Cytokine 54 (2011) 305–314

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Upregulation of PIAS1 protects against sodium taurocholate-induced severe acute pancreatitis associated with acute lung injury

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ARTICLE INFO

Article history:
Received 2 October 2010
Received in revised form 12 January 2011
Accepted 18 February 2011
Available online 17 March 2011

Keywords:
Pancreas
Lung
Protein inhibitor of activated STAT 1
Inflammation

ABSTRACT

The regulator of cytokine signaling known as protein inhibitor of activated STAT-1 (PIAS1) is increasingly understood to have diverse regulatory functions for inflammation, but its effect in inflammatory conditions such as severe acute pancreatitis (SAP) has not previously been reported. The aim of this study was to investigate the effect of upregulation of PIAS1 on SAP associated with acute lung injury (ALI), and its subsequent effect on disease severity. Sprague–Dawley rats were given an IV injection of adenovirus serotype 5 (Ad5)/F35-PIAS1, Ad5/F35-vector or saline before induction of SAP. The control group received only a sham operation. Lung and pancreas samples were harvested 16 h after induction. The protein levels of PIAS1 in tissue were investigated. The severity of pancreatic injury was determined by a histological score of pancreatic injury, serum amylase, and pancreatic water content. The lung injury was evaluated by measurement of pulmonary microvascular permeability, lung myeloperoxidase activity and malondialdehyde levels. The survival rates of rats were also analyzed. The results found that in Ad5/F35-PIAS1 treated rats, serum tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 levels were decreased but showed no influence on the levels of IL-10, and the severity of pancreatic tissue injury was less compared with either untreated SAP or Ad5/F35-vector treated rats ($P < 0.01$). The administration of Ad5/F35-PIAS1 in SAP-induced rats downregulated the activity of the signal transducer and activator of transcription-1 (STAT1) pathway and the expressions of matrix metalloproteinase-9 (MMP-9) and intercellular adhesion molecule (ICAM)-1 protein in lung. Thus, compared with the untreated SAP rats, the inflammatory response and the severity of ALI decreased, and the survival rates increased ($P < 0.01$). These findings suggest that PIAS1 could augment anti-inflammatory activity by inhibiting STAT1, thus attenuating the severity of SAP associated with ALI.

1. Introduction

Acute pancreatitis is the sudden onset of inflammation of the pancreas, affecting ~30–40 individuals per 100,000. It carries an overall mortality rate of 10–15% [1], but the rate approaches 30–40% for especially severe cases [2]. Deaths associated with severe acute pancreatitis (SAP) are mainly due to its major complication, the development of multiple organ dysfunction syndromes (formerly known as multiple organ failure). Acute lung injury (ALI) is a major component of this complication, clinically manifested as acute respiratory distress syndrome [3]. Despite significant advances in understanding the pathogenesis of ALI in SAP and its management, the mortality rate remains unacceptably high.

Studies have shown that pancreatic damage due to SAP leads to the release of systemic inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β. These cytokines may result in distant organ damage and the development of ALI. They therefore play a key role in the pathogenesis of SAP with ALI, and are ultimately responsible for the majority of deaths associated with this condition [4]. Related studies found that the transcriptional induction of genes involved in the release of inflammatory cytokines associated with SAP is controlled by various regulated factors, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway [5].

A family of negative regulators of the JAK/STAT signaling pathway is that of the protein inhibitor of activated STAT (PIAS), with four members: PIAS1, PIAS3, PIASx, and PIASy. Gene activation analyzes show that PIAS1 selectively regulates a subset of STAT1-dependent genes, with a notable preference for inflammatory response [6]. Related studies found that PIAS1-null mice demonstrated a hypersensitivity to lipopolysaccharide-induced septic shock, and others indicate that PIAS1 is involved into in response to inflammatory stimuli, such as TNF-α and lipopolysaccharide [7].

PIAS1 participates in anti-inflammatory responses by regulating the transcription factor, nuclear factor-κ-light-chain-enhancer of

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doi:10.1016/j.cyto.2011.02.016

activated B cells. This transcription factor is involved in the induction of approximately 200 genes, many of which are involved in inflammatory responses [8–10]. In addition, PIAS1 has also been suggested to promote the sumoylation of the transcription factor peroxisome proliferator-activated receptor-γ, resulting in the trans-repression of inflammatory gene activation [11]. These findings support a hypothesis that a focus on the regulation of PIAS1 might be a novel therapeutic strategy for the treatment of inflammatory disorders. However, past studies have not fully addressed whether the administration of PIAS1 would prove equally efficacious if administered upon the onset of SAP. As well, the precise mechanisms determining the effect of PIAS1 on SAP relative to the degree of lung injury are largely unknown.

The purpose of this study was to elucidate the up-regulating effect of PIAS1 on SAP associated with ALI. We performed a series of studies to explore for a new therapeutic agent for SAP, including total water content, scoring of lung injury, neutrophil count in bronchoalveolar lavage fluid (BALF), lung tissue myeloperoxidase (MPO) activity, malondialdehyde (MDA) levels and inflammatory mediators levels, and a survival analysis of rats with SAP.

2. Materials and methods

2.1. Animals and reagents

The male Sprague–Dawley rats, 150–200 g, obtained from the animal experimental department of Ruijin Hospital were randomly divided into the following groups.

In the SAP group ($n = 20$), 24 h prior to the start of the experiments the rats were deprived of food but allowed access to water. The rats were anesthetized by intraperitoneal injection of pentobarbital (30%, 0.15 mL/100 g). SAP was induced by retrograde infusion of 3.5% sodium taurocholate (1.5 mL/kg, Sigma–Aldrich, St. Louis, MO, USA) into the pancreatic duct transduodenally for 2–3 min via a 24-gauge angiocath using a constant infusion rate of 100 μL/min under laparotomy. The abdominal wounds were closed and the rats returned to their cages with free access to water and food after surgery.

In treated groups, we used replication-defective adenovirus serotype 5/F35 (Ad5/F35) as the vector. Ad5/F35-PIAS1 was constructed by Benyuanzhengyang Bio. (Shanghai, China) using the previously described method [12]. PIAS1 cDNA, containing the full-length translated regions, were sub-cloned into the PDC316-MCMV-EGFP transfer plasmid. This plasmid was cotransfected into 293 cells, along with a fragment of the plasmid containing the Ad5/F35 adenoviral vector. Additionally, an Ad5/F35 containing an empty expression cassette was constructed for use as a control (Ad5/F35-vector). All of the viral constructs were similar with the exception of the transgene, and the production and purification procedures were identical. The dosage of Ad5/F35 delivery to these animals was based on our previous studies' results (Un-published data). Ad5/F35-PIAS1 or Ad5/F35-vector (3.6×10^8 PFU/100 g in 25 μL sterile PBS) was delivered by IV injection through the penile vein in rats at 10 min, on day 1, or on day 2 prior to SAP induction ($n = 10$, 10 and 10, respectively for each treatment). For these treatment groups, SAP was induced with 3.5% sodium taurocholate as described above. Based on the results of PIAS1 protein expression levels, on day 2 before SAP induction, rats who had received the Ad5/F35-PIAS1 or Ad5/F35-vector treatment were selected for future study.

In the control group ($n = 10$), rats were given only a sham operation, consisting of administration of an equal volume of PBS (with no SAP induction, Ad5/F35-PIAS1 or Ad5/F35-vector treatment).

Blood samples, pancreatic and lung tissues were harvested at 16 h after the onset of induction according to our previous methods [13]. The serum was separated from blood by a 1600*g*, 10 min centrifugation for examination. The experiments were conducted according to the Guidelines of the Shanghai Animal Use and Care Committee and the National Animal Welfare Law.

2.2. Morphological examination

Pancreatic and lung tissue samples were fixed in 4% formaldehyde overnight and subsequently dehydrated through a graded ethanol series. After impregnation in paraffin wax, tissue samples were cut into blocks (4 μm). Pancreatic and lung tissues were stained with hematoxylin–eosin (H&E) and examined by light microscopy. Sections were examined by an experienced morphologist, who was blinded to the sample identity, for tissue injury. For this study, five randomly chosen microscopic fields were examined for each tissue sample and given a histological score for injury according to the previously described method [14,15].

2.3. Wet/dry weight ratio of pancreatic or lung tissue

Pancreatic or lung tissue edema was evaluated by tissue water content. A portion of the pancreatic or lung tissue was taken immediately after sacrifice, to trim fat and weigh. Tissue water content was determined by calculating the wet weight/dry weight ratio according to the formula: [(wet weight − dry weight)/dry weight] × 100%, where the wet weight was the initial weight of the respective tissue and the weight after incubation at 72 °C for 24 h was the dry weight.

2.4. Biochemical assays

Serum amylase level was determined by a Beckman CX7 Chemistry Analyzer (Beckman Coulter, Fullerton, CA, USA). Serum TNF-α, IL-1β, IL-6 and IL-10 levels were evaluated by enzyme-linked immunosorbent assay (ELISA kit, Mai Bio, Shanghai, China).

2.5. Lung tissue MPO and MDA assays

To carry out the assays, 1 g lung tissue samples were thawed, homogenized in 1 M PBS (pH 7.4) and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was assayed for MPO activity and MDA concentration with test kits (Jianchen Bio, Nanjing, China). All procedures were done in accordance with the manufacturer's instructions.

2.6. Neutrophil counting in bronchoalveolar lavage fluid (BALF)

Rats were anesthetized with intraperitoneal pentobarbital, and a median sternotomy allowed for exposure of both lungs. The trachea was exposed and inserted with an IV infusion needle. After ligating the hilum of the right lung, the left lung was lavaged five times with 1 mL ice-cold PBS. The recovery ratio of the fluid was about 90%. The BALF was then centrifuged at 1000*g* for 10 min to separate cells from the supernatant. Cells were suspended in saline and the number of neutrophils was counted with a hemocytometer.

2.7. Western blot assay

Tissue samples were washed twice with PBS, homogenized with a tissue homogenizer in RIPA buffer (Biyuntian Shanghai, China), and centrifuged at 12,000*g* at 4 °C for 10 min. The supernatants were collected and stored at −80 °C. Protein concentration of each sample was determined by bicinchoninic acid protein assay (Biyuntian, Shanghai, China). Each sample was adjusted up to the desired protein content of 40 μg, denatured in loading buffer (62 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.003% bromophenol blue) and separated by electrophoresis on a

6% SDS–polyacrylamide gel at 100 V for 120 min. The separated proteins were transferred to a polyvinylidene difluoride membrane by using transfer buffer (39 mM glycine, 48 mM Tris pH 8.3, 20% methanol) at 200 mA for 90 min. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)-0.1% Tween for 1 h at room temperature, washed three times for 10 min in TBS-0.1% Tween. They were then incubated with a primary antibody, either STAT1, phosphorylated STAT1 (P-STAT1; Cell Signaling Biotechnology, Danvers, MA, USA, diluted to 1:1000), PIAS1, intracellular adhesion molecule (ICAM)-1, or matrix metalloproteinase-9 (MMP-9; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, diluted to 1:500), each in TBS-0.1% Tween, overnight at 4 °C. After being washed 3 × for 10 min in TBS-0.1% Tween, the membranes were incubated with a second antibody: peroxidase-conjugated goat anti-rabbit IgG, rabbit anti-goat IgG or rabbit anti-mouse IgG (Kangcheng, Shanghai, China) for 1 h at room temperature. After washing, the membranes were detected by enhanced chemiluminescence methods (Amersham, Piscataway, NJ, USA). The PIAS1, STAT1, P-STAT1, ICAM-1 and MMP-9 proteins were quantified by scanning densitometry using a bio-image analysis system (Bio-Rad, Baltimore, MD, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein was determined in a similar manner with anti-GAPDH antibody (Sigma, St. Louis, MO, USA, diluted to 1:500) as an endogenous control for other proteins.

2.8. Immunohistochemical localization of PIAS1

A volume of 4 μm paraffin embedded pancreatic or lung tissue sections was deparaffinized, mounted on poly-L-lysine-coated glass slides, and incubated with PIAS1 antibody (1:50 dilution). Protein

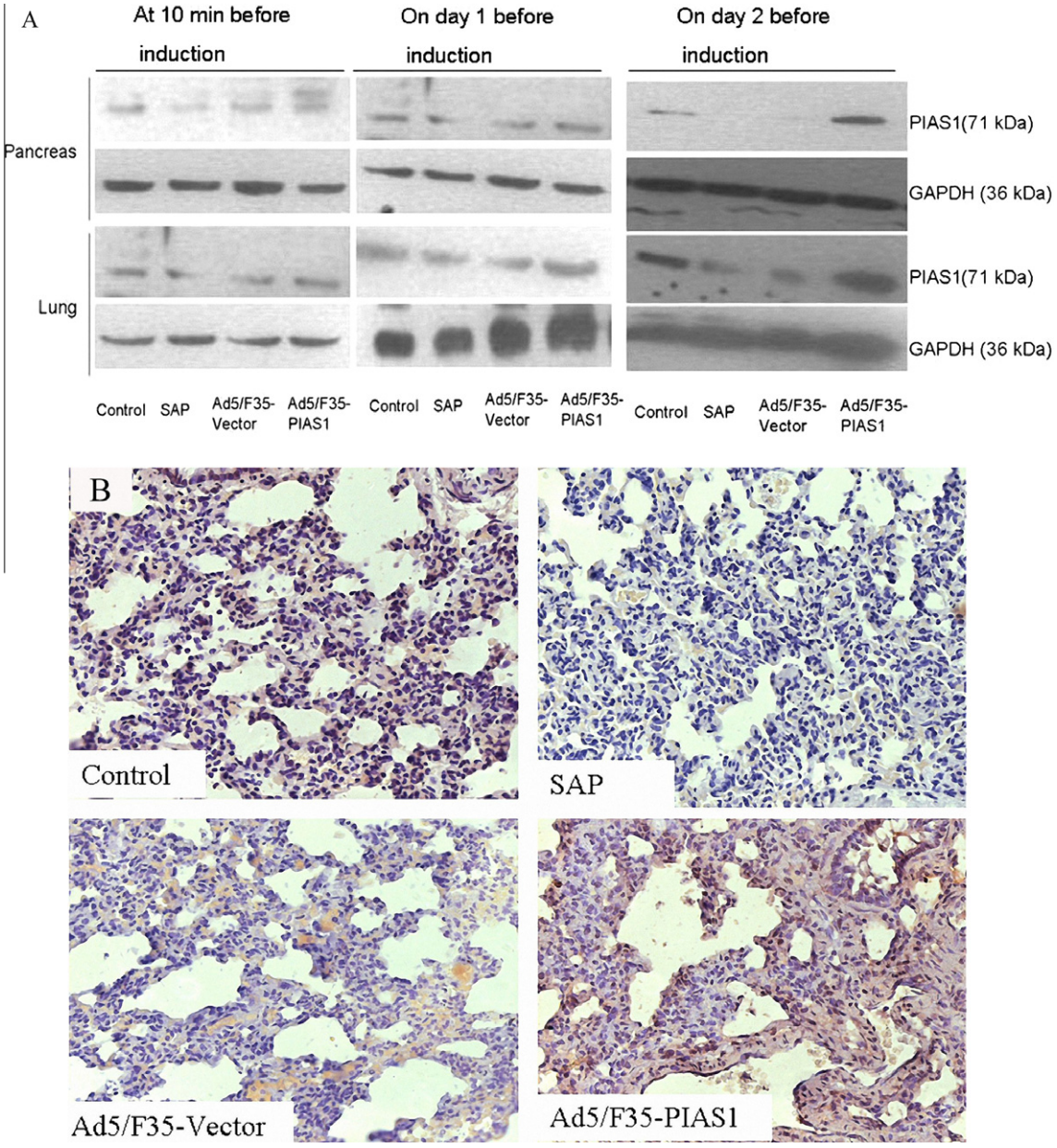

Fig. 1. Representative Western blot of proteins in lung and pancreatic tissues, detected with PIAS1 and GAPDH antibodies (A). Treatments administered at different time points prior to SAP induction. Ad5/F35-PIAS1or Ad5/F35-vector treated rats, SAP and control rats. Immunohistochemical localization of PIAS1 expression was observed in lung (B) and pancreatic tissues (C) in the Ad5/F35-PIAS1 or Ad5/F35-vector treated rats on day 2 before SAP induction, SAP and control rats: lung and pancreatic cells exhibiting level of PIAS1 protein expression in Ad5/F35-PIAS1 treated rats, slight PIAS1 staining was found in control rats; PIAS1 protein expression was not remarkable in SAP and Ad5/F35-vector treated rats. 200×.

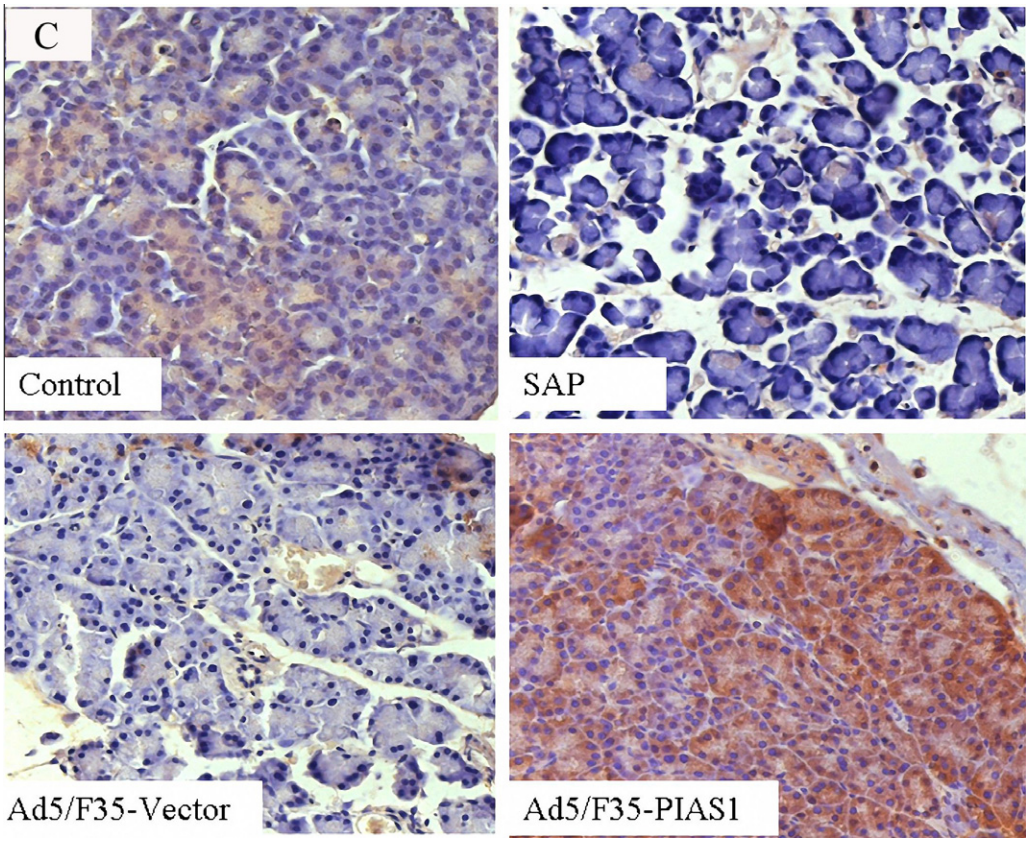

Fig. 1 (*continued*)

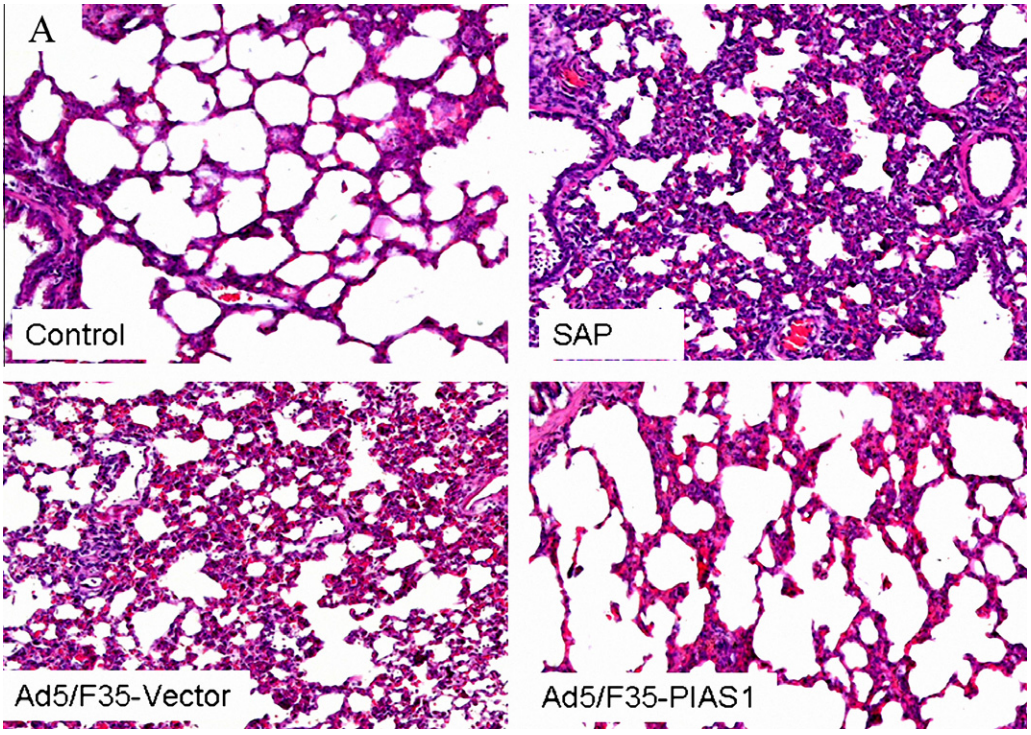

Fig. 2A. Histological examination of the lung in each group (H&E staining). There were no remarkable pathologic changes in control rats. In the untreated SAP and Ad5/F35-vector treated rats, the lungs show widespread alveolar wall thickness caused by edema, severe hemorrhage in the alveolus, alveolus collapse and obvious inflammatory cell infiltration. In Ad5/F35-PIAS1 treated rats, the only changes in the lung were edema, and mild hemorrhage in the alveolus. 100×.

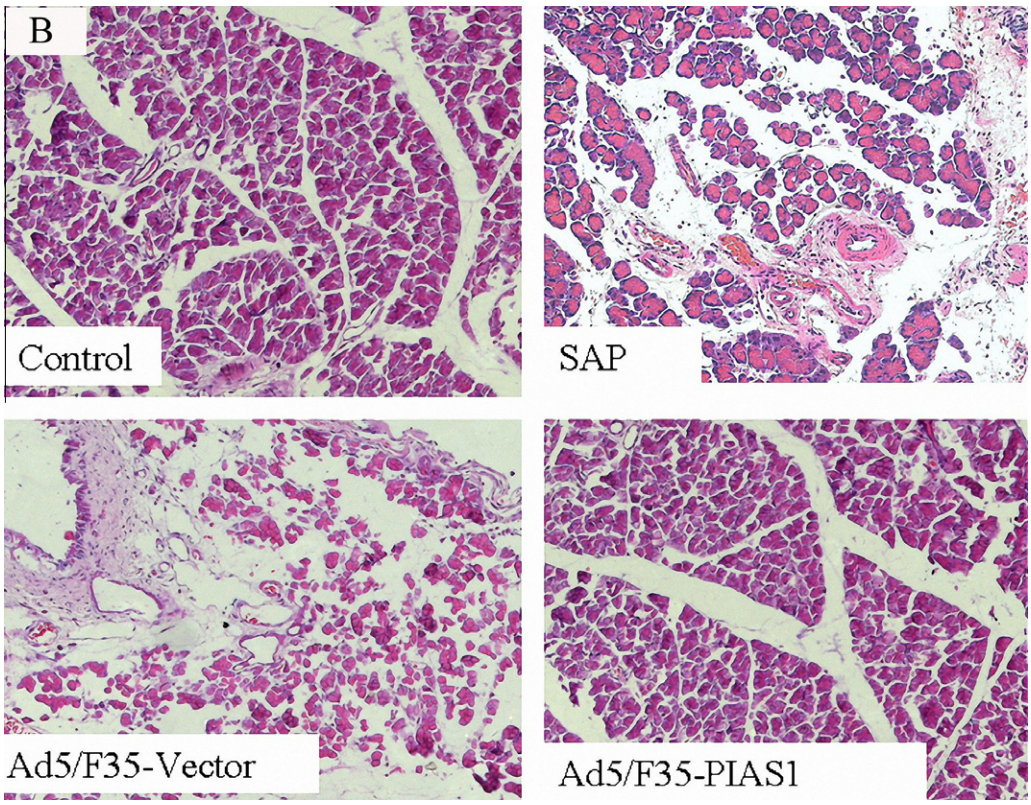

Fig. 2B. Histological examination of the pancreas in each group (H&E staining). There were no remarkable pathologic changes in control rats. Interstitial edema was observed in Ad5/F35-PIAS1 treated rats. Broad necrosis of acinar cells, erythrocyte effusion and interstitial edema were recognized in rats with SAP and Ad5/F35-vector treated rats. 100×.

Table 1
Wet/dry weight ratio of pancreatic tissue, serum amylase levels and histological scores of pancreatic injury in each experimental group.

Groups	Control	SAP	Ad5/F35-vector treated	Ad5/F35-PIAS1 treated
Wet/dry weight ratio of pancreatic tissue (%)	0.64 ± 0.12	1.61 ± 0.37[a,b]	1.54 ± 0.51[a,b]	0.96 ± 0.42
Serum amylase (U/L)	1386 ± 259	2353 ± 703[a,b]	2251 ± 681[a,b]	1594 ± 281[a]
Total score of pancreatic injury	1.10 ± 0.31	8.20 ± 1.98 [a,b]	7.5 ± 1.35 [a,b]	5.2 ± 1.61 [a]
Edema	1.10 ± 0.31	2.00 ± 0.00	1.90 ± 0.31	1.90 ± 0.31
Vascular change	0.00 ± 0.00	2.80 ± 0.78	2.20 ± 0.78	1.60 ± 0.84
Fat necrosis	0.00 ± 0.00	1.80 ± 0.42	1.80 ± 0.42	0.90 ± 0.31
Acrinar necrosis	0.00 ± 0.00	2.30 ± 0.82	1.80 ± 0.91	1.10 ± 0.73
Calcification	0.00 ± 0.00	0.80 ± 0.78	0.80 ± 0.63	0.40 ± 0.51

Values are means ± SD.
[a] Compared to the control group: $P < 0.01$.
[b] Compared to the Ad5/F35-PIAS1 treated group: $P < 0.01$.

antigen expression was detected using biotin-conjugated goat anti-rabbit IgG (Maixin Biotech, Fuzhou, China) and was visualized by a 10 min application of 3,3-diaminobenzidine. The sections were counterstained with hematoxylin to identify nuclei and observed under a light microscope. Random observation of five high microscope views was used to evaluate staining results. Brown staining cells were defined as positive protein expression cells.

2.9. Survival studies

Additional groups of 10 rats each were treated as described above, and were included in survival studies. The rats were allowed free access to food and water, and the survival time was recorded for 48 h.

2.10. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistics were done using Statistical Package for the Social Sciences (SPSS) software, version 10.5. The one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests was used for comparisons. The survival curve was estimated using the Kaplan–Meier method. The log-rank test was used to compare survival time in each group. The life table method was used to evaluate the survival rate. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Expression of PIAS1 protein

On the Western blot, PIAS1 protein was very weakly distinguishable in lung and pancreas between the untreated SAP and Ad5/F35-vector treated rats. However, treatment with Ad5/F35-PIAS1 resulted in a much greater increase in the PIAS1 protein expression level in lung and pancreas, and more so when the treatment was administered on day 2 than at either 10 min or 1 day before SAP induction (Fig. 1A). The expressions of PIAS1 were also

Table 2
Wet/dry weight ratio of lung, histological scores of lung injury in each experimental group.

Groups	Control	SAP	Ad5/F35-vector treated	Ad5/F35-PIAS1 treated
Wet/dry weight ratio of lung tissue (%)	0.83 ± 0.33	2.47 ± 0.41[a,b]	2.33 ± 0.50[a,b]	1.40 ± 0.24[a]
Total score of lung injury	0.80 ± 0.63	11.40 ± 1.89[a,b]	10.40 ± 1.57[a,b]	5.80 ± 1.22[a]
Alveolar congestion	0.70 ± 0.48	3.00 ± 0.81	2.80 ± 1.03	1.50 ± 0.52
Hemorrhage	0.00 ± 0.00	2.8 ± 0.91	2.30 ± 0.48	1.20 ± 0.63
Infiltration of neutrophils	0.10 ± 0.31	3.10 ± 0.87	3.00 ± 0.81	1.60 ± 0.69
Thickness of alveolar wall	0.00 ± 0.00	2.50 ± 0.70	2.30 ± 0.82	1.60 ± 0.51

Values are means ± SD.
[a] Compared to the control group: $P < 0.01$.
[b] Compared to the Ad5/F35-PIAS1 treated group: $P < 0.01$.

Table 3
Changes of inflammatory response of lung tissues in each experimental group.

Groups	Control	SAP	Ad5/F35-vector treated	Ad5/F35-PIAS1 treated
MPO (U/g)	0.85 ± 0.67	2.98 ± 1.43[a,b]	2.82 ± 1.79[a,b]	1.18 ± 0.12
MDA (nmol/mg)	1.60 ± 0.95	4.32 ± 2.30[a,b]	4.21 ± 1.37 [a,b]	1.60 ± 0.73
Neutrophil counting in BALF (10^3/L)	7.22 ± 7.34	75.90 ± 38.20[a,b]	67.80 ± 38.15[a,b]	33.10 ± 11.47

Values are means ± SD.
[a] Compared to the control group: $P < 0.01$.
[b] Compared to the Ad5/F35-PIAS1 treated group: $P < 0.01$.

Table 4
Serum TNF-α, IL-1β, IL-6, and IL-10 levels in each experimental group.

Groups	Control	SAP	Ad5/F35-vector treated	Ad5/F35-PIAS1 treated
TNF-α (pg/mL)	48.60 ± 17.36	93.10 ± 23.79[a,b]	104.40 ± 43.36[a,b]	57.80 ± 18.58
IL-1β (pg/mL)	38.60 ± 20.51	86.30 ± 36.02[a,b]	99.50 ± 30.91[a,b]	55.70 ± 21.13
IL-6 (pg/mL)	57.30 ± 18.77	155.90 ± 36.14[a,b]	134.30 ± 50.55[a,b]	87.80 ± 20.73
IL-10 (pg/mL)	78.70 ± 18.17	55.20 ± 13.96[a]	52.54 ± 13.92[a]	50.01 ± 15.28[a]

Values are means + SD.
[a] Compared to the control group: $P < 0.01$.
[b] Compared to the Ad5/F35-PIAS1 treated group: $P < 0.01$.

investigated by immunohistochemical staining in lung and pancreatic tissue of all rats. The results showed that in the Ad5/F35-PIAS1 treated rats, there was remarkable positive staining for PIAS1 in endothelial cells of lung and pancreatic acinar cells. However, PIAS1 was weakly expressed in control rats, and there was no remarkable PIAS1 staining in either the untreated SAP or Ad5/F35-vector treated rats (Fig. 1B and C).

3.2. Histological changes of pancreatic and lung tissue

Lung tissue from control rats showed a normal structure and no histopathological changes under a light microscope. In untreated SAP and Ad5/F35-vector treated rats, the lung tissue stained with H&E indicated a widespread increase in alveolar wall thickness caused by edema, severe hemorrhage in the alveolus, alveolus collapse and obvious inflammatory cell infiltration. In the Ad5/F35-PIAS1 treated rats, the histopathological changes of lung tissue were minor compared with those in the untreated SAP and Ad5/F35-vector treated rats, especially for hemorrhage in the alveolus (Fig. 2A). The infiltration of neutropils and mononuclear cells, interstitial edema and focal necrotic areas were seen in the pancreatic tissue of both untreated SAP and Ad5/F35-vector treated rats. The morphological changes in pancreatic tissue of Ad5/F35-PIAS1 treated rats included intralobular edema, inflammatory infiltrate, and acinar cell necrosis, but these were greatly reduced (characterized only by slightly interstitial edema), and without obvious parenchyma necrosis and hemorrhage. There were no observable pancreatitis pathologic changes in control rats (Fig. 2B).

The severity of pancreatic injury in rats based on histological score of pancreatic injury, the ratio of wet /dry weight of pancreatic tissue and serum amylase levels, are summarized in Table 1. The results showed that the levels of these markers for inflammation were significantly lower in Ad5/F35-PIAS1 treated rats as compared to those of the untreated SAP and Ad5/F35-vector treated rats ($P < 0.01$). However, the histological score of pancreatic injury and serum amylase levels in the Ad5/F35-PIAS1 treated rats were higher than for the control rats ($P < 0.01$). The levels of the above-mentioned markers showed no statistically significant difference between the untreated SAP and Ad5/F35-vector treated rats ($P > 0.05$).

The tests for levels of severity of lung injury in rats, including the histological score of lung injury and the ratio of wet/dry weight of lung tissue, are summarized in Table 2. Compared to the control rats, the histological score of lung injury and ratio of wet/dry weight of lung were significantly increased in both the untreated SAP and Ad5/F35-vector treated rats ($P < 0.01$). The increase in histological score of lung injury and ratio of wet/dry weight of lung was significantly reduced in the Ad5/F35-PIAS1 treated rats ($P < 0.01$) compared to either untreated SAP or Ad5/F35-vector treated rats, although still higher than in the control rats ($P < 0.01$). The levels of the above-mentioned markers showed no statistical difference between the untreated SAP and Ad5/F35-vector treated rats ($P > 0.05$).

3.3. Detection of inflammatory response of lung

The MPO activity and concentrations of MDA in the lung were increased in the untreated SAP and Ad5/F35-vector treated rats compared to that of the controls rats ($P < 0.01$, respectively), but there was no significant different between the SAP and Ad5/F35-

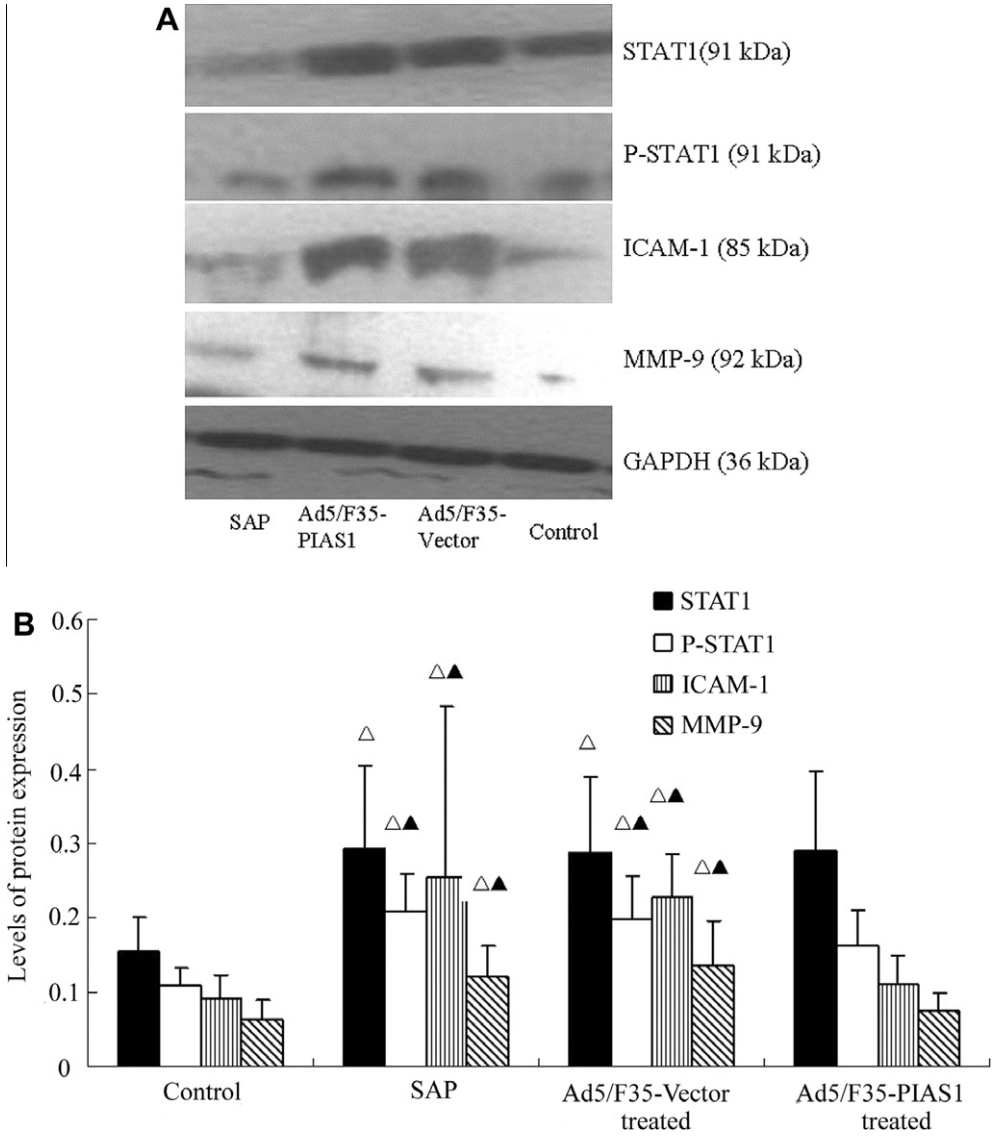

Fig. 3. Representative Western blot of proteins in lung detected with STAT1, P-STAT1, ICAM-1, MMP-9 and GAPDH antibodies in each group. The graph shows a densitometric analysis of Western blot; the ratios of STAT1, P-STAT1, ICAM-1, MMP-9 to GAPDH band density was determined for each group. △ Compared to the control group: $P < 0.01$; ▲ compared to the Ad5/F35-PIAS1 treated group: $P < 0.01$.

vector treated rats ($P > 0.05$). However, in Ad5/F35-PIAS1 treated rats, the upregulation of PIAS1 protein markedly decreased the MPO activity and MDA concentration (vs. the untreated SAP and Ad5/F35-vector treated rats, $P < 0.01$; vs. the control rats, $P > 0.05$, respectively). The number of neutrophils in BALF showed increases in rats who received the untreated SAP and Ad5/F35-vector treatment, as compared with that seen in the lungs of Ad5/F35-PIAS1 treated and control rats ($P < 0.01$). There were no significant difference between the untreated SAP and Ad5/F35-vector treated rats ($P > 0.05$) (Table 3).

3.4. Comparison of serum TNF-α, IL-1β, IL-6 and IL-10 levels

The TNF-α, IL-1βand IL-6 serum levels were significantly decreased in Ad5/F35-PIAS1 treated rats in comparison with those of SAP and Ad5/F35-vector treated rats ($P < 0.01$), and there was no difference between the Ad5/F35-PIAS1 treated and control rats ($P > 0.05$). In addition, the serum levels of TNF-α, IL-1βand IL-6 showed no significant difference between the untreated SAP and Ad5/F35-vector treated rats ($P > 0.05$). Anti-inflammatory cytokine IL-10 levels were greatly deceased in untreated SAP rats compared to control rats ($P < 0.01$), and were also lower in Ad5/F35-PIAS1 or Ad5/F35-vector treated rats than control rats ($P < 0.01$, respectively). There was no difference in IL-10 levels in Ad5/F35-PIAS1 or Ad5/F35-vector treated rats compared to that of untreated SAP rats ($P > 0.05$) (Table 4).

3.5. Pulmonary expression of inflammatory-regulated mediators

Western blot analysis of inflammatory-regulated mediators is shown in Fig. 3. To gain direct evidence for the mechanism leading to the reduction of the inflammatory response of lung induced by PIAS1 in rat with SAP, we determined the levels of un-phosphorylated and phosphorylated STAT1 protein in lung tissue. The phosphorylation of STAT1 protein (as assayed with P-STAT 1 antibody) in the lungs of untreated SAP and Ad5/F35-vector treated rats was enhanced compared to that of control rats ($P < 0.01$). In contrast, the treatment of SAP rats with Ad5/F35-PIAS1 led to the abrogation of phosphorylation of the STAT1 protein ($P < 0.01$, compared to untreated SAP and Ad/F35-vector treated rats), there

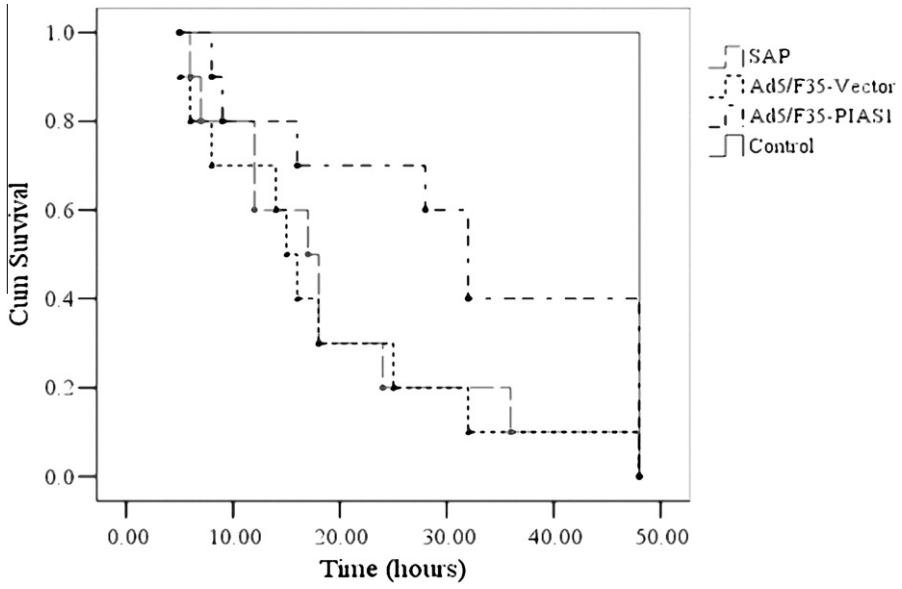

Fig. 4. Alterations in the survival rate during 48 h after induction in each group. There were 10 animals in each group. The survival rate was estimated by the Kaplan–Meier method and compared by log-rank test.

was no difference in this regard when compared with the control rats ($P > 0.05$). However, the un-P-STAT1 protein levels (as assayed with STAT 1 antibody) for all SAP-induced rats were higher compared to that of control rats ($P < 0.01$). ICAM-1 protein expression in the lung revealed that its 85-kDa protein expression levels were increased in the untreated SAP and Ad5/F35-vector treated rats, compared with either the Ad5/F35-PIAS1 treated or control rats ($P < 0.01$). Moreover, the increase of MMP-9 protein expression in lung was much more marked in the untreated SAP and Ad5/F35-vector treated rats compared to that of the Ad5/F35-PIAS1 treated and control rats ($P < 0.01$). The levels of ICAM-1 and MMP-9 proteins showed no difference between either the Ad5/F35-PIAS1 treated and control rats or between the Ad5/F35-vector treated and untreated SAP rats ($P > 0.05$).

3.6. Survival study

The average survival time of SAP-induced rats receiving Ad5/F35-PIAS1 was 31.70 ± 5.16 h (95% confidence interval (CI): 21.47–41.82). The average survival time of SAP-induced rats receiving Ad5/F35-vector was 19.80 ± 4.17 h (95% CI: 11.62–27.97). As for the untreated SAP rats, the average survival time was 18.70 ± 4.19 h (95% CI: 10.48–26.91). The 48 h survival rate was significantly improved in SAP rats with Ad5/F35-PIAS1 treatment compared with the untreated SAP and Ad5/F35-vector treated rats ($P < 0.05$). No statistically significant difference was seen in the survival rate between the untreated SAP and Ad5/F35-vector treated rats (Fig. 4).

4. Discussion

The use of Ad5-based gene therapy offers several significant advantages over the administration of recombinant proteins. For example, the pretreatment of mice with Ad5 delivery-IL-10 reduced weight loss, attenuated the release of inflammatory cytokines, and reduced mortality in mice with sepsis; pretreatment with a control Ad5 delivery did not significantly exacerbate the inflammatory response. The results suggest that the injection of Ad5 delivery-IL-10 could reduce the severity of sepsis in rats [16]. Therefore, the gene transfer has been proposed as a novel method to produce cytokine inhibitors or antagonists in inflammation, in the present study the delivery of adenovirus-based gene was used for the severe inflammatory disease SAP to explore its effect of action.

Expression with Ad5 vectors is rapid, with appearance of the protein usually occurring within hours; peak expression occurs within 1–3 days after Ad5 instillation [16]. Some studies were performed in which the Ad5-delivered gene therapy was administered before the onset of the inflammatory state [17,18]. Recently, a hybrid Ad5 vector was developed, in which the fiber protein from Ad35 was substituted for the original fiber. This vector binds in a Coxsackie virus and adenovirus receptor (CAR)-independent manner, and has successfully been used to infect suspension cells. And the ability of Ad5/F35 to deliver transgenes to cells with high efficiency and low toxicity provides an improved means of studying the consequences of transient gene expression in cells [19].

This study explored the effect of using Ad5/F35-based gene therapy to deliver PIAS1 in SAP-induced rats. Firstly, the time to administrate Ad5/F35-PIAS1 seems to be very important, since it takes time to transfect and express protein. The route and time course of Ad5/F35 delivery to these animals was based on previous studies that have also been shown that cytokine production after the administration of Ad5-cytokine gene persists for 4 days [20]. According to the results of PIAS1 protein expression, the current study demonstrates that Ad5/F35 transfer of PIAS1 is an effective method to transfect the pancreas and lung for up to 3 days in rats when delivered by IV injection. The expression of PIAS1 protein in lung and pancreatic tissue was greatly increased in rats who were treated with Ad5/F35-PIAS1 on day 2 before SAP induction compared to those treated 10 min or 1 day prior to SAP induction. Therefore, the rats received with Ad5/F35-PIAS1 on day 2 before SAP induction was used for future investigation. And these findings represent the first demonstration of prophylactic strategy of Ad5/F35 gene transfer in experimental SAP. Secondly, the early intervention is necessary in the treatment of SAP, since SAP develops fast and the consequent systemic responses will increase mortality, thus making therapies more difficult and more costly. Here, we presented the first evidence that early prophylactic administration of Ad5F35-PIAS1 could ameliorate the extent of injury of pancreatic tissue in SAP-induced rats and decrease mortality. Thirdly, the administration of the Ad5F35-vector had no effect on the injury

extent of pancreatic tissue in SAP-induced rats. Finally, because of the results mentioned above, it was necessary to further evaluate the effective mechanisms of up-regulation of PIAS1 on SAP-induced rats associated with ALI.

ALI is an important problem affecting the severity of SAP [21]. It is believed that the excess production of inflammatory mediators released by macrophages, neutrophils and other cells of the immune system in a cascade network is the underlying mechanism causing ALI in SAP [22,23]. As SAP progresses, neutrophils are also transmigrated across endothelial surfaces and into the tissues by inflammatory mediator induction, where they exert their toxic effects, resulting in microvascular dysfunction and local inflammatory response. These responses include microvascular disorder, alveolar capillary barrier leakage, interstitial and alveolar edema, and eventual cell death [24,25].

The wet weigh/dry weight ratios of lung tissue for each group were measured for assessment of changes in lung vascular permeability. This study found that the Ad5/F35-PIAS1 treated rats showed a much lower ratio of wet to dry weight in lungs than in the untreated SAP rats. Furthermore, when compared to the untreated SAP and Ad5/F35-vector treated rats, the Ad5/F35-PIAS1 treated rats had lower scores for lung injury, including measurements of alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in airspace or vessel wall, and thickness of the alveolar wall. According to the MPO assay measurement of neutrophil accumulation, and the BALF count of neutrophils, the untreated SAP rats had a higher neutrophil accumulation than either the control or Ad5/F35-PIAS1 treated rats. MDA is one of the final products of lipid peroxidation, and its concentration is directly proportional to the cell damage caused by reactive oxygen metabolites [26]. In the present study, the increase in pulmonary MDA production following SAP induction was reduced by Ad5/F35-PIAS1 prophylactic administration, suggesting that Ad5/F35-PIAS1 is involved in an anti-inflammatory effect. These results lend support to the use of Ad5/F35-PIAS1 as an anti-inflammatory therapy for SAP associated with ALI.

The STAT1 signaling pathway, one of the most important intracellular signaling pathways in mammals, is involved in the regulation of the inflammatory response. Various forms of stimuli can activate it and thereafter the phosphorylation of STAT1 regulates a variety of downstream proteins. Many studies have shown that STAT1 regulates the transcription of cytokine genes [27]. Our previous studies found that there was a positive interaction between the activity of STAT1, which regulates the expression of inflammatory cytokines such as TNF-α, IL-1β and IL-6, and the severity of SAP. The inhibitor of STAT1 (Rapamycin) decreased the levels of these inflammatory cytokines, thus lessening the severity of SAP (Un-published data).

Meanwhile, TNF-α and IL-1β are important inflammatory cytokines participating in the pathogenesis of SAP, directly injuring cells and causing necrosis, inflammation and edema [28]. IL-6 is a multifunctional cytokine released by macrophages and is an accurate early predictor of acute pancreatitis severity, with a sensitivity range of 89–100% [29]. The current study found that the early administration of Ad5/F35-PIAS1 might block the induction of TNF-α and IL-1β. Previous study has also shown that IL-10 is an important anti-inflammatory cytokine and plays a role as a self-defense mechanism, limiting the intensity of the inflammatory process. Several studies concluded that IL-10 plays an important protective role for pancreatic cell injury in SAP [30], but Ad5/F35-PIAS1 gene prophylactic administration had no significant influence on the release of IL-10 in the course SAP in this study.

As secondary chemotactic factors of inflammatory-regulated mediators, ICAM-1 and MMP-9 initiate a cascade reaction, accumulate neutrophilic leukocytes and stimulate the production of reactive oxygen species and other pro-inflammatory factors, attack and degrade membranes, damage membrane stability and causes lung vascular permeability [31–33]. In our experiments, the elevated levels of ICAM-1 and MMP-9 were found in SAP. The Ad5/F35-vector were administered IV have no significantly promotion in lung inflammation were observed in this study. Furthermore, the pretreatment of Ad5/F35-PIAS1 was able to inhibit the activation of STAT1, down-regulated the levels of downstream inflammatory-regulated mediators including ICAM-1 and MMP-9. As these mediators are known mediators of inflammatory injury, the reduction in their levels may be important in attenuating the endothelial permeability of lung tissue and lessening the severity of ALI of SAP. These findings suggest that pretreatment with Ad5/F35-based gene therapy expressing PIAS1 can be safely performed during acute inflammation and can reduce the magnitude of the subsequent inflammatory process. As a final result, the survival time was prolonged in Ad5/F35-PIAS1 treated rats compared with SAP rats.

In our study, the results suggest that the prophylactic administration of PIAS1 inhibited the activation of STAT1 and down-regulated downstream inflammatory cytokines, relieved endothelial permeability, attenuated ALI and increased the survival rate in SAP-induced rats. PIAS1 also inhibited the inflammation and lessened the pancreatic injury of SAP. It might be helpful to use PIAS1 in SAP as a therapeutic strategy for the future experiment and we believe this study will be particularly relevant to clinical settings.

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