Single immunoglobulin and Toll-interleukin-1 receptor domain containing molecule protects against severe acute pancreatitis *in vitro* by negatively regulating the Toll-like receptor-4 signaling pathway: A clinical and experimental study

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Abstract. Single immunoglobulin and Toll-interleukin-1 receptor domain-containing molecule (SIGIRR) is a specific inhibitor of IL-1R and Toll-like receptor (TLR) signaling and considered a potential target for the treatment of inflammatory diseases. Pathogenic mechanisms associated with the TLR4 signaling pathway have a critical role in the development of severe acute pancreatitis (SAP). The aim of the present study was to determine the role of SIGIRR in the regulation of TLR4 signaling during the progression of SAP. Pancreatitis-associated ascitic fluid (PAAF) was collected from patients with SAP. Murine RAW264.7 macrophages were transfected with a SIGIRR overexpression plasmid and co-cultured with the PAAF from the donors in order to evaluate the effect of SIGIRR *in vitro*. The mRNA expression of TLR4, SIGIRR and other key downstream signaling molecules was quantified using semi-quantitative PCR with agarose gel electrophoresis. Furthermore, the levels of pro-inflammatory cytokines in the culture supernatant were detected using ELISA. In contrast to SIGIRR, the mRNA expression levels of TLR4, myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase-1 (IRAK-1) and TNF receptor-associated factor-6 (TRAF-6) were significantly increased in RAW264.7 cells following treatment with PAAF. Furthermore, TLR4, MyD88, IRAK-1 and TRAF-6 mRNA levels were significantly downregulated following SIGIRR overexpression and PAAF treatment in RAW264.7 cells. The levels of IL-2, IL-12, IL-17 and IFN-γ in the culture supernatant were also significantly decreased, while IL-10 levels were increased. Overall, SIGIRR negatively regulated the TLR4 signaling pathway to protect against the development of SAP in an *in vitro* model. Therefore, SIGIRR may represent a promising therapeutic target for SAP.

Introduction

Severe acute pancreatitis (SAP) is the most serious type of pancreatitis. SAP is frequently accompanied by systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS) and is associated with high morbidity and mortality rates (1). Although gallstones are the most common cause of pancreatitis, and increase in alcohol misuse has been linked to the increased incidence of pancreatitis in several European countries and the USA (2). The pathogenic mechanisms underlying the development of SAP have been extensively studied but remain incompletely understood. Pancreatitis-associated ascitic fluid (PAAF) contains high concentrations of pancreatic enzymes, free fatty acids, inflammatory cytokines and endotoxins (3). The removal of PAAF by abdominal paracentesis drainage and peritoneal lavage exerts a beneficial effect on patients with SAP, reducing multiple organ failure and mortality rates (4,5). However, the

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mechanisms underlying the effectiveness of this procedure have remained elusive.

Toll-like receptors (TLRs) are innate pattern-recognition receptors that detect distinct pathogen-associated molecules and induce the transcription and release of inflammatory cytokines (6). TLR4 in particular recognizes lipopolysaccharide present on Gram-negative bacteria (7). TLR4 is also required for the phagocytosis of *E. coli* in murine macrophages (8). Stimulation of TLR4 is required for the activation of pro-inflammatory pathways, thereby inducing the production of inflammatory cytokines in a variety of cell types (9). Excess release of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, is the underlying mechanism of MODS in patients with SAP (10). Several studies have confirmed that TLR4-mediated activation of the NF-κB signaling pathway is involved in pancreatic injury in a mouse model of SAP (11,12). Thus, inhibition of TLR4-activated NF-κB signaling may represent a potential approach for the prevention of SAP (11).

Single immunoglobulin and Toll-interleukin-1 receptor domain-containing molecule (SIGIRR), which is also known as Toll-IL-1R (TIR)-8 or IL-1R8, is a member of the IL-1R family (13). It is widely expressed in several epithelial tissues, particularly in epithelial cells of the kidney, digestive tract, liver, lung and lymphoid organs. Among leukocytes, SIGIRR is expressed in monocytes, B and T lymphocytes, dendritic cells and natural killer cells (14). SIGIRR functions as a negative regulator of IL-1R and TLR signaling by inhibiting TIR domain-containing receptors and adapter proteins, thus blocking Akt, JNK, mitogen-activated protein kinases, TGF-β-activated kinase 1, mTOR and NF-κB activation (15). SIGIRR may have a beneficial or detrimental role in the innate immunity against pathogens, emerging as a key modulator of the equilibrium between protective immune responses and inflammation and host injury (16).

PAAF is one of the initial presentations of acute pancreatitis (17), and PAAF-induced inflammatory responses are involved in the development of SAP (18). Furthermore, inflammatory cytokines involved in the TLR4 signaling pathway are associated with pancreatic injury (11). However, whether an association exists between PAAF and the activation of the TLR4-mediated inflammatory pathways in patients with SAP has remained to be determined. In addition, little is known about the role of SIGIRR in SAP. Therefore, in the present study, it was hypothesized that PAAF-mediated activation of the TLR4 signaling pathway contributes to the development of SAP. Accordingly, SIGIRR may negatively regulate this process to protect against SAP. The aim of the present study was to investigate the role of SIGIRR in regulating the TLR4 signaling pathway in an *in vitro* SAP model of PAAF-treated macrophages. To the best of our knowledge, the present study was the first to examine the association between SIGIRR and SAP *in vitro*. The results may provide insight into the pathogenesis of SAP and help identify therapeutic targets for this condition.

Materials and methods

Sources of PAAF. PAAF was obtained by collection of abdominal drainage fluid from two patients with untreated SAP under aseptic conditions who were admitted to the First Affiliated Hospital of Nanchang University (Nanchang, China) in January 2019. The diagnosis was based on at least two of the following parameters: i) Amylasemia >3 times the upper limit of normal; ii) abdominal pain compatible with acute pancreatitis; and iii) computed tomography or magnetic resonance imaging features compatible with acute pancreatitis. The diagnostic criteria for SAP were according to the 2012 revision of the Atlanta Classification of Acute Pancreatitis (1). The general laboratory characteristics of the PAAF from the two patients are presented in Table I. A total of 50 ml PAAF was collected from both patients, then pooled in a centrifuge tube and centrifuged at 11,180 x g for 15 min at 4°C. The PAAF supernatant was collected and stored at -80°C after sequential filtration through membranes with pore sizes of 3.0, 2.0, 1.0 and 0.45 μm, as well as 0.22 μm. Written informed consent for the use of PAAF samples was obtained from both patients enrolled in this study. The study was approved by The Ethics Committee of The First Affiliated Hospital of Nanchang University (Nanchang, China; approval no. EL20180027) and was performed in compliance with the Declaration of Helsinki.

Cell culture and treatment. RAW264.7 murine macrophage cells were obtained from Nanjing KeyGen Biotech Co., Ltd. and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Zhejiang Tianhang Biotechnology Co., Ltd.) at 37°C with 5% CO_2. RAW264.7 cells in the logarithmic growth phase were treated with titrated doses of PAAF (1.25, 2.5, 5.0 and 10.0% diluted in RPMI-1640 medium) harvested from the patients with SAP for 6, 12 or 24 h. Untreated cells were used as a negative control.

Plasmid extraction and transfection. The GT110 *E. coli* strains containing the pUNO plasmid or pUNO-mSIGIRR plasmid (4199 bp) with two restriction sites were purchased from InvivoGen. Plasmids were extracted from GT110 cells in the logarithmic growth phase using a Plasmid Mini kit (Tiangen Biotech Co., Ltd.), then identified by digestion with the *Eco*RI restriction endonuclease (Thermo Fisher Scientific, Inc.) at 37°C for 3 h, resulting in two fragments. RAW264.7 cells were separately transfected with pUNO or pUNO-mSIGIRR (InvivoGen) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. After 24 h, the transfected RAW264.7 cells in the logarithmic growth phase were transferred to six-well tissue culture plates at a density of $2x10^5$ cells/well. Certain designated wells of transfected cells were treated with 5% PAAF for 24 h. Furthermore, untreated and untransfected cells were used as negative controls.

Semi-quantitative reverse transcription (RT-PCR). Total RNA was extracted from RAW264.7 cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed with PrimeScript RT Reagent kit (Takara Bio, Inc.) to obtain complementary DNA according to the manufacturer's protocol. Briefly, genomic DNA was eliminated using gDNA Eraser treatment for 2 min at 42°C. RT was then carried out at 37°C for 15 min, followed by 85°C for 5 sec.

Table I. General laboratory characteristics of pancreatitis-associated ascitic fluid.

Parameter	Patient A	Patient B
Disease information		
Age	47	51
Sex	Male	Female
Severity	Severe	Severe
Disease duration	5 days	4 days
Relevant medical history	None	None
Routine analysis		
Colour	Red	Yellow
Turbidity	Turbid	Slightly turbid
Clot	Flocculation	None
Rivalta test	Positive	Positive
Nucleated cells ($1/\mu l$)	6,720	3,800
Segmented neutrophils (%)	90	70
Lymphocytes (%)	10	28
Biochemistry		
Total bilirubin (μmol/l)	27.9	37.9
Total proteins (g/l)	38.5	44.5
Albumin (g/l)	28.6	32.9
Globulin (g/l)	9.9	11.6
Glucose (mmol/l)	5.8	14.3
Lactate dehydrogenase (U/l)	895	1718
Amylase (U/l)	1,438	1,248
Adenosine deaminase (U/l)	1	2
Cytokines		
IL-1 (pg/ml)	231	257
IL-6 (pg/ml)	1,051	998

The resulting cDNA which was then used as a template for amplification of the target genes with 2X Taq PCR MasterMix (Tiangen Biotech Co., Ltd.) including TLR2, TLR4, myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase-1 (IRAK-1) and TNF receptor-associated factor-6 (TRAF-6) and SIGIRR. Primer sequences specific for these genes are presented in Table II. The thermocycling conditions for each target gene are described in Table III. All RT-PCRs were set up in triplicate. Amplification products were resolved by electrophoresis using 1% agarose gels stained with ethidium bromide. Electrophoresis images were acquired using the ChemiDoc MP system (Bio-Rad Laboratories, Inc.) and the gray-scale value of bands was determined to quantify mRNA expression levels using the BandScan software (version 5.0; Glyko, Inc.).

ELISA. The concentrations of IL-2, IL-4, IL-10, IL-12, IL-17 and IFN-γ in the culture supernatant of RAW264.7 cells were determined using ELISA kits (cat. nos. BMS601 for IL-2; BMS613 for IL-4; BMS614/2 for IL-10; BMS616 for IL-12; BMS6001 for IL-17; BMS606 for IFN-γ; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. All assays were repeated at least three times.

Statistical analysis. Continuous variables are presented as the mean ± standard deviation of at least three independent experiments. Multigroup comparisons were performed using two-way ANOVA, followed by Tukey's Honestly Significant Difference test. Paired data were analyzed using two-way repeated-measures ANOVA, followed by Tukey's post hoc test. Statistical analysis was performed using SPSS 24.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of PAAF on TLR4 signaling and SIGIRR expression in PAAF-treated macrophages. RAW264.7 cells were treated with 1.25, 2.5, 5 or 10% PAAF for 6, 12 or 24 h. TLR2 mRNA expression was not significantly altered following PAAF treatment (Fig. 1A). By contrast, TLR4 mRNA levels in macrophages significantly were increased in response to incubation with 2.5-10% PAAF for 24 h (Fig. 1B). Furthermore, in cells stimulated with 5% PAAF for 24 h, for instance, the mRNA levels of TLR4, MyD88, IRAK-1 and TRAF-6 increased in a time-dependent manner (Fig. 1B-E; $P<0.05$ vs. the same PAAF concentration in the 6-h group). However, the expression of TLR4 and downstream key molecules did not further increase with increasing PAAF concentration. Compared with 5% PAAF stimulation, the mRNA levels of TLR2, TLR4, MyD88, IRAK-1 and TRAF-6 were decreased following stimulation with 10% PAAF in the same subgroup at 24 h.

In addition, SIGIRR mRNA expression was reduced with increasing PAAF concentration, but only in a significant manner following 24 h of incubation (Fig. 1F; $P<0.01$ vs. the same PAAF concentration in 6 h group). Therefore, the optimal concentration of 5% PAAF and a stimulus duration of 24 h were used in subsequent experiments.

Effect of SIGIRR on the TLR4 signaling pathway in PAAF-treated macrophages. SIGIRR overexpression plasmid was successfully constructed and validated using RT-PCR and restriction enzyme digestion (Fig. 2). A clear 123-bp band was observed for the PCR product from the pUNO-mSIGIRR plasmid, but not the empty plasmid, which was consistent with the expected size of the SIGIRR PCR product (Fig. 2A). In addition, the pUNO-mSIGIRR plasmid was cleaved into two fragments using a single restriction enzyme, a 1,911-bp SIGIRR-containing fragment and a 2,288-bp vector fragment (Fig. 2B). SIGIRR gene expression was increased in macrophages transfected with pUNO-mSIGIRR as compared with that in untransfected macrophages and macrophages transfected with the empty vector (Fig. 2C).

Transfection with pUNO-mSIGIRR resulted in significantly increased levels of SIGIRR as compared with those in untransfected macrophages and the empty vector group, regardless of PAAF stimulation ($P<0.05$ vs. the control group; Fig. 3A). By contrast, the mRNA levels of key molecules in the TLR4 signaling pathway, including TLR4, MyD88, IRAK-1 and TRAF-6, were significantly decreased in the SIGIRR-transfected group stimulated with 5% PAAF ($P<0.01$ vs. the control group with 5% PAAF). There was a further decrease in the presence of PAAF in the SIGIRR group ($P<0.05$ vs. SIGIRR group without 5% PAAF). However, the presence

Table II. Primer sequences used for semi-quantitative PCR and resulting product sizes.

Target gene	Primer sequence (5'-3')	Product size (bp)
β-actin	Forward: TGGAATCCTGTGGCATCCATGAAAC Reverse: TAAAACGCAGCTCAGTAACAGTCCG	234
SIGIRR	Forward: GTGGCTGAAAGATGGTCTGGCATTG Reverse: CAGGTGAAGGTTCCATAGTCCTCTGC	123
TLR4	Forward: CAGCTTCAATGGTGCCATCA Reverse: CTGCAATCAAGAGTGCTGAG	438
TLR2	Forward: TCAAAAGTCGATCGGCGACAT Reverse: TACCCAGCTCGCTCATCACGT	340
MyD88	Forward: AGAGCTGCTGGCCTTGTTA Reverse: TCATCTCCTGCACAAACTCG	265
IRAK-1	Forward: GCCTCAACGACTGGACATTC Reverse: GCCTCTTCTTGGCCCGACGGT	589
TRAF-6	Forward: GAGGAGATCCAGGGCTACGA Reverse: ATGTACTTGATGATCCTCGA	292

IL-1R, IL-1 receptor; SIGIRR, single immunoglobulin and Toll-interleukin-1 receptor domain containing molecule; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK-1, IL-1R-associated kinase-1; TRAF-6, TNF receptor-associated factor-6; bp, base pairs.

Table III. Semi-quantitative PCR thermocycling conditions.

Genes	Initial denaturation, temperature (˚C)/ duration (min)	Denaturation, temperature (˚C)/ duration (min)	Annealing temperature (˚C)/ duration (min)	Extension, temperature (˚C)/ duration (min)	Terminal extension, temperature (˚C)/ duration (min)	Number of cycles
β-actin	94/5	94/0.5	56/0.5	72/1	72/5	30
SIGIRR	94/5	94/0.5	56/0.5	72/1	72/5	30
TLR4	94/5	94/0.5	48/0.5	72/1	72/5	30
TLR2	94/5	94/0.5	53/0.5	72/1	72/5	30
MyD88	94/5	94/0.5	49/0.5	72/1	72/5	30
IRAK-1	94/5	94/0.5	54/0.5	72/1	72/5	30
TRAF-6	94/5	94/0.5	47/0.5	72/1	72/5	30

IL-1R, IL-1 receptor; SIGIRR, single immunoglobulin and Toll-interleukin-1 receptor domain containing molecule; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK-1, IL-1R-associated kinase-1; TRAF-6, TNF receptor-associated factor-6.

of PAAF caused an increase both in the control and vector groups ($P<0.05$ vs. the same sub-group without 5% PAAF; Fig. 3B-E). However, there was no significant change in the mRNA levels of TLR2 (Fig. 3F).

Effect of SIGIRR overexpression on cytokine secretion in PAAF-treated macrophages. The levels of the pro-inflammatory cytokines IL-2, IL-12, IL-17 and IFN-γ were significantly decreased in the supernatant of PAAF-treated macrophages transfected with SIGIRR overexpression vector ($P<0.05$ vs. the control group; Fig. 4A-D). Conversely, increased concentrations of the anti-inflammatory cytokine IL-10 were observed in the SIGIRR group with or without 5% PAAF stimulation for 24 h ($P<0.01$ vs. the control or vector group with 5% PAAF for 24 h; Fig. 4E). However, for IL-2 and IL-12, the levels increased in the presence of PAAF. This effect was

also observed in the control group (IL-2, IL-12, IL-17, and IFN-γ; $P<0.05$ vs. the control group without 5% PAAF for 24 h). However, there were no significant changes in IL-4 expression (Fig. 4F).

Discussion

Previous studies have documented the importance of PAAF-induced inflammatory responses in the development of acute pancreatitis (19-22). Inflammatory responses mediated by the TLR4 signaling pathway are associated with SAP (11,23-25). SIGIRR overexpression inhibits TLR-induced cytokine production in macrophages, while SIGIRR knockdown results in increased cytokine production following TLR stimulation (13,15,26,27). TLR-mediated activation of NF-κB promotes the transcription of genes
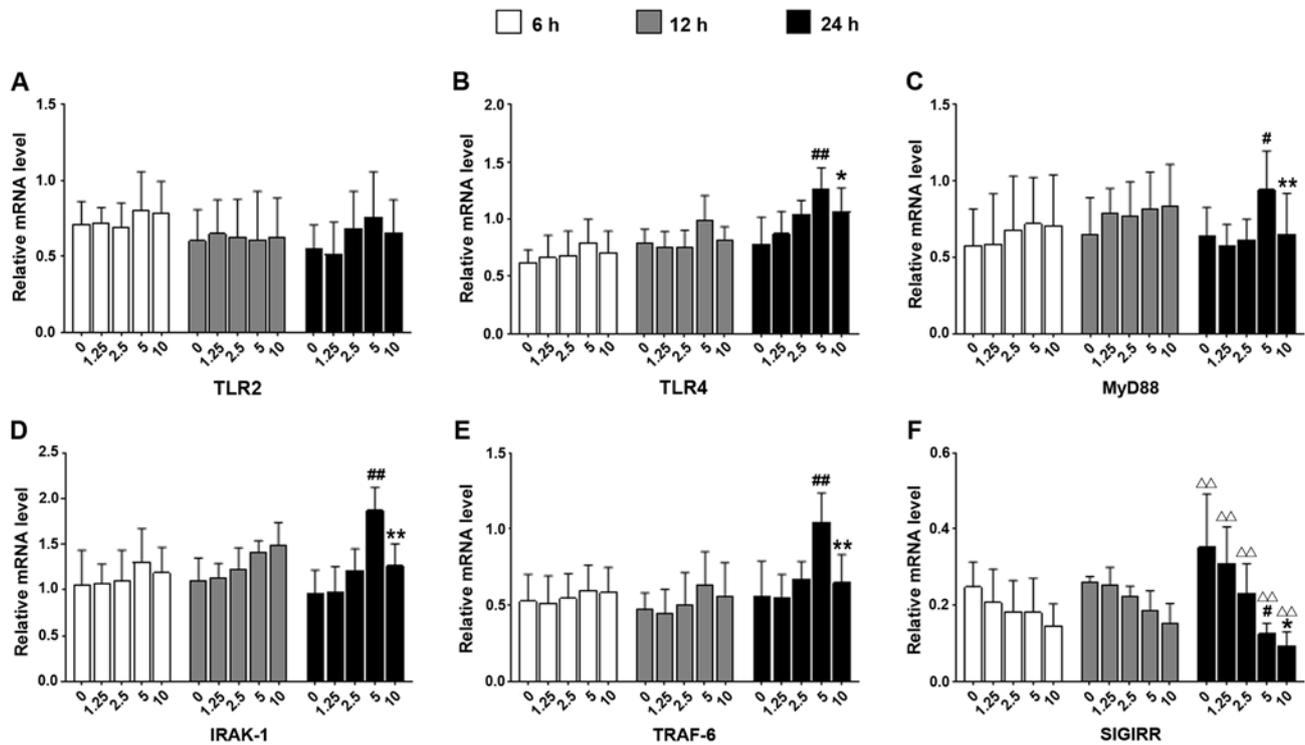
Figure 1. Effect of PAAF on the TLR4 signaling pathway and expression of SIGIRR in macrophages *in vitro*. The RAW264.7 cells were stimulated with 1.25, 2.5, 5 and 10% PAAF. The mRNA levels of (A) TLR2, (B) TLR4, (C) MyD88, (D) IRAK-1, (E) TRAF-6 and (F) SIGIRR were determined. Values are expressed as the mean ± standard deviation from six independent experiments. #P<0.05, ##P<0.01 vs. corresponding concentration at the 6-h time-point. *P<0.05, **P<0.01 vs. the same group treated with 5% PAAF at 24 h. △△P<0.01 vs. the same PAAF concentration in the 6 h group. IL-1R, IL-1 receptor; SIGIRR, Single Ig and Toll-IL-1 receptor domain containing molecule; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK-1, IL-1R-associated kinase-1; TRAF-6, TNF receptor-associated factor-6; PAAF, pancreatitis-associated ascitic fluid.

encoding pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, IL-12 and IFN-γ, their protein secretion and inflammation (28), which is an important mechanism of SIRS in acute pancreatitis (10).

PAAF contains high concentrations of pancreatic enzymes, free fatty acids, inflammatory cytokines and endotoxins. PAAF resembles the environment in the pancreas during SAP that may contain inflammatory messengers. Thus, in the present study, it was used to trigger TLR4 signaling/inflammatory response in macrophages as an *in vitro* model of SAP (19,20). However, whether a direct association exists between PAAF and the activation of the TLR4 signaling pathway in patients with SAP has remained elusive. In addition, the potential protective effects of SIGIRR have remained to be evaluated in an *in vitro* model of SAP. Therefore, the aim of the present study was to determine the effect of PAAF on TLR4 signaling and the expression of SIGIRR in macrophages *in vitro*. Furthermore, the effect of SIGIRR on the TLR4 signaling pathway in macrophages stimulated with PAAF was assessed. PAAF was collected aseptically from two patients with untreated SAP who were diagnosed using the criteria of the 2012 revision of the Atlanta Classification of Acute Pancreatitis (1). The effect of PAAF treatment on SIGIRR-overexpressing murine RAW264.7 macrophages was evaluated. The major results of the present study were that TLR4, MyD88, IRAK-1 and TRAF-6 mRNA levels in RAW264.7 cells increased following PAAF stimulation, compared with those in untreated cells. However, these molecules were significantly downregulated in SIGIRR-overexpressing RAW264.7 cells stimulated with

PAAF as compared with those in untransfected and untreated cells. Furthermore, the concentrations of IL-2, IL-12, IL-17 and IFN-γ in the culture supernatant decreased, while IL-10 levels increased.

PAAF contributes to the pathogenesis of acute pancreatitis, as it stimulates the synthesis of TNF-α by pancreatic acinar cells and induces macrophage activation (19). PAAF increases the production of pro-inflammatory cytokines by interfering with pro-inflammatory and anti-inflammatory signaling pathways (29) involved in the pathogenesis of SIRS and MODS in patients with acute pancreatitis (30). In addition, the expression of TLR4 in pancreatic tissue was significantly increased in murine models of SAP, compared with control mice receiving normal saline injections (31,32). However, the molecular mechanisms underlying the role of SIGIRR in regulating the TLR4 signaling pathway in an *in vitro* SAP model of PAAF-treated macrophages have remained largely elusive. In the present study, TLR4 and key downstream molecules were upregulated in PAAF-stimulated macrophages, except for TLR2. Awla *et al* (33) demonstrated that TLR4, but not TLR2, regulates inflammation and tissue damage in individuals with acute pancreatitis induced by a retrograde infusion of taurocholate. A similar explanation for this result is that PAAF may be involved in the development of acute pancreatitis and may be involved in the activation of the TLR4, but not TLR2, signaling pathway. In the present study, the expression of key molecules in the TLR4 signaling pathway was upregulated after treatment with PAAF up to a concentration of 5%. The expression of TLR4 also increased in a time-dependent
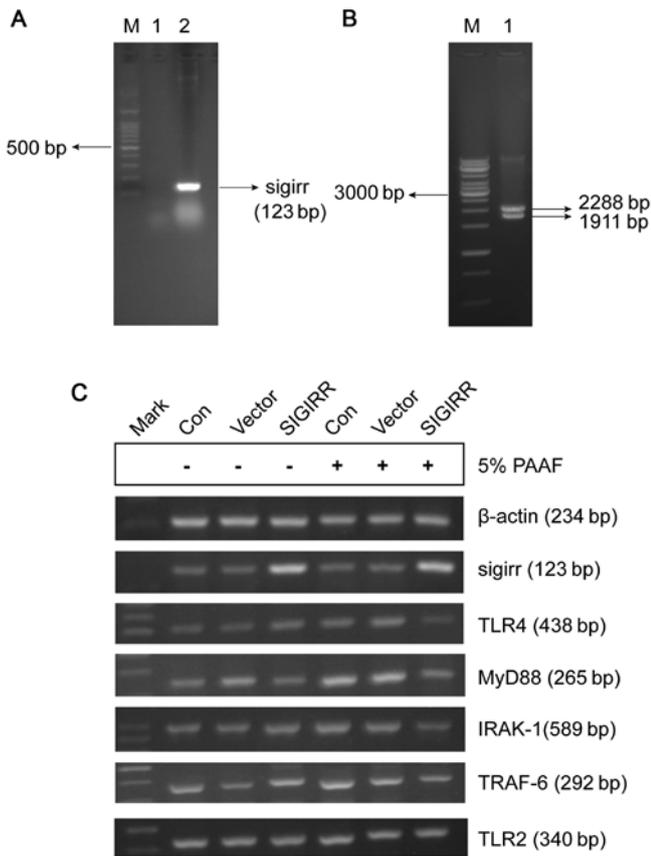
Figure 2. Plasmid transfection and identification. (A) SIGIRR gene products in pUNO-mSIGIRR plasmids and pUNO plasmids were amplified and detected by PCR. Lanes: M, 100-bp DNA ladder; 1, SIGIRR PCR product from empty plasmids; 2, SIGIRR PCR product from SIGIRR-containing plasmids. (B) Results of restriction enzyme digestion. The SIGIRR-containing plasmid was cleaved into a 1,911-bp gene-containing fragment and a 2,288-bp vector fragment. (C) Representative agarose gel electrophoresis of SIGIRR, TLR4, MYD88, IRAK-1, TRAF-6 and TLR2 expression. RAW264.7 macrophages were transfected with pUNO-mSIGIRR overexpression vector and treated with 5% PAAF. Control cells were left untransfected and the vector group was transfected with empty pUNO plasmid. Con, control; Mark, marker; IL-1R, IL-1 receptor; SIGIRR, single immunoglobulin and Toll-interleukin-1 receptor domain containing molecule; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK-1, IL-1R-associated kinase-1; TRAF-6, TNF receptor-associated factor-6.

manner. Thus, the ascites from patients with SAP activate the TLR signaling pathway in macrophages, which may have an important role in the development of SIRS and SAP.

Of note, SIGIRR mRNA was downregulated in PAAF-stimulated macrophages. Previous studies have suggested that SIGIRR acts as a key modulator of inflammation in several pathological conditions, ranging from infectious and sterile inflammation, to autoimmunity and cancer-related inflammation (16,34). SIGIRR exerts a protective effect on keratitis and gastroenteritis by negatively regulating the activation of inflammatory cytokines in the TLR4 signaling pathway (35,36). PAAF contains a large number of ligands for TLRs, such as IL-1 and LPS, which are able to directly activate TLR signaling pathways (20). In this study, PAAF could inhibit the negative regulation of TLR signaling pathways by downregulating the expression of SIGIRR, which may be one of the mechanisms underlying the early hyperactivation of TLR signaling pathways in patients with SAP. Thus, it may be hypothesized that SIGIRR

was able to attenuate PAAF-mediated activation of the TLR4 signaling pathway in the present study.

In SIGIRR-overexpressing macrophages, the mRNA levels of TLR4 and key downstream molecules in the signaling pathway decreased following PAAF treatment. Furthermore, the levels of the pro-inflammatory factors IL-2, IL-12, IL-17, and IFN-γ also decreased as compared with those in untransfected cells. IL-2, IL-12 and IFN-γ promote T-helper type 1 (Th1) cell-mediated immune responses and exacerbate inflammation, which has a role in the onset and development of SAP (37). IL-17 levels increase significantly in individuals with early-stage acute pancreatitis and are a prognostic factor for SAP (38). IL-10 is an anti-inflammatory cytokine that inhibits the production of the pro-inflammatory cytokines IL-1, TNF-α and IL-6 in response to LPS stimulation but also inhibits the activation of Th1 cells and the synthesis of IL-6 and IL-8 by downregulating major histocompatibility complex class II on monocytes and macrophages, thus inhibiting a cascade of inflammatory mediators and blocking or delaying the occurrence of SIRS (39). Thus, the results of the present study suggest that ascites from patients with SAP promote the production of pro-inflammatory cytokines and inhibit the secretion of anti-inflammatory cytokines in macrophages. In addition, SIGIRR is involved in acute pancreatitis and negatively regulates the TLR4 signaling pathway, which is partially consistent with the results of a previous study by our group (40).

The expression levels of TLR4 and its key downstream molecules did not increase with the increase in the concentration of PAAF. When the concentration of PAAF used to stimulate cells reached 10%, the expression level of these factors did not increase, but decreased. The potential explanation for this result is that high concentrations of PAAF may inhibit the proliferation and function of macrophages. Hyperactivation of the TLR signaling pathway has been demonstrated to occur in the early stages of SAP in several studies (11,31). In the present study, PAAF stimulation for 6 h altered the expression of TLR4, but not that of SIGIRR, MyD88, IRAK-1 and TRAF-6, further confirming an important role of TLR4 in pancreatitis. In addition, the expression of these molecules was increased by PAAF in the control and vector-transfected cells but decreased in SIGIRR-overexpressing RAW264.7 cells, suggesting that SIGIRR influence the expression of molecules in the TLR4 signaling pathway in the absence of exogenous stimulation. Thus, the inhibitory effect of SIGIRR on mediators of the TLR signaling pathway was hypothesized to mainly occur in the presence of exogenous stimuli. These results further support the hypothesis that SIGIRR suppresses inflammatory responses and alleviates the inflammatory injury in tissues by exerting a negative regulatory effect on pancreatitis.

Of note, the present preliminary study has certain limitations. The major limitation is that the protein concentrations of key molecules in the TLR4 signaling pathway were not detected. In addition, the mRNA expression levels of TLR4 and key downstream molecules and concentrations of inflammatory factors were not detected in the peripheral blood from the patients. Despite these limitations, the present study certainly improves the current understanding of the association between PAAF and the TLR4 signaling pathway, as well as the role of SIGIRR in the PAAF-mediated activation of the TLR4 signaling pathway.
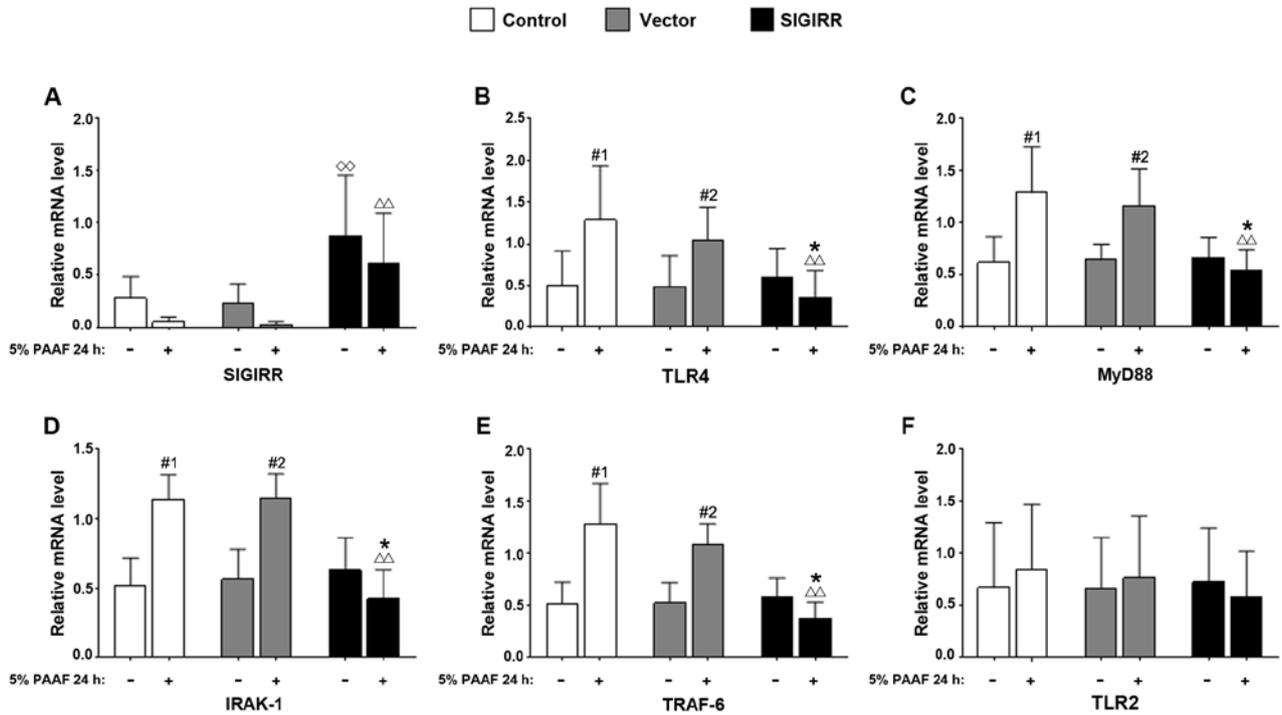
Figure 3. Effect of SIGIRR on the TLR4 signaling pathway in macrophages with PAAF stimulation. RAW264.7 macrophages were transfected with either the pUNO-mSIGIRR or pUNO vector and treated for 24 h with 5% PAAF. Control cells were left untransfected. The transcription levels of (A) SIGIRR, (B) TLR4, (C) MyD88, (D) IRAK-1, (E) TRAF-6 and (F) TLR2 were measured by semi-quantitative reverse transcription PCR. Values are expressed as the mean ± standard deviation from six independent experiments. $^{\diamond\diamond}$P<0.01 vs. control group-5% PAAF, $^{\triangle\triangle}$P<0.01 vs. control group + 5% PAAF. *P<0.05 vs. SIGIRR group-5% PAAF. $^{\#1}$P<0.05 vs. control group-5% PAAF. $^{\#2}$P<0.05 vs. vector group-5% PAAF. IL-1R, IL-1 receptor; SIGIRR, single immunoglobulin and Toll-interleukin-1 receptor domain containing molecule; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK-1, IL-1R-associated kinase-1; TRAF-6, TNF receptor-associated factor-6; PAAF, pancreatitis-associated ascitic fluid.
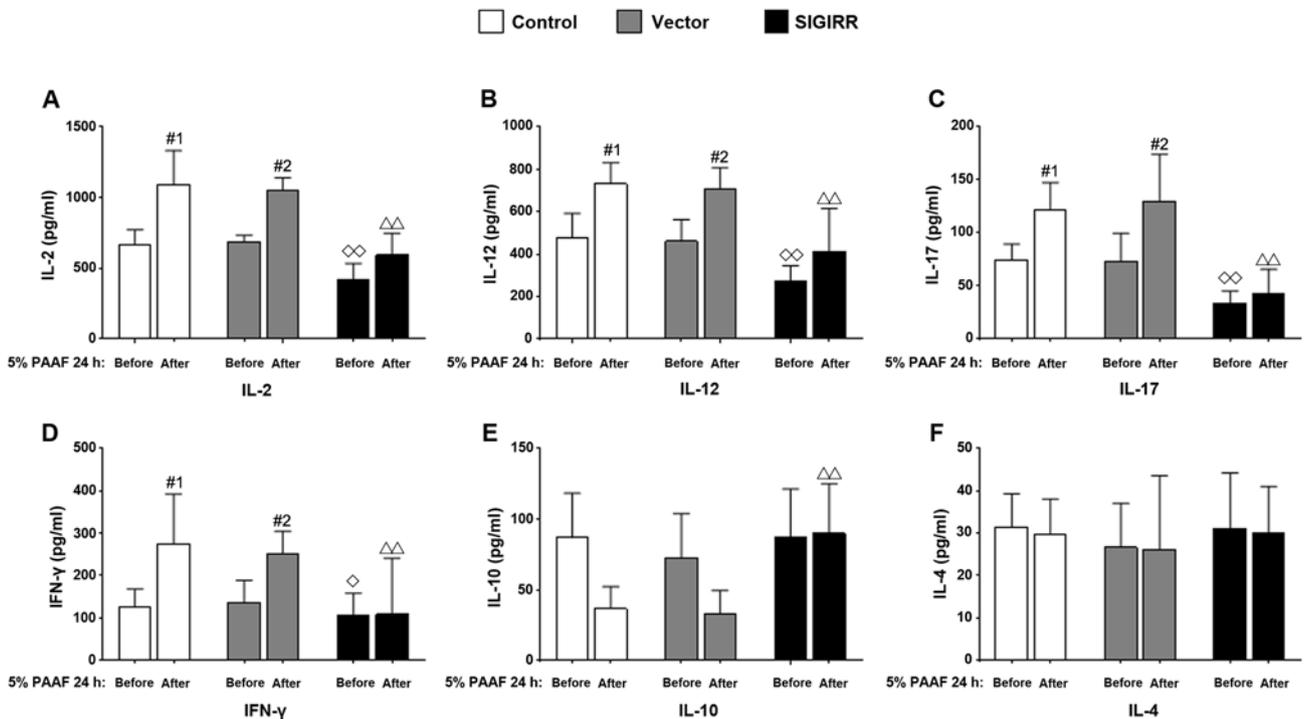

Figure 4. Inflammatory cytokine levels in SIGIRR-overexpressing macrophages following PAAF stimulation. RAW264.7 macrophages were transfected with pUNO-mSIGIRR overexpression vector and treated with 5% PAAF. Control cells were left untransfected and the vector group was transfected with empty pUNO plasmid. The concentrations of (A) IL-2, (B) IL-12, (C) IL-17, (D) IFN-γ, (E) IL-10 and (F) IL-4. ELISAs were performed with the culture supernatant of the same well at different time-points. 'Before' refers to culture supernatant at the beginning of the incubation, whereas 'after' refers to supernatant from the end of the 24-h incubation. Values are expressed as the mean ± standard deviation from five independent experiments. $^{\diamond}$P<0.05, $^{\diamond\diamond}$P<0.01 vs. control group-5% PAAF, $^{\triangle\triangle}$P<0.01 vs. control group + 5% PAAF. $^{\#1}$P<0.05 vs. control group-5% PAAF. $^{\#2}$P<0.05 vs. vector group-5% PAAF. SIGIRR, single immunoglobulin and Toll-interleukin-1 receptor domain containing molecule; PAAF, pancreatitis-associated ascitic fluid.

In summary, the present study was the first *in vitro* study on the effects of SIGIRR as an inhibitor of the TLR4 signaling pathway in macrophages stimulated with PAAF. The negative regulatory effect of SIGIRR on the *in vitro* model of PAAF-mediated activation of the TLR4 signaling pathway may be a mechanism of action and a potential therapeutic target for SAP. Further studies are necessary to determine whether the function of endogenously expressed SIGIRR is compromised in patients with acute pancreatitis. An understanding of the mechanisms by which SIGIRR regulates inflammation may lead to novel therapeutic opportunities for immune-mediated diseases, including acute pancreatitis.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and YX designed the study. RZ and CS performed the experiments. LL, QL and NZ collected and interpreted the patient data regarding severe acute pancreatitis and pancreatitis-associated ascitic fluid. CS performed the statistical analysis. CS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The First Affiliated Hospital of Nanchang University (approval no. EL20180027) and was carried out in compliance with the Helsinki Declaration. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Banks PA, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG and Vege SS; Acute Pancreatitis Classification Working Group: Classification of acute pancreatitis-2012: Revision of the Atlanta classification and definitions by international consensus. Gut 62: 102-111, 2013.
2. Roberts SE, Akbari A, Thorne K, Atkinson M and Evans PA: The incidence of acute pancreatitis: Impact of social deprivation, alcohol consumption, seasonal and demographic factors. Aliment Pharmacol Ther 38: 539-548, 2013.
3. Gou S, Yang C, Yin T, Liu T, Wu H, Xiong J, Yang Z and Wang C: Percutaneous catheter drainage of pancreatitis-associated ascitic fluid in early-stage severe acute pancreatitis. Pancreas 44: 1161-1162, 2015.
4. Li Z, Xia C, Zhang L, Zhang Y, Liu Z and Qiu F: Peritoneal lavage for severe acute pancreatitis: A meta-analysis and systematic review. Pancreas 45: 806-813, 2016.
5. Huang Z, Yu SH, Liang HY, Zhou J, Yan HT, Chen T, Cheng L, Ning L, Wang T, Luo ZL, *et al*: Outcome benefit of abdominal paracentesis drainage for severe acute pancreatitis patients with serum triglyceride elevation by decreasing serum lipid metabolites. Lipids Health Dis 15: 110, 2016.
6. De Nardo D: Toll-like receptors: Activation, signalling and transcriptional modulation. Cytokine 74: 181-189, 2015.
7. Molteni M, Gemma S and Rossetti C: The role of toll-like receptor 4 in infectious and noninfectious inflammation. Mediators Inflamm 2016: 6978936, 2016.
8. Skjesol A, Yurchenko M, Bösl K, Gravastrand C, Nilsen KE, Grøvdal LM, Agliano F, Patane F, Lentini G, Kim H, *et al*: The TLR4 adaptor TRAM controls the phagocytosis of Gram-negative bacteria by interacting with the Rab11-family interacting protein 2. PLoS Pathog 15: e1007684, 2019.
9. Wang Y, Cui Y, Cao F, Qin Y, Li W and Zhang J: Ganglioside GD1a suppresses LPS-induced pro-inflammatory cytokines in RAW264.7 macrophages by reducing MAPKs and NF-κB signaling pathways through TLR4. Int Immunopharmacol 28: 136-145, 2015.
10. Minkov GA, Halacheva KS, Yovtchev YP and Gulubova MV: Pathophysiological mechanisms of acute pancreatitis define inflammatory markers of clinical prognosis. Pancreas 44: 713-717, 2015.
11. Li G, Wu X, Yang L, He Y, Liu Y, Jin X and Yuan H: TLR4-mediated NF-κB signaling pathway mediates HMGB1-induced pancreatic injury in mice with severe acute pancreatitis. Int J Mol Med 37: 99-107, 2016.
12. Zhong K: Curcumin mediates a protective effect via TLR-4/NF-κB signaling pathway in rat model of severe acute pancreatitis. Cell Biochem Biophys 73: 175-180, 2015.
13. Drexler SK, Kong P, Inglis J, Williams RO, Garlanda C, Mantovani A, Yazdi AS, Brennan F, Feldmann M and Foxwell BM: SIGIRR/TIR-8 is an inhibitor of Toll-like receptor signaling in primary human cells and regulates inflammation in models of rheumatoid arthritis. Arthritis Rheum 62: 2249-2261, 2010.
14. Fawley J, Cuna A, Menden HL, McElroy S, Umar S, Welak SR, Gourlay DM, Li X and Sampath V: Single-immunoglobulin interleukin-1-related receptor regulates vulnerability to TLR4-mediated necrotizing enterocolitis in a mouse model. Pediatr Res 83: 164-174, 2018.
15. Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, Towne J, Sims JE, Stark GR and Li X: SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol 4: 920-927, 2003.
16. Molgora M, Supino D, Mantovani A and Garlanda C: Tuning inflammation and immunity by the negative regulators IL-1R2 and IL-1R8. Immunol Rev 281: 233-247, 2018.
17. Landahl P, Ansari D and Andersson R: Severe acute pancreatitis: Gut barrier failure, systemic inflammatory response, acute lung injury, and the role of the mesenteric lymph. Surg Infect (Larchmt) 16: 651-656, 2015.
18. Zhou J, Huang Z, Lin N, Liu W, Yang G, Wu D, Xiao H, Sun H and Tang L: Abdominal paracentesis drainage protects rats against severe acute pancreatitis-associated lung injury by reducing the mobilization of intestinal XDH/XOD. Free Radic Biol Med 99: 374-384, 2016.
19. Satoh A, Shimosegawa T, Masamune A, Fujita M, Koizumi M and Toyota T: Ascitic fluid of experimental severe acute pancreatitis modulates the function of peritoneal macrophages. Pancreas 19: 268-275, 1999.

20. Ramudo L, Manso MA and De Dios I: Biliary pancreatitis-associated ascitic fluid activates the production of tumor necrosis factor-alpha in acinar cells. Crit Care Med 33: 143-148, 2005.
21. Gutierrez PT, Folch-Puy E, Bulbena O and Closa D: Oxidised lipids present in ascitic fluid interfere with the regulation of the macrophages during acute pancreatitis, promoting an exacerbation of the inflammatory response. Gut 57: 642-648, 2008.
22. Pérez S, Finamor I, Martí-Andrés P, Pereda J, Campos A, Domingues R, Haj F, Sabater L, de-Madaria E and Sastre J: Role of obesity in the release of extracellular nucleosomes in acute pancreatitis: A clinical and experimental study. Int J Obes (Lond) 43: 158-168, 2019.
23. Hietaranta A, Mustonen H, Puolakkainen P, Haapiainen R and Kemppainen E: Proinflammatory effects of pancreatic elastase are mediated through TLR4 and NF-kappaB. Biochem Biophys Res Commun 323: 192-196, 2004.
24. Xue J and Habtezion A: Carbon monoxide-based therapy ameliorates acute pancreatitis via TLR4 inhibition. J Clin Invest 124: 437-447, 2014.
25. Pan LF, Yu L, Wang LM, He JT, Sun JL, Wang XB, Wang H, Bai ZH, Feng H and Pei HH: Augmenter of liver regeneration (ALR) regulates acute pancreatitis via inhibiting HMGB1/TLR4/NF-κB signaling pathway. Am J Transl Res 10: 402-410, 2018.
26. Xiao H, Yin W, Khan MA, Gulen MF, Zhou H, Sham HP, Jacobson K, Vallance BA and Li X: Loss of single immunoglobulin interleukin-1 receptor-related molecule leads to enhanced colonic polyposis in Apc(min) mice. Gastroenterology 139: 574-585, 2010.
27. Zhao J, Zepp J, Bulek K and Li X: SIGIRR, a negative regulator of colon tumorigenesis. Drug Discov Today Dis Mech 8: e63-e69, 2011.
28. Anders HJ: Pseudoviral immunity-a novel concept for lupus. Trends Mol Med 15: 553-561, 2009.
29. Wang J, Xu P, Hou YQ, Xu K, Li QH and Huang L: Pancreatitis-associated ascitic fluid induces proinflammatory cytokine expression in THP-1 cells by inhibiting anti-inflammatory signaling. Pancreas 42: 855-860, 2013.
30. Hongliang T, Rong Z, Xiaojing W, Rao S, Lun L, Jinhui T, Nong C and Kehu Y: The effects of continuous blood purification for SIRS/MODS patients: A systematic review and meta-analysis of randomized controlled trials. ISRN Hematol 2012: 986795, 2012.
31. Sharif R, Dawra R, Wasiluk K, Phillips P, Dudeja V, Kurt-Jones E, Finberg R and Saluja A: Impact of toll-like receptor 4 on the severity of acute pancreatitis and pancreatitis-associated lung injury in mice. Gut 58: 813-819, 2009.
32. Sawa H, Ueda T, Takeyama Y, Yasuda T, Shinzeki M, Nakajima T and Kuroda Y: Role of toll-like receptor 4 in the pathophysiology of severe acute pancreatitis in mice. Surg Today 37: 867-873, 2007.
33. Awla D, Abdulla A, Regnér S and Thorlacius H: TLR4 but not TLR2 regulates inflammation and tissue damage in acute pancreatitis induced by retrograde infusion of taurocholate. Inflamm Res 60: 1093-1098, 2011.
34. Riva F, Bonavita E, Barbati E, Muzio M, Mantovani A and Garlanda C: TIR8/SIGIRR is an interleukin-1 receptor/Toll like receptor family member with regulatory functions in inflammation and immunity. Front Immunol 3: 322, 2012.
35. Huang X, Hazlett LD, Du W and Barrett RP: SIGIRR promotes resistance against *Pseudomonas aeruginosa* keratitis by down-regulating type-1 immunity and IL-1R1 and TLR4 signaling. J Immunol 177: 548-556, 2006.
36. Stahl M, Ries J, Vermeulen J, Yang H, Sham HP, Crowley SM, Badayeva Y, Turvey SE, Gaynor EC, Li X and Vallance BA: A novel mouse model of *Campylobacter jejuni* gastroenteritis reveals key pro-inflammatory and tissue protective roles for Toll-like receptor signaling during infection. PLoS Pathog 10: e1004264, 2014.
37. Gong D, Zhang P, Ji D, Chen Z, Li W, Li J, Li L and Liu Z: Improvement of immune dysfunction in patients with severe acute pancreatitis by high-volume hemofiltration: A preliminary report. Int J Artif Organs 33: 22-29, 2010.
38. Dai SR, Li Z and Zhang JB: Serum interleukin 17 as an early prognostic biomarker of severe acute pancreatitis receiving continuous blood purification. Int J Artif Organs 38: 192-198, 2015.
39. Keceli M, Kucuk C, Sozuer E, Kerek M, Ince O and Arar M: The effect of interleukin-10 on acute pancreatitis induced by cerulein in a rat experimental model. J Invest Surg 18: 7-12, 2005.
40. Liu J, Chen Y, Liu D, Liu W, Hu S, Zhou N and Xie Y: Ectopic expression of SIGIRR in the colon ameliorates colitis in mice by downregulating TLR4/NF-κB overactivation. Immunol Lett 183: 52-61, 2017.