse loss of acinus cells
1,5	Destruction of 12%, 5% of the parenchyma
2	Destruction of 25% of the parenchyma
2,5	Destruction of 37%, 5% of the parenchyma
3	Destruction of >50% of the parenchyma
Immune cell infiltration	
Score	Explanation
0	<100 leucocytes/HPF
0,5	100-200 leucocytes/HPF
1	201-300 leucocytes/HPF
1,5	301-400 leucocytes/HPF
2	401-500 leucocytes/HPF
2,5	501-600 leucocytes/HPF
3	>600 leucocytes/HPF
Vacuolization	
Score	Explanation
0	No vacuolization
0,5	Less than sixth of cells with vacuoles

(Continues)

T A B L E 1 (Continued)

Vacuolization	
Score	Explanation
1	Between one and two sixth
1,5	Between two and three sixth
2	Between three and four sixth
2,5	Between four and five sixth
3	Between five and six sixth

Pancreatic stellate cells express secreted protein acidic and rich in cysteine in the acute phase of pancreatitis

As described previously SPARC$^{-/-}$ mice differ phenotypically from their SPARC$^{+/+}$ littermates.[27,28] In line with these studies, 20 weeks old SPARC$^{-/-}$ mice had a significant lower body weight compared to age matched SPARC$^{+/+}$ mice before pancreatitis induction and after recovery (Suppl. Figure 4). However, as shown before, pancreatic development does not differ between SPARC$^{+/+}$ and SPARC$^{-/-}$ mice with no signs of exocrine or endocrine insufficiency or organ atrophy.[24]

Secreted protein acidic and rich in cysteine is known to be expressed in inflammatory processes.[16,17] Therefore, it is not surprising that immunohistochemistry staining revealed a high SPARC expression 8 h after the last cerulein injection that decreased within the following week in SPARC$^{+/+}$ (Figure 3a,b). Interestingly, SPARC was solely expressed in the periacinar space in spindle-shaped cells. This finding suggested the expression of SPARC in PSCs. This hypothesis was corroborated by α-SMA staining and confirmed by SPARC-immunoblot in PSCs and acinar cells revealing robust SPARC immunoreactivity in PSCs but not acinar cells (Figure 3c,d).
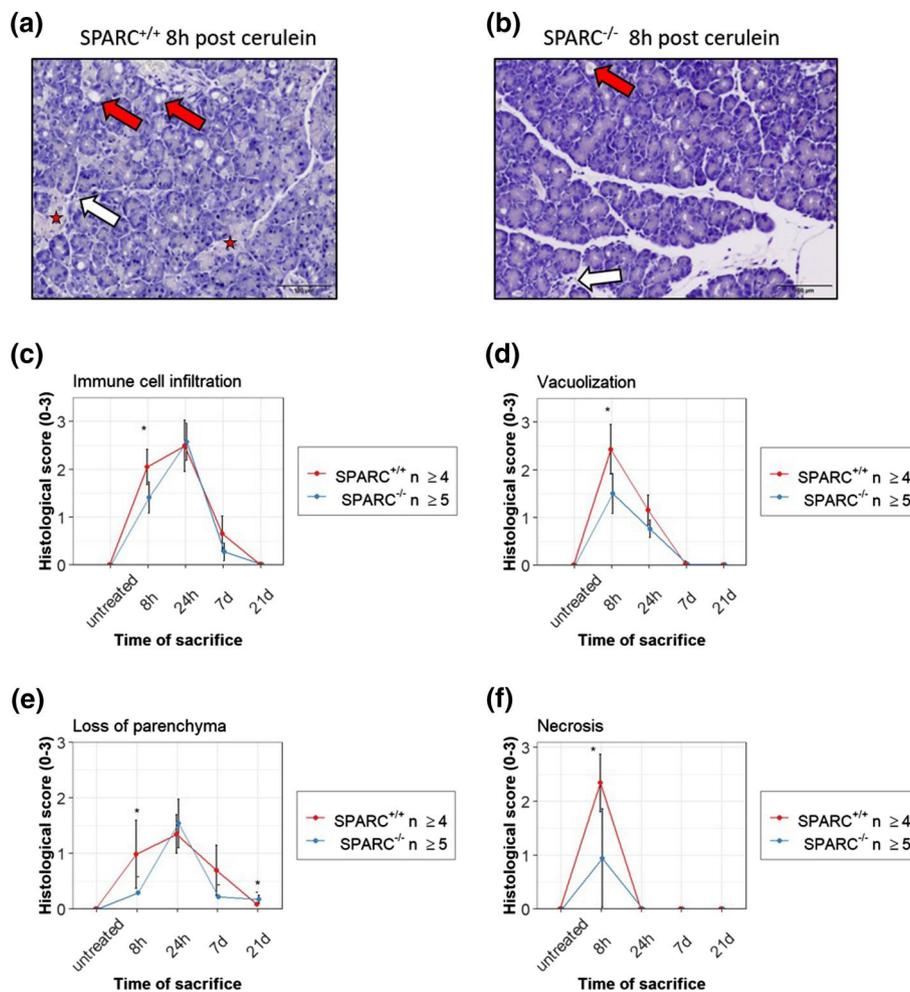
F I G U R E 1 Severity of Acute pancreatitis (AP) in SPARC$^{+/+}$ and SPARC$^{-/-}$ mice. H&E staining for (a) SPARC$^{+/+}$ and (b) SPARC$^{-/-}$ pancreatic tissue 8h after the last cerulein injection. Necrotic area is marked by red stars. Green arrows indicate vacuoles and white arrows point to immune cells. Severity of pancreatitis was evaluated by applying an established histopathology score. The following parameters were analysed: (c) Immune cell infiltration, (d) Vacuolization, (e) Loss of parenchyma, (f) Necrosis. Means and standard deviations were presented for each time point, n = four to seven SPARC$^{+/+}$ mice and five to seven SPARC$^{-/-}$ mice. Wilcoxon test was performed with false discovery rate (FDR)-adjustment of p-values, p-value < 0.02 *
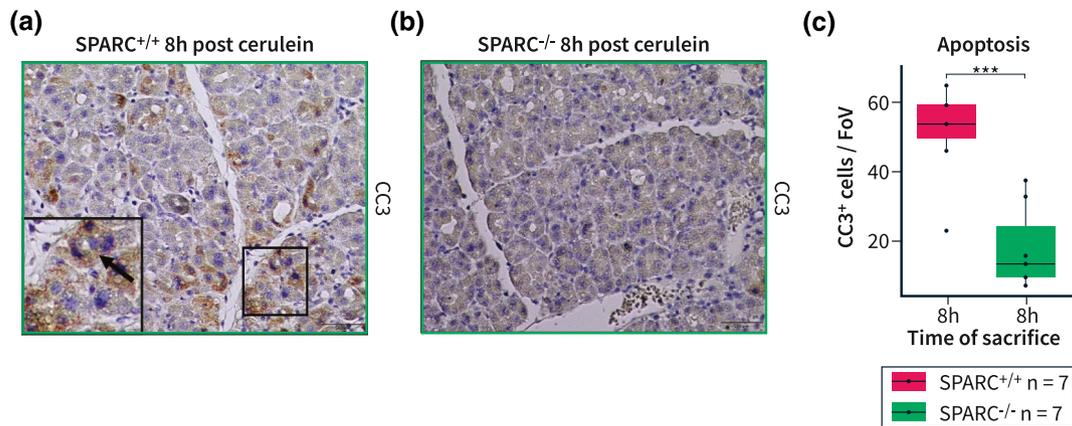
FIGURE 2 Immunohistochemistry for cleaved caspase-3 (CC3). 8 h after the last cerulein injection pancreata derived from (a) SPARC$^{+/+}$ and (b) SPARC$^{-/-}$ mice were harvested and stained for the apoptosis marker CC3. (c) CC3^{+} cells per fields of view were compared (n = 7 per group). t-test was applied, p-value < 0.001 ***
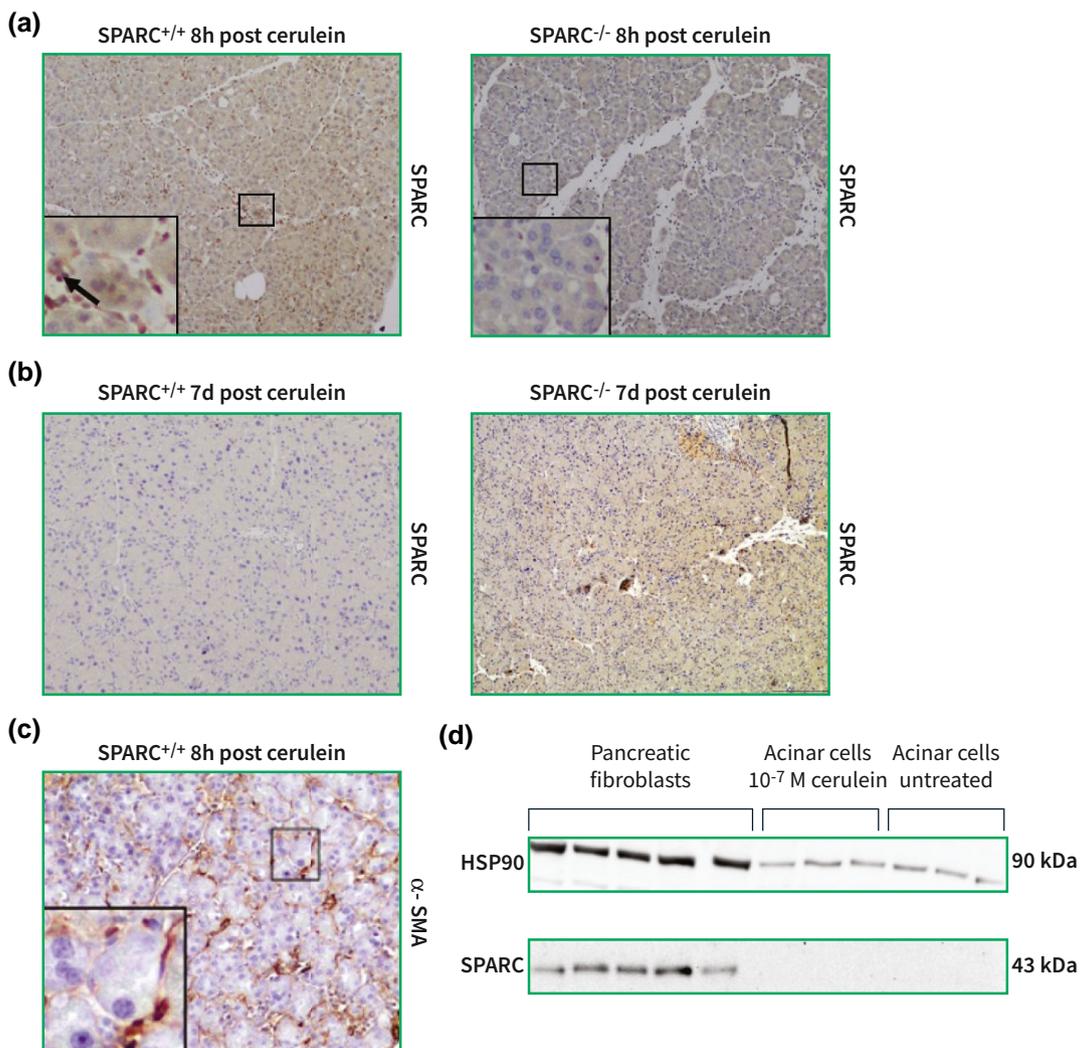
**FIGURE 3** SPARC expression in pancreatic fibroblasts upon cerulein induced Acute pancreatitis (AP). SPARC$^{+/+}$ and SPARC$^{-/-}$ mice were treated with cerulein for 2 consecutive days and pancreata were harvested after 8 h, 7 and 21 days after the last cerulein injection. (a) 8 h after the last cerulein injection multiple SPARC^{+} cells were observed in the periacinar space (arrow) SPARC$^{+/+}$ mice but not SPARC$^{-/-}$ littermates. (b) After 7 days almost no secreted protein acidic and rich in cysteine (SPARC) expression was detected in both subgroups. (c) Immunohistochemistry for α-SMA shows activated pancreatic stellate cells (PSCs) in periacinar space in SPARC$^{+/+}$ mice. (d) Immunoblot against SPARC shows robust SPARC expression in PSCs but no expression in cerulein-treated and untreated acinar cells

## Dynamics of serum parameters in acute phase of pancreatitis

Serum samples were collected at endpoint and analysed for amylase, lipase, and glucose levels, respectively. Interestingly, untreated SPARC$^{+/+}$ mice differed significantly in terms of amylase but not lipase serum levels compared to untreated SPARC$^{-/-}$ mice (Suppl. Figure 5a,b). Therefore, to compare the differences upon cerulein treatment, enzyme levels were normalized to the mean of untreated animals. In analogy to the histological grading, SPARC$^{+/+}$ mice showed significantly elevated amylase levels compared to their SPARC$^{-/-}$ littermates 8h after the last cerulein injection (Figure 4a). For lipase levels a similar tendency, though not statistically significant, was observed (Figure 4b). These fold changes of both enzymes significantly correlated with the histopathological necrosis score (Figure 4c,d). To further investigate the endocrine function, blood glucose was measured at different time points after pancreatitis induction. Here, both groups showed the same dynamics without any significant differences (Figure 4e).

Cerulein induced amylase secretion in acinar cells is independent of secreted protein acidic and rich in cysteine genotype

Cerulein treatment of isolated acinar cells from SPARC$^{+/+}$ and SPARC$^{-/-}$ mice leads to vesicle development at the basal site of cells within 30 min indicating a stimulation of the acinar exocrine function (Figure 5a,b). To investigate the impact of SPARC expression, acinar cells from $n = 3$ SPARC$^{+/+}$ and $n = 3$ SPARC$^{-/-}$ mice were isolated. Following stimulation, acinar cells were analysed for amylase content, and the proportion of newly secreted amylase was determined. Interestingly, the amylase release was dependent on the cerulein dose but independent of the SPARC genotype (Figure 5c). Considering the fact that we detected SPARC protein predominantly in periacinar cells, most likely PSCs, upon pancreatitis induction (Figure 3c,d), these results further support the notion that SPARC derived from the inflammatory pancreas microenvironment during the acute phase of cerulein-induced pancreatitis is involved in mediating the more severe phenotype in SPARC$^{+/+}$ mice.
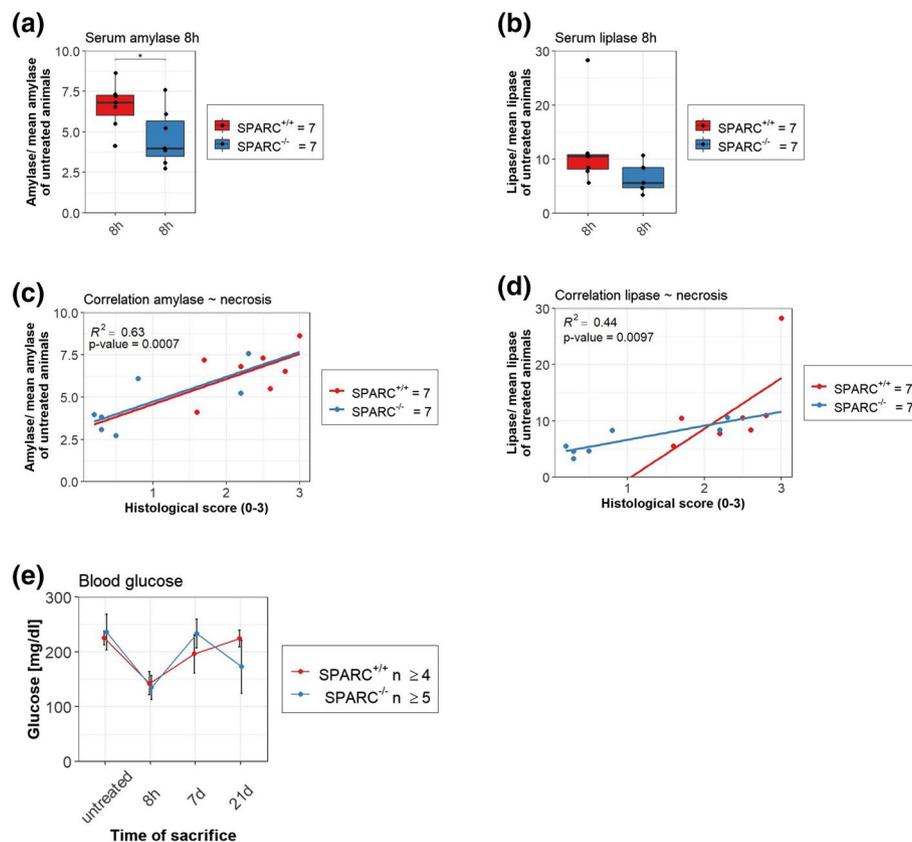
FIGURE 4 Dynamics of serum parameters in the acute phase of pancreatitis. SPARC$^{+/+}$ and SPARC$^{-/-}$ were sacrificed 8 h after the last cerulein injection and serum samples were analysed for (a) amylase and (b) lipase ($n = 7$ per group). Correlation was calculated between fold changes of (c) amylase and (d) lipase and necrosis 8 h after the last cerulein treatment. (e) Glucose levels at different time points after cerulein treatment. For (a), (b) and (e) t-test was performed and correlation in (c) and (d) was calculated with spearman method, p-value < 0.05 *
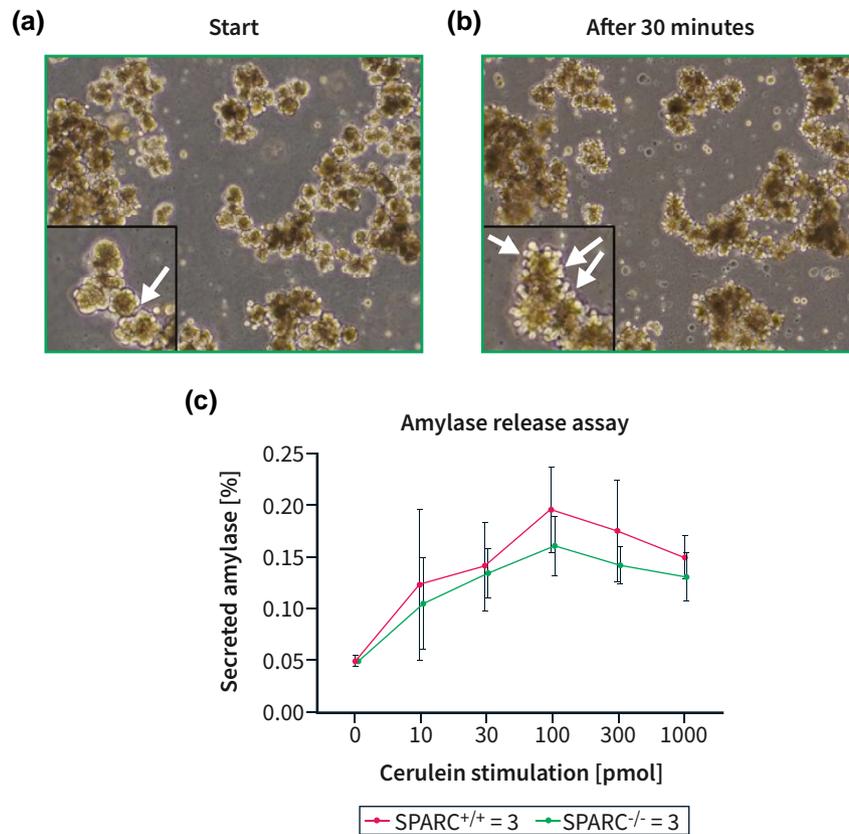
FIGURE 5 Cerulein induced amylase secretion is independent of secreted protein acidic and rich in cysteine (SPARC) expression. Primary isolated acinar cells derived from SPARC$^{+/+}$ and SPARC$^{-/-}$ mice were stimulated with 10-7 pmol cerulein and pictures were taken (a) immediately after stimulation and (b) 30 min later (secretory vesicles marked with white arrows). (c) Proportion of secreted amylase under different cerulein stimulations was similar in SPARC$^{+/+}$ and SPARC$^{-/-}$ mice ($n = 3$ per group). Wilcoxon test was performed with False discovery rate (FDR) adjustment of ($p > 0.549$)

Secreted protein acidic and rich in cysteine mediated severity of Acute pancreatitis is associated with increased levels of Chemokine Ligand 2 in CD45$^+$ positive immune cells.

Our data suggest that SPARC expression in periacinar cells may be causally involved in the severity of acute experimental pancreatitis in mice. To further investigate the regulatory mechanism of SPARC dependent pancreatic inflammation, we systematically assessed intrapancreatic cytokines using proteome profiler. To this end, pancreatic tissue lysates from SPARC$^{+/+}$ and SPARC$^{-/-}$ mice (each $n = 3$) were analysed. Out of 40 cytokines, nine cytokines were detected in pancreatic tissue lysates (Suppl. Figure 6A). Interestingly, only the C-C Motif CCL2 was significantly different between SPARC$^{+/+}$ and SPARC$^{-/-}$ mice upon cerulein induction (Figure 6a, Suppl Figure 6B). To validate this finding, Quantikine® ELISA was subsequently performed revealing a highly significant increase of CCL2 levels in SPARC$^{+/+}$ ($n = 6$) compared to SPARC$^{-/-}$ mice ($n = 6$) (Figure 6b). In line with the results from the histopathological evaluation upon cerulein induction, the increase of CCL2 levels was only seen during the acute and early phase of cerulein induced pancreatitis (8h after the last injection), whereas no differences were seen in untreated mice or after 7 days in SPARC$^{+/+}$ and SPARC$^{-/-}$ mice (Suppl. Figure 7).

To further investigate the cellular origin of CCL2, we performed Co-IF staining. To this end, pancreata were stained with antibodies against CD45 and CCL2. Interestingly, the majority of CD45$^+$ immune cells were also positive for CCL2 (Figure 6c), and a significantly increased expression was seen in pancreata from SPARC$^{+/+}$ mice (Figure 6d).

In conclusion, our results show that SPARC expression is induced during early AP in periacinar stellate cells resulting in an increased pancreatic damage (necrosis) and immune cell migration. In addition, immune cell derived CCL2 levels were significantly elevated in SPARC$^{+/+}$ mice compared to SPARC$^{-/-}$ mice pointing towards a potential link between SPARC and CCL2 in mediating the severity of AP.

Secreted protein acidic and rich in cysteine ablation results in reduced collagen I/III upon chronic cerulein stimulation

Secreted protein acidic and rich in cysteine is known as a modulator of ECM remodelling. To this end the induction in PSCs might result in
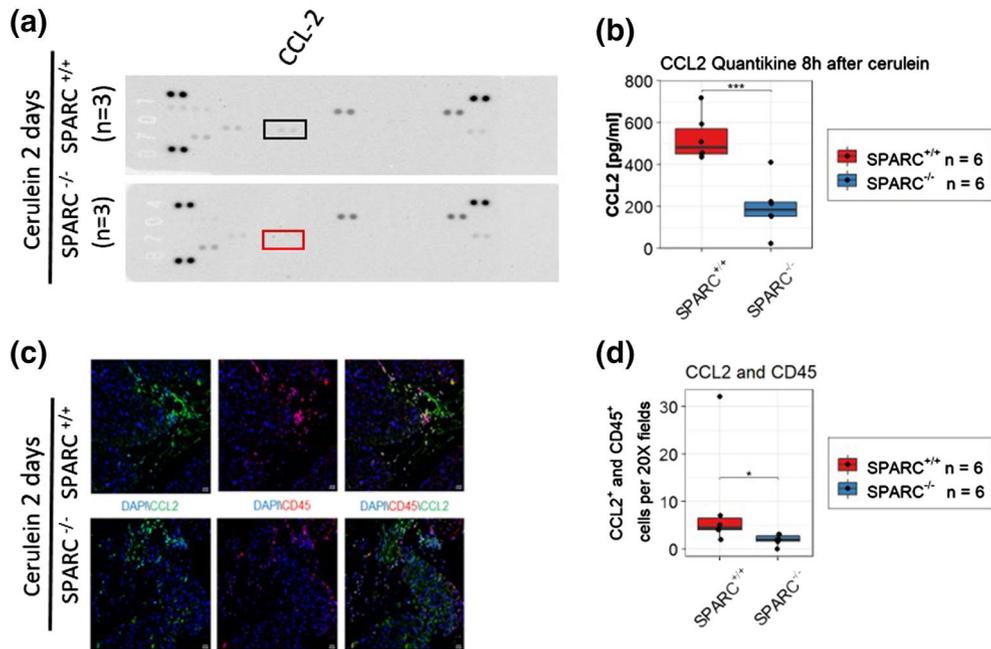
FIGURE 6 Elevated Chemokine Ligand 2 (CCL2) concentration in pancreatic immune cells in SPARC^{+/+} mice. (a) Representative pancreatic tissue lysates of SPARC^{+/+} and SPARC^{−/−} mice (3 pooled samples) were analysed with proteome profiler revealing a distinctly elevated expression of CCL2 in SPARC^{+/+} mice (in total $n = 6$ mice per group). (b) Quantikine® ELISA was performed measuring the concentration of CCL2 per SPARC^{+/+} and SPARC^{−/−} tissue lysates ($n = 6$ per group). For statistical testing, the t-test was applied. (c) co-immunofluorescence (Co-IF) for CD45^{+} and CCL2 showed co-localization of both markers. The cell number of CCL2^{+} and CD45^{+} cells was significantly increased in SPARC^{+/+} compared to SPARC^{−/−} mice. For quantification cells were counted in 5 high-power fields (HPF) per mouse and medians are depicted. Wilcoxon test was performed, p-values < 0.05*
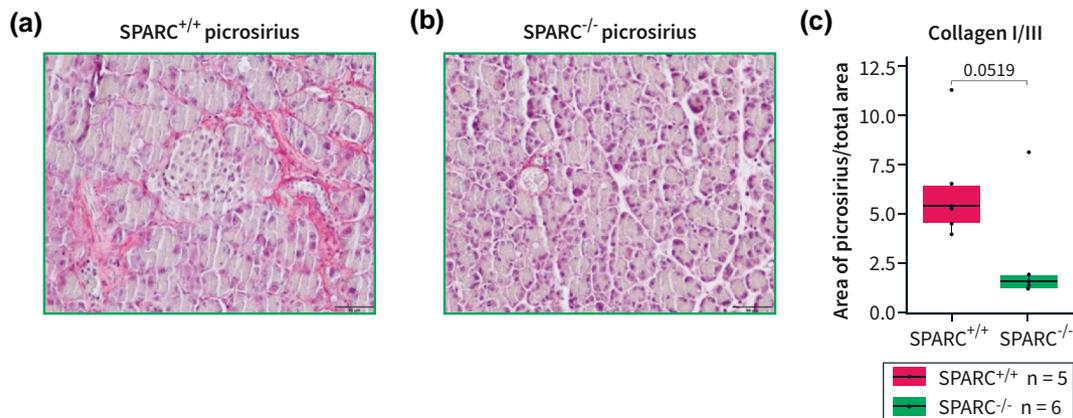
FIGURE 7 Increased collagen deposition due to prolonged cerulein treatment. Picrosirius staining for (a) SPARC^{+/+} and (b) SPARC^{−/−} pancreatic tissue after 4 weeks of cerulein injection. (c) Area of picrosirius was measured compared to total area ($p = 0.052$). Wilcoxon-test was performed

higher collagen production in a chronic inflammatory setting. A progressive organ fibrosis is a hallmark of CP. Indeed, Hu et al recently identified SPARC as a candidate marker for the progression from RAP to CP.[20] To further elucidate the role of SPARC in this disease continuum and in organ fibrosis, we treated SPARC^{+/+} and SPARC^{−/−} with cerulein trice a week for 4 consecutive weeks. Harvested pancreata were stained for picrosirius to evaluate collagen deposition (Figure 7a,b). To this end, SPARC^{+/+} mice showed a clear, yet not significant, increase of collagen deposits compared to

SPARC^{−/−} littermates (Figure 7c). This result may indeed provide first evidence that SPARC may mediate organ fibrosis in CP.

DISCUSSION

Here, we aim to address the functional role of SPARC during acute experimental pancreatitis. To this end, we employed an established cerulein-induced AP model in genetically engineered mice with

germline knock-out of the SPARC gene. Secreted protein acidic and rich in cysteine is a matricellular protein with a range of diverse function during wound healing, fibrosis and tumor progression.[29] In particular, the role of peritumoral SPARC expression during pancreatic tumor development, tumor progression and therapeutic resistance has been addressed in several preclinical and clinical studies.[18,24,30,31] SPARC is predominantly expressed by PSCs from the tumor microenvironment, whereas the SPARC promoter is aberrantly hypermethylated in tumor cells resulting in a loss of expression in pancreatic tumor cells.[32] Notably, only few data are currently available on the expression and function of SPARC in AP and CP.[33,34] A clinical pilot study in acute biliary pancreatitis found elevated serum SPARC levels that correlated with the clinical course of the disease, highlighting a potential role of SPARC during AP.[19] Indeed, a more recent systematic comparison of transcriptomic profiles from total pancreas, PSCs, acinar cells and immune cells identified SPARC as an important candidate marker for activated PSCs in AP. Moreover, SPARC was found to be differentially expressed in RAP, and highly expressed in two clinical datasets of CP.[20] However, whether the increasing SPARC expression from AP to RAP and CP in activated PSCs reflects the increasing number of persistently activated PSCs, or whether SPARC has functional implications has not been addressed experimentally before.

Here, we show for the first time that SPARC expression is induced in PSCs during experimental AP in mice and determines the early severity of the disease. Upon cerulein stimulation, SPARC$^{+/+}$ mice showed significantly more pancreatic damage as evidenced by elevated serum amylase levels, pancreatic apoptosis and necrosis as well as increased immune cell infiltrations compared to SPARC knock-out littermates. Remarkably, the observed differences between SPARC$^{+/+}$ and SPARC$^{-/-}$ mice during the acute and early phase of experimental pancreatitis completely resolved after 7 days, and no increased SPARC expression could be detected anymore. Mechanistically, we could show that genetic ablation of SPARC was associated with significantly reduced levels of intrapancreatic CCL2 derived from CD45^{+} immune cells in the pancreas microenvironment. Chemokine Ligand 2 is a proinflammatory inducible chemokine that belongs to the CC family and can recruit several subsets of immune cells, in particular macrophages, to the site of inflammation. For cerulein-induced AP, it was shown that CCL2-dependent monocyte/macrophage migration is required for pancreatitis induction.[35] In addition, Frossard et al. showed that genetic deletion of CCL-2 attenuated the course of experimental pancreatitis in mice further supporting the notion that CCL-2 is a key chemokine in modulating the severity of AP.[36] For the first time, we establish a potential link between SPARC and CCL2 for the induction of AP. Based on our observations, we speculate that PSC-derived but not acinar-cell derived SPARC induces CCL2 expression in immune cells that in turn is able to activate and recruit macrophages to the site of pancreatic inflammation. As PSCs are only briefly activated during the induction of AP in this model, it seems plausible that SPARC ablation only transiently attenuates the severity of experimental AP

in the acute early phase. However, recent data from RAP and CP suggest that SPARC could indeed be one of the key players that mediate the transition from AP to CP upon persistent PSC activation eventually resulting in pancreatic remodelling with loss of acinar cells and fibrosis.[20] Our data derived from prolonged cerulein treatment (4 weeks) may support this hypothesis. Here SPARC$^{-/-}$ mice show reduced levels of collagen deposition compared to their SPARC$^{+/+}$ littermates. Interestingly, it was shown that SPARC is able to shift the balance towards proinflammatory M1 polarization of macrophages thus promoting heart fibrosis.[37] These observations are in line with our findings of more severe inflammation in SPARC$^{+/+}$ animals. Furthermore, necrotic acinar cells may further induce SPARC expression in inflammatory cells in the pancreas that perpetuate the SPARC expression.

Our study has several limitations. First, our results are limited to the cerulein-induced model, however, other models such as the L-Arginine model or the pancreas-duct ligation model may show distinct results. However, experimental data show that CCL2 axis seems to be equally relevant for the induction of pancreatitis in the L-Arginine model.[35] Second, we show that genetic SPARC ablation attenuates the severity of experimental AP compared to SPARC$^{+/+}$ mice that are essentially wildtype for SPARC. Additional models with constitutive overexpression of SPARC may be helpful to further elucidate and support the notion that endogenous SPARC expression levels determine the severity of AP.

In summary, we show for the first time that SPARC is transiently induced in experimental AP in pancreatic fibroblasts. Ablation of SPARC results in a robust reduction of pancreatic necrosis, apoptosis and inflammation, and potentially links SPARC with induction of CCL2 induction which is known to be required for pancreatic inflammation in AP.

CONFLICTS OF INTEREST
The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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