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
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. EL20184027) 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% CO2. 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 EcoRI 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 2x10³ 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/µl)           | 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 III. 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
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...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

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: TGGATCCCTTGACCATCCATGAAAC  |
|             | Reverse: TAAAACGCAGCTCAACAGCTCCG     | 234              |
| SIGIRR      | Forward: GTGGCCTGAAAGATGGTGATGCGATGTTG |
|             | Reverse: CAGGTGAAAGGTCTCCATAGTCTCTCG  | 123              |
| TLR4        | Forward: CAGCTTCAATGGTGTCACATCA      |
|             | Reverse: CTGCAATCAGAAGTGTGCTGAG      | 438              |
| TLR2        | Forward: CTCCCCAGCTGCTCAGC        |
|             | Reverse: TACCCAGCTGCTCATCAGT        | 340              |
| MyD88       | Forward: AGAGCGGTCTGCGCTTTGTA       |
|             | Reverse: TCATTCTCCTGACAAACTCG       | 265              |
| IRAK-1      | Forward: GCCTCTACGACTGGACATTC       |
|             | Reverse: GCCTCTCCTGCTGCAGC          | 589              |
| TRAF-6      | Forward: GAGGAGATCCAGGGGCTACAGA     |
|             | Reverse: ATGTACTTGATGATCCTCAGA      | 292              |

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; bp, base pairs.
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 expression of key molecules in the TLR4 signaling pathway was upregulated after treatment with PAAF up to a concentration of 5%.

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. 

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, the expression of key molecules in the TLR4 signaling pathway was 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
In this study, PAAF could inhibit the negative IL-1 and LPS, which are able to directly activate TLR signaling. PAAF contains a large number of ligands for TLRs, such as inflammatory cytokines in the TLR4 signaling pathway (35,36). This study suggested that SIGIRR acts as a key modulator of inflammatory 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. ◊◊P<0.01 vs. control group-5% PAAF, ∆∆ 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. ◊◊P<0.01 vs. control group-5% PAAF, ∆∆P<0.01 vs. control group + 5% PAAF. #3P<0.05 vs. control group-5% PAAF. #4 P<0.05 vs. control group + 5% PAAF. #5 P<0.05 vs. control 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 experiments. 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.

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