The Role of Sphingosine Kinase 1 in Patients With Severe Acute Pancreatitis

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Objective: To investigate the role of sphingosine kinase 1 (SphK1)/sphingosine 1-phosphate (S1P) signaling in inflammatory response in severe acute pancreatitis (SAP).

Background: SAP is an acute inflammatory process of the pancreas, which may lead to systemic inflammatory response syndrome and multiorgan dysfunction syndrome. SphK1 and its product S1P have been implicated in inflammatory response and various immune cell functions. However, the potential role for SphK1/S1P in inflammatory response in SAP is still unclear.

Methods: Twenty-two patients with SAP were enrolled in this study. SphK1 expression on peripheral neutrophils, monocytes, and lymphocytes was evaluated by flow cytometry. SphK enzymatic activity in neutrophils and lymphocytes was measured using a radiometric assay. The expression of $S1P_1$ and $S1P_3$ mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The serum levels of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1β), and IL-6 were measured by ELISA.

Results: The expression of SphK1 and SphK activity were markedly increased in peripheral immune cells in the early stage of SAP and then reduced in the restoration stage in the patients. Moreover, we found that the level of $S1P_3$ mRNA in peripheral neutrophils and lymphocytes of SAP patients was significantly elevated in the early stage as compared with the healthy volunteers, and it reduced in the restoration period. SphK1 expression on human peripheral neutrophils, monocytes, and $CD4^+$ T lymphocytes were positively correlated with the APACHE (Acute Physiological and Chronic Health Evaluation) II scores in patients with SAP. The levels of serum proinflammatory cytokines including TNF-α, IL-1β, and IL-6 showed similar shifts with intracellular SphK1 expression in SAP patients.

Conclusions: The authors identified a link between the SphK1 expression on peripheral immune cells and the severity of SAP. Observations showed a possible immunomodulating role for SphK1/S1P signaling in inflammatory response in SAP, suggesting that regulation of SphK1/S1P pathway may represent novel targets in the treatment of SAP.

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Severe acute pancreatitis (SAP) is an acute inflammatory process of the pancreas that leads to systemic inflammatory response syndrome (SIRS) and multiorgan dysfunction syndrome (MODS) with high morbidity and mortality.[1] A third of patients with SAP develop infectious complications that may explain up to 20% to 60% of deaths.[2–8] Systemic inflammation in SAP is considered to contribute to the development of MODS and subsequent mortality.[9–11]

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Previous studies have shown that pancreatic injury is activated by inflammatory cells including neutrophils, macrophages, and lymphocytes and that it is mediated by the release of proinflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8, which are correlative with the severity and prognosis of acute pancreatitis.[12,13] Despite intense research in receiving anticytokine treatment, the mortality rates are still high, suggesting that other mediators might be involved in the pathogenesis of SAP. Although a great deal is known regarding etiology of SAP, the molecular and pathophysiological mechanisms underlying it remain unclear.[14]

Sphingosine kinase (SphK) is an intracellular signaling enzyme that catalyzes the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P).[15] SphK1, 1 of 2 SphK isoforms, is merging as an important mediator in inflammatory responses and has been extensively implicated in these processes. SphK1 is activated by several proinflammatory stimuli including endotoxin,[16] TNF-α,[17,18] and IL-1β.[18] Other inflammatory signaling molecules, such as interferon-γ (IFN-γ), IgE, and C5a, have also been shown to activate SphK1, further suggesting the importance of SphK1 in the inflammatory response.[19] SphK1 mRNA synthesis is increased under inflammatory conditions.[18] The activation of SphK1/S1P pathway is required for the downstream signaling, which would induce the production of proinflammatory cytokines.[16–20] And the pivotal roles for SphK1 and S1P in inflammatory response have been documented.[20] Recently, it has become clear that SphK1 regulates endotoxin signaling and sepsis-induced inflammatory responses and inhibition of SphK1 is a potential therapy for septic shock.[16] The ability of SphK1 to mediate inflammatory responses prompted us to investigate its role in inflammation in SAP patients.

In addition to their roles in inflammatory signaling, SphK1 and S1P have also been implicated in regulating immune cells such as neutrophils, monocytes, and lymphocytes involved in inflammatory response.[16,21–23] Several proinflammatory stimuli, including TNF-α, C5a, and immune complexes, activate SphK1 in human neutrophils and macrophages.[16,21–23] It has been shown that SphK1 regulates neutrophil priming to provide an essential defense against infections.[24] SphK1 is considered to act through S1P to modulate lipopolysaccharide (LPS)-induced activation of mitogen activated protein kinase (MAPK) and nuclear factor κB(NFκB) in inflammatory cascade in neutrophils and in macrophages.[25–27] SphK1 is a critical regulator in TNF-α–mediated proinflammatory responses in human monocytes.[28] SphK1/S1P participates in several inflammatory responses, including leukocyte chemotaxis[27,28] and cytokine production, and blockade of SphK activity inhibits such responses.[27–30] However, the role of SphK1/S1P in regulating immune cell functions in inflammatory response in SAP has not yet been defined.

We report here that SphK1 expression and its activity are upregulated on peripheral neutrophils, monocytes, and lymphocytes in SAP patients. We further identify that SphK1 expression on neutrophils, monocytes, and $CD4^+$ T lymphocytes is positively correlative with severity of SAP disease. SphK1 may participate in modulating inflammatory response in SAP via $S1P_3$ signaling. These results implicate a possible role for the SphK1/S1P pathway in inflammatory response in SAP.

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METHODS

Patients

Twenty-two SAP patients admitted to Jinling Hospital from January 2009 to October 2010 were involved. SAP is defined as the presence or absence of organ failure; local complications such as pancreatic necrosis abscess, or pseudocyst; or both.[31] All patients enrolled were evaluated according to the Acute Physiological and Chronic Health Evaluation (APACHE) II score. The clinical data including etiology, ICU (intensive care unit) days, and outcome were recorded as well. Twelve healthy volunteers matched for age and sex served as control subjects. The protocol of this study was approved by the Human Subjects Institutional Committee of Jinling Hospital. All patients and volunteers gave informed consent before entry into the study.

Reagents

Fluorescein isothiocyaniate (FITC)–conjugated anti-CD4, Phycoerythrin (PE)-conjugated anti-CD8, PerCP-conjugated anti-CD3, FITC-conjugated anti-CD16, FITC-conjugated anti-CD14, and PE-anti-human leucocyte antigen-DR(HLA-DR) antibodies were all purchased from Becton Dickinson (BD Biosciences, San Jose, CA). The monoclonal antibody against human SphK1 was obtained from Cell Signaling Technology (Beverly, MA). Secondary antibodies conjugated to Allophycocyanin (APC) were from Jackson ImmunoResearch Lab (Baltimore, PA). D-Erythro-[3–^{3}H] sphingosine was a product of Perkin Elmer Life Sciences Inc (Boston, MA).

Determination of Clinical Parameters

Serum levels of amylase, lipase, and C-reactive protein (CRP) in SAP patients were measured using the Olympus AU400 Automated Chemistry Analyzer (Olympus, Tokoyo, Japan) at 1-day intervals from either a peripheral or a central vein. White blood cell differential counts were conducted routinely for patients with SAP by a COULTER LH instrument (Beckman-Coulter Inc, Fullerton, CA).

Human lymphocyte Subpopulations

Lymphocyte subpopulations in peripheral blood were detected by flow cytometry according to manufacturer's instructions. 0.1 mL of whole blood was incubated with 10 μL of CD3/CD4/CD8 monoclonal antibodies for 15 minutes at room temperature in the dark. The red blood cells were then lysed using FACS lysing solution (BD Biosciences, San Jose, CA) for 15 minutes. After centrifuged at 800 g for 5 minutes, the cell pellets were washed 3 times and immediately run on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using CellQuest software (BD Biosciences, San Jose, CA) and the results were expressed as the percentages of CD4-, CD8-positive cells in the lymphocytes.

HLA-DR Expression on Monocytes

The whole blood was sampled as that for lymphocyte subpopulations analysis. Blood was incubated with monoclonal antibodies against human HLA-DR and CD14. A mouse IgG2b isotype conjugated with PE was used as negative control. And a monocyte gate was set by the CD14^{+} group. HLA-DR expression on monocytes was determined using flow cytometry and the data were analyzed with CellQuest software.

Assay of TNF-α, IL-1β, and IL-6 in Serum

Serum was collected from the peripheral venous blood of the patients and healthy subjects. The levels of TNF-α, IL-1β, and IL-6 were measured by ELISA kits (R&D Systems, Abingdon, UK) in accordance with the manufacturer's protocols. The lower limit of detection for TNF-α, IL-1β, and IL-6 was 10 pg/mL.

Flow Cytometry Analysis of SphK1 Expression

The expression of SphK1 on neutrophils, monocytes, and lymphocytes was determined by 4-color flow cytometry analysis. Briefly, the whole blood was incubated with CD16, CD14, and CD3/CD4/CD8 monoclonal antibodies, respectively. The erythrocytes were lysed using FACS lysing solution. Cells were then fixed with formalin and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA). After stained with human SphK1 monoclonal antibody, followed by APC-conjugated anti-mouse IgG, cells were analyzed with a FACSCalibur flow cytometer. Data were analyzed using CellQuest software, and the results were expressed as percentage and mean fluorescence intensity (MFI) of SphK1 positive cells. The correlation between SphK1 expression on peripheral neutrophils, monocytes, and lymphocytes and the APACHE II scores of SAP patients were analyzed using Pearson test.

Isolation of Primary Human Neutrophils and Lymphocytes

Neutrophils and lymphocytes from the peripheral blood were isolated by density gradient centrifugation (Ficoll–Hypaque medium: Pharmacia, Piscataway, NJ) according to the manufacturer's procedures. The cells (2 × 10^{6} cells/mL) were resuspended in RPMI-1640 medium containing 10% fatal bovine serum and frozen in liquid nitrogen until use.

SphK Activity Assay

SphK activity in the neutrophils and lymphocytes was measured as described previously with minor modification.[32] In brief, cells were resuspended in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 20 mM $ZnCl_2$, 1 mM sodium orthovanadate, and 15 mM sodium fluoride and 0.1% protease inhibitor mixture solution, and sonicated (4× 10-s bursts, Ultrasonic disrupter Virsonic 100, Virtis Co, Gardiner NY). The cell lysates were centrifuged at 10000 g for 20 minutes and the supernatants were subjected to SphK activity assay. [3–^{3}H]sphingosine was used as substrate and the radioactive S1P formed was determined using a Beckman LS6500 Scintillation Counter. SphK activity was expressed as pmol/mg/min.

Total RNA Extraction

Total RNA was extracted from isolated neutrophils or lymphocytes of SAP patients and healthy subjects using TRIzol reagent (Invitrogen, Carlsbad, CA) following the instructions. The extracted RNA samples were treated with DNaseI (Ambion, Austin, TX). The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm using a Beckman spectrophotometer (Beckman-Coulter Inc, Fullerton, CA).

RT-PCR Analysis

cDNA was synthesized using an A3500 reverse transcript system (Promega, Madison, WI). The reaction mixture included 1 μg of total RNA, 4 μL $MgCl_2$ (25 mM), 2 μL 10× reverse transcriptase buffer, 1 μL dNTPs (10 mM), 1 μL random primers (500 μg/mL), 1 μL avian myeloblastosis virus (AMV) reverse transcriptase (10 U/μL), and 0.5 μL recombinant RNase inhibitor (40 U/μL). Reverse transcription was performed at 37°C for 60 minutes followed by 95°C for 5 minutes. PCR amplication was performed with 50 μL mixture containing 5 μL 10× PCR buffer, 1 μL dNTP mixture (2.5 mM each), 1 μL each primer (10 nM), 0.5 μL Taq polymerase (5 U/μL), 39.5 μL sterile water, and 2 μL cDNA template. The primer sets were shown in Table 1. The conditions for thermal cycling were as follows: predenature at 95°C for 5 minutes; 40 cycles of 95°C for 15 seconds, annealing for 30 seconds, and 72°C for 30 seconds. PCR

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TABLE 1. Primers Used for RT-PCR

mRNA Targets	Primer Sequence: Sense	Primer Sequence: Antisense	Amplicon (bp)
hS1PR1	5'-TATCAGCGCGGACAAGGAGAACAG-3'	5'-ATAGGCAGGCCACCCAGGATGAG-3'	429
hS1PR3	5'-CTGCCTGCACAATCTCCCTGACTG-3'	5'-GGCCCGCCGCATCTCCT-3'	394
hGAPDH	5'-GATGACATCAAGAAGGTGGTGAA-3'	5'-GTCTTACTCCTTGGAGGCCAT GT-3'	246

products were analyzed on a 2% (W/V) agarose gel electrophoresis. The gels were then stained with ethidium bromide (500 ng/mL) and photographed by the ChemiDOC XRS instrument (Bio-Rad). Band intensities were quantified with QuantityOne analysis software (Bio-Rad). The levels of $S1P_1$ and $S1P_3$ mRNA expression in the neutrophils and lymphocytes were normalized to GAPDH mRNA expression.

Statistical Analysis

All data were presented as mean ± SD or percentage as indicated. Statistical analysis was performed with SPSS software (SPSS Inc, version 13.0, Chicago, IL). And the Mann-Whitney *U* test was used to compare continuous variables. A $P < 0.05$ was considered statistically significant. For detection of correlation, we performed linear regression analysis using Pearson test.

TABLE 2. Clinical Characteristic in 22 Patients With SAP and 12 Healthy Subjects

Characteristic	SAP (n = 22)	Healthy Controls (n = 12)
Median, age (range), yrs	44.1 (24–71)	42.5 (26–65)
Sex		
Male/female	11/11	7/5
Etiology		
Biliary	14 (64%)	
Alcohol	2 (9%)	
Hyperlipemia	3 (14%)	
Other	3 (14%)	
APACHE II score	10.6 ± 2.4	
ICU stay (d)	18 ± 5	
Mortality	1 (4.5%)	

RESULTS

Clinical Characteristics in SAP Patients

Twenty-two SAP patients aged 24 to 71 years (median age 44.1 years with 11 women and 11 men) were studied. Healthy controls consisted of 7 male and 5 female with an average age of 42.5 years (26–65 years). Clinical characteristics of the patients enrolled were presented in Table 2. The causes of SAP were identified as follows: biliary origin in 14 (64%), alcoholism in 2 (9%), hyperlipidemia in 3 (14%), and others in 3 (14%). The average APACHE II score of patients on admission were 10.6 ± 2.4 (range, 6–17). Five of them underwent surgery after 2 weeks of hospitalization and 1 died after surgery due to the fulminate MODS. The mean ICU stay of SAP patients was 18 ± 5 days. All patients were treated according to a standardized interdisciplinary management protocol, including fasting, fluid infusion, intravenous antacid, somatostatin, prophylactic antibiotics, organ function protection, and intensive care.

Biochemical Data

Figure 1 showed the shifts of the routine laboratory parameters including serum amylase, lipase, and CRP in the patients with SAP. Serum levels of amylase and lipase were significantly augmented on days 1 and 3 and then reduced to normal on day 5 of SAP. Serum CRP values were also markedly elevated and reached to peak on day 3 in SAP patients (Fig. 1C). The levels of serum CRP were still much higher in the patients than that in the controls (<10 mg/L) until day 14.

The Peripheral Blood Cell Counts

The peripheral leucocyte and neutrophils were significantly raised in SAP patients as compared with healthy controls ($P < 0.01$) and held during the first week of SAP (Figs. 2A, B). Subsequently, neutrophils and leucocytes decreased to normal. In contrast, lymphocyte and monocyte counts in the peripheral blood markedly reduced in patients in the early stage of SAP and raised gradually thereafter (Figs. 2C, D). On day 7, all the patients were found to recover from the lymphopenia and monocytopenia.

Analysis of Lymphocyte Subpopulations

The results of lymphocyte subpopulations analysis were shown in Figs. 3A and B. In the early stage, the percent of $CD4^+$ T lymphocytes was 27.5% ± 7.0% and it was much lower compared to the healthy controls (37.5 ± 3.9%, $P < 0.01$) (Fig. 3A). The percent of $CD4^+$ T cell was significantly elevated in the restoration stage. There was no significant difference, however, in the percent of $CD8^+$ T lymphocytes between SAP patients and healthy controls (Fig. 3A). The ratio of $CD4^+/CD8^+$ lymphocytes was significantly reduced in the early stage in SAP patients ($P < 0.05$) (Fig. 3B).

HLA-DR Expression on Monocyte in SAP Patients

Monitoring of HLA-DR expression is a useful marker for identifying monocyte function that is closely correlated with the clinical course in AP. We measured the expression of HLA-DR on monocytes in SAP patients in the early and restoration stage (Fig. 3C). In the early stage of SAP, the percentage of HLA-DR expression on monocytes was 31.1% ± 13.6%, which was lower than healthy controls (91.2% ± 5.1%, $P < 0.01$). HLA-DR expression on monocytes increased to 73.4% ± 13.1% in SAP patients. We then analyzed the correlation between HLA-DR expression and APACHE II scores in patients. And a negative correlation was identified between HLA-DR expression and APACHE II scores in SAP patients ($r = -0.489$, $P = 0.021$) (Fig. 3D).

Changes in Serum Cytokine Levels

Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are produced predominantly by activated leukocytes such as neutrophils, monocytes, and macrophages. Thus we investigated the changes of serum cytokine levels in SAP patients. The results showed that the concentration of serum TNF-α, IL-1β, and IL-6 were increased in the early stage by 14.7, 15.2, and 15.2 folds, respectively ($P < 0.01$) (Fig. 4). Moreover, the serum concentration of the cytokines markedly reduced in the restoration stage ($P < 0.01$, as compared to the early stage), and it was still higher than healthy controls ($P < 0.05$).

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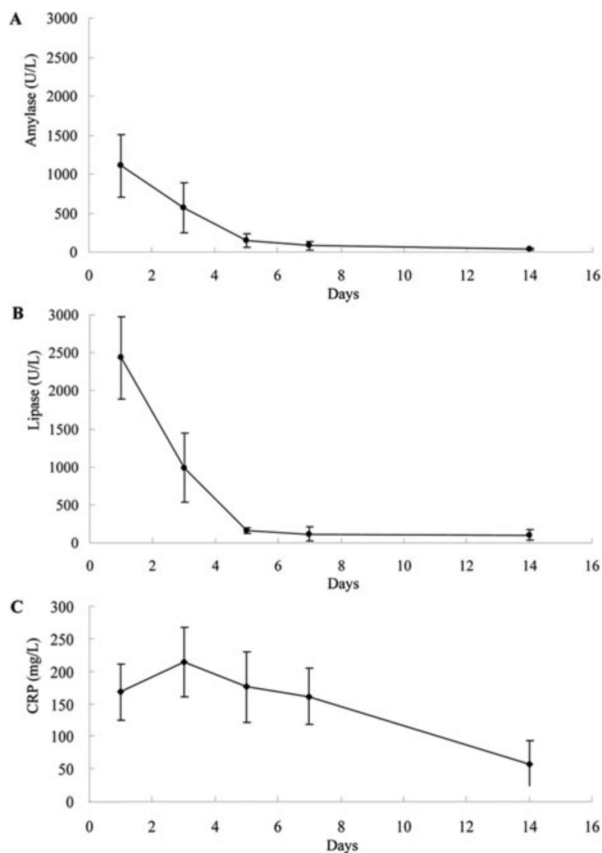

FIGURE 1. Serum amylase (A), lipase (B), and C-reactive protein (CRP) (C) levels in SAP. The 3 laboratory parameters from the onset of the pancreatitis and throughout the 14 days study period were measured. Data were presented as mean ± SD.

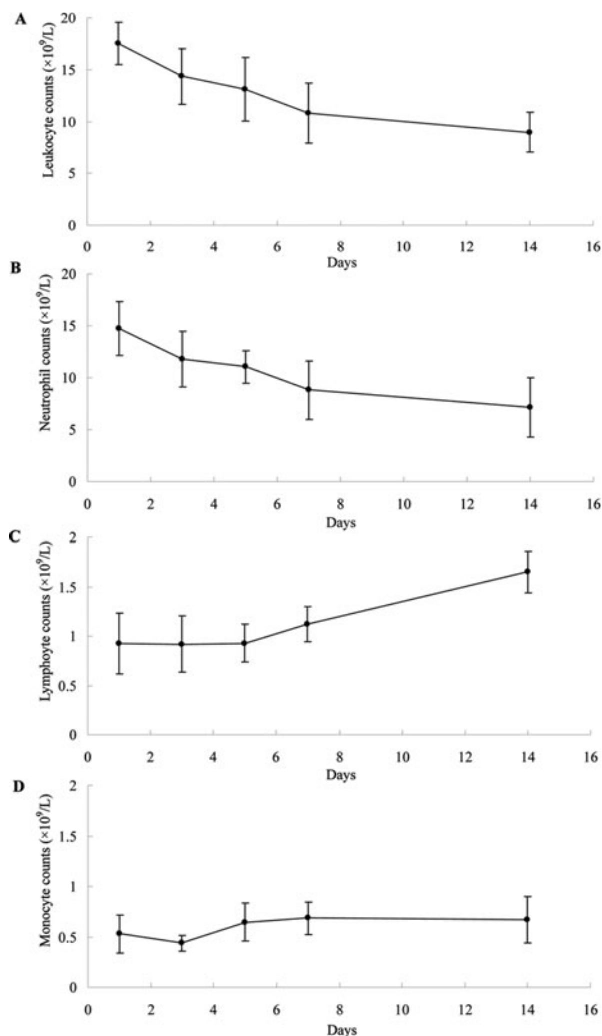

FIGURE 2. The peripheral blood cell counts in SAP patients. A significant increase in the number of peripheral leucocyte (A) and neutrophils (B), and dramatic reduction in lymphocyte and monocyte counts were observed at the onset of SAP. The counts of the peripheral blood cells recovered to normal on day 7.

Activation of SphK1 Expression in SAP Patients

Previous studies demonstrated that SphK1 expression was strongly upregulated by extracellular bacteria, LPS, and TNF-α on human neutrophils and macrophages.[16] We investigated for the first time the expression of SphK1 in peripheral neutrophils, monocytes, and lymphocytes in SAP patients and analyzed the correlation between SphK1 expression and severity of SAP. In peripheral neutrophils, the level of SphK1 expression was markedly elevated at the attack of SAP, which was much higher than that of the controls (62.4 ± 8.5 vs 39.4 ± 5.1, $P < 0.01$) (Figs. 5A, B). The MFI in neutrophils was decreased to 47.6 ± 5.7 when the patients were recovered from SAP ($P < 0.01$). But it was still higher compared with healthy controls ($P < 0.05$). SphK1 expression on neutrophils was positively correlative with APACHE II scores of SAP patients ($r = 0.701$, $P < 0.001$, Fig. 5C).

The expression of SphK1 on the monocytes was also found to be significantly increased in the early stage (45.9 ± 7.5 vs 28.9 ± 4.0, $P < 0.01$) (Figs. 5D, E). There was a significant decrease in the expression of SphK1 on the monocytes in SAP patients in the restoration stage ($P < 0.05$). A significant positive correlation was presented between SphK1 expression on the monocytes and APACHE II scores ($r = 0.545$, $P < 0.01$, Fig. 5F).

We next determined the changes of SphK1 expression on the lymphocytes (Fig. 6C). In the early stage of SAP, the percent of SphK1$^+$ cells in CD4$^+$ and CD8$^+$ T lymphocytes were respectively 17.5% ± 2.9% and 14.9% ± 2.7%, which were higher than in the healthy controls (6.4% ± 1.5% and 8.2% ± 1.1%, $P < 0.01$). Similarly, SphK1 expression on CD4$^+$ and CD8$^+$ T lymphocytes was also augmented at the early stage of SAP (43.0 ± 5.1 vs 29.4 ± 3.3, $P < 0.01$; 40.8 ± 4.0 vs 33.9 ± 3.0, $P < 0.05$; compared with healthy controls) (Fig. 6D). The percent and MFI of SphK1 on T lymphocytes were all recovered to levels of controls at the restoration stage. The expression of SphK1 on CD4$^+$ T lymphocytes was positively correlated with the APACHE II scores ($r = 0.624$, $P < 0.01$, Fig. 6E). However, there was no correlation between SphK1 expression on CD8$^+$ T lymphocytes and APACHE II scores of SAP patients ($r = 0.368$, $P > 0.05$, Fig. 6F).

Our findings showed that expression of SphK1 on peripheral neutrophils, monocytes, and lymphocytes was strongly upregulated in the early stage. We identified that there was positive correlation between the SphK1 expression in peripheral neutrophils, monocytes, and CD4$^+$ T lymphocytes and APACHE II scores in SAP patients,

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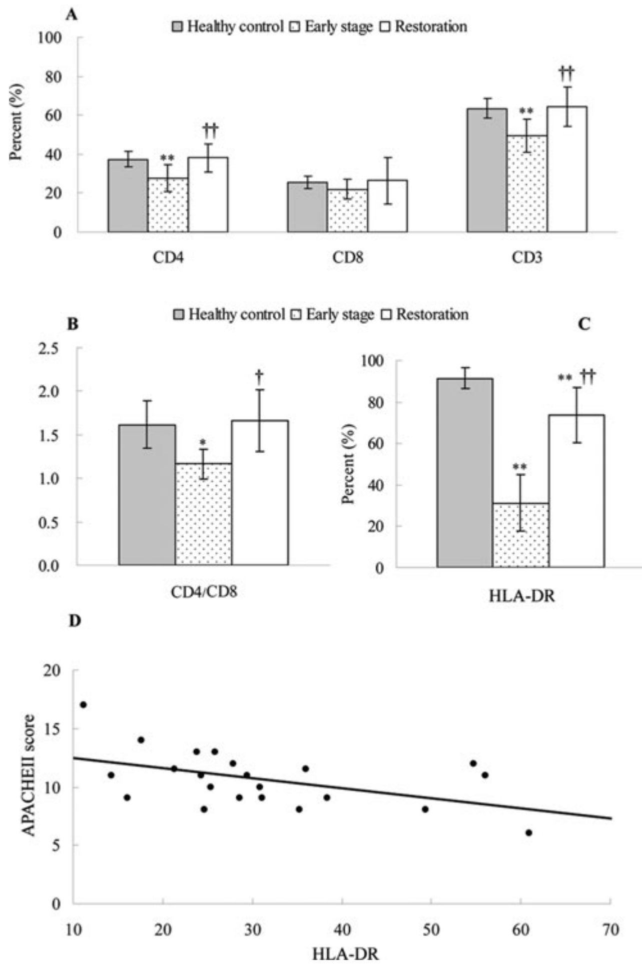

FIGURE 3. Changes in lymphocyte subpopulations and HLA-DR expression in SAP patients. A, Proportion of $CD4^+$ and $CD8^+$ T lymphocytes in SAP patients. B, Comparison of percentages changes in the ratio of $CD4^+/CD8^+$ lymphocytes. C, HLA-DR downregulation on monocytes as determined by flow cytometry. Percentages indicate the proportion of monocytes positive for HLA-DR antigens. D, Negative correlation between APACHE II values and the proportion of HLA-DR-positive monocytes. $^{**}P < 0.01$, $^{*}P < 0.05$ (early stage vs control) and ‡‡$P < 0.01$, ‡$P < 0.\ 05$ (restoration vs early stage).

suggesting that SphK1 expression may be served as a novel predictor for the diagnosis of the severity of SAP.

Upregulation of SphK Activity in SAP Patients

SphK activity, quantitated as the phosphorylation of sphingosine to S1P, was determined in SAP patients. As shown in Figure 7, direct measurement of SphK activity showed that the enzyme was activated in SAP patients in the early stage, which was in consistence with the elevated expression of SphK1. In the neutrophils from the patients, SphK activity was increased by 52.3% as compared with the healthy volunteers. Similar responses were presented in the lymphocytes, in which SphK activity also increased at the early stage of SAP. But there was no significant difference in SphK activity between SAP patients and the healthy controls. Furthermore, in the neutrophils of SAP patients, a reduction in SphK activity was noticed in the restoration stage. Similarly, the results demonstrated that SphK activity in the lymphocytes recovered to normal level when the patients discharged from hospital. The decrease in SphK activity was in line with the deduced expression of SphK1, indicating that changes in SphK activity in SAP patients may be ascribed to decreased SphK1 expression.

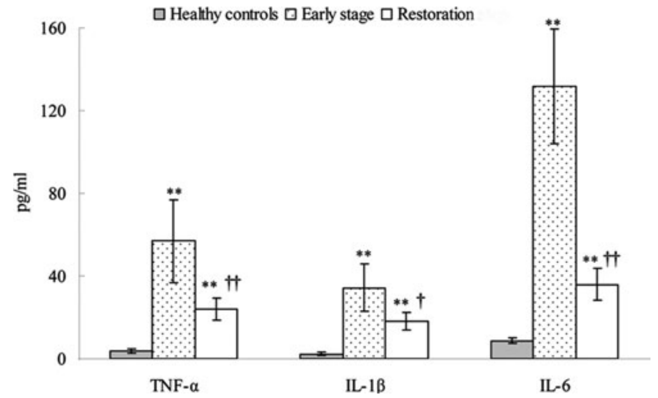

FIGURE 4. The levels of the proinflammatory mediators (TNF-α, IL-1β, and IL-6) in the serum of SAP patients. SAP patients demonstrate higher levels of TNF-α, IL-1β, and IL-6 during hospitalization. $^{**}P < 0.01$ (early stage vs control) and ‡‡$P < 0.01$, ‡$P < 0.05$ (restoration vs early stage).

The Expression of S1P Receptors mRNA in SAP Patients

To investigate the signaling mechanisms of S1P involved in SAP, we examined the expression of $S1P_1$ and $S1P_3$ mRNA in isolated neutrophils and lymphocytes by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. As shown in Fig 8A, the expression of $S1P_3$ mRNA in neutrophils was markedly increased by 1.64 folds in the early stage of SAP in comparison to the healthy volunteers ($P < 0.01$). Thereafter, the level of $S1P_3$ mRNA was lowered in neutrophils from SAP patients in the restoration ($P < 0.05$). Similar results were presented in the lymphocytes (Fig. 8B). Elevated expression of S1P3 mRNA was found in lymphocytes in the early stage, which was 1.42 folds higher than that in the healthy controls ($P < 0.05$). The $S1P_3$ mRNA level in lymphocytes was markedly reduced in the restoration stage ($P < 0.05$). Differing from $S1P_3$, the expression of $S1P_1$ mRNA in the neutrophils and the lymphocytes had no significant difference between the SAP patients and the healthy controls. These data indicated that $S1P_3$ may be involved in the inflammatory response in SAP.

DISCUSSION

The studies have shown that SphK1 and its product S1P are served as important mediators and closely involved in inflammation and inflammatory disease including sepsis,[16] asthma,[30] rheumatoid arthritis (RA),[33] and inflammatory bowel disease (IBD).[34,35] SphK1 has been established to play critical roles in modulating sepsis-induced inflammatory response. The blockade of SphK1 inhibits the secretion of inflammatory mediators such as TNF-α, IL-1β, and IL-6 in mice with sepsis.[16] The expression of SphK1 was increased in IBD and *SPHK1* gene deficiency could strikingly decrease the systemic inflammatory response in a mice model with dioctyl sodium sulfosuccinate (DSS)-induced colitis.[35] The specific role of SphK1/S1P involved in inflammatory response is unclear in SAP. In the present study, we investigated the expression of SphK1 and S1P receptors in the immune-effector cells including neutrophils, monocytes, and lymphocytes, in an effort to identify the role for SphK1/S1P in

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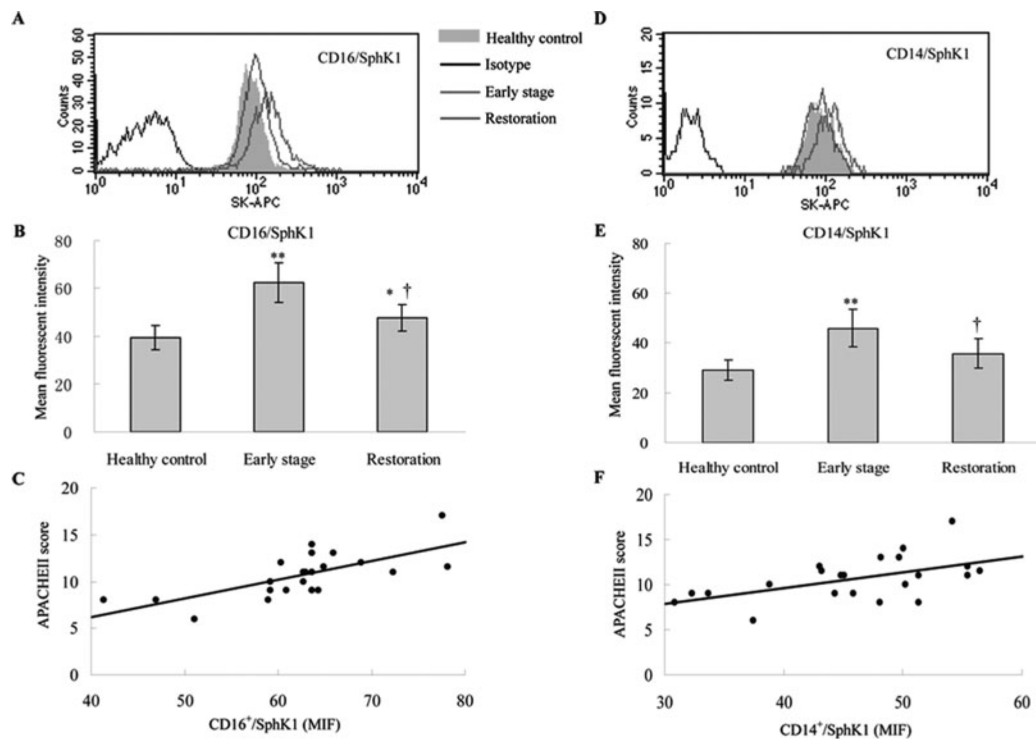

FIGURE 5. The elevated SphK1 expression in SAP patients and the positive correlation between the SphK1 expression in neutrophils and monocytes and APACHE II score. A, D, Flow cytometric analysis of intracellular SphK1 expression in neutrophils (A) and monocytes (D). Neutrophils and monocytes were stained with FITC-CD16 and FITC-CD14 antibody, respectively. Isotype control indicates staining of cells with an isotype control antibody. B, E, Mean fluorescent intensity of SphK1 positive cells. $^{**}P < 0.01$, $^{*}P < 0.05$ (early stage vs control) and $^{\ddagger}P < 0.05$ (restoration vs early stage). C, F, Analysis of correlations showed that SphK1 expression in peripheral neutrophils and monocytes were positively associated with APACHE II scores in patients with SAP ($P < 0.01$).

modulating inflammatory response in SAP. The findings raised a possibility that the role of SphK1 in inflammatory response may be mediated via $S1P_3$ signaling in SAP. And it is possible that this inflammatory response in SAP is mediated via SphK1/S1P signaling. Moreover, the data showed that SphK1 expression on human peripheral neutrophils, monocytes, and $CD4^+$ T lymphocytes is positively correlated with the APACHE II scores in patients with SAP.

The role of SphK1 and S1P in inflammatory disease has been established.[20] Inflammatory processes involved in the progression of an asthmatic attack are regulated by SphK1 and S1P.[36,37] SphK1 is activated in synoviocytes from RA patients and S1P is elevated in the synovium of RA patients.[38] SphK1 has a protective function in LPS- and platelet-activating factor-induced lung inflammation.[39,40] SphK1 participates in modulating systemic and local inflammatory response and it is as a potential therapeutic target for IBD.[34,35] These findings indicate a key role for the SphK1/S1P pathway in the pathology in inflammatory disease. Here we report for the first time that SphK1 is activated in SAP and its expression is recovered to normal levels in the restoration stage. Furthermore, we demonstrate a significantly positive correlation between SphK1 expression and the severity of SAP. These findings provided clues that SphK1 expression on peripheral immune-effector cells may play a beneficial role in the diagnosis of SAP.

SphK activation has been demonstrated to play a role in the inflammatory responses triggered by several immune-effector cells,[22,30,41] and it has pleiotropic effects on macrophages and monocytes[42,43] and neutrophils.[27,29,44] Recently, it has been shown that TNFα-triggered proinflammatory responses in human monocytes were dependent on SphK1 activity.[16] The activation of SphK1 is required for an oxidative burst in C5a-primed neutrophils.[27] SphK1 is closely involved in activation of immune cells in vitro[20] and the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 is reduced in human neutrophils through an inhibition of SphK1.[44] In addition, SPHK1$^{-/-}$ mice showed a marked reduction in neutrophil infiltration into the colon in DSS-induced colitis.[35] However, the role of SphK1 activation on immune effector cells is uncertain in SAP. In this study, SphK1 is upregulated in peripheral neutrophils, monocytes, and lymphocytes in the early stage in SAP patients. $S1P_3$ expression in peripheral neutrophils and lymphocytes displays a significant increase in transcript level in SAP. The activation of SphK1/$S1P_3$ signaling is simultaneously presented with the excessive excretion of proinflammatory cytokines in SAP patients. These data indicate that SphK1/S1P signaling may be implicated in immune-effector cells in inflammatory response for SAP patients. Our results suggest that there might be a role for SphK1/S1P signaling that is similar to what has been found in animal studies in other inflammatory conditions such as sepsis,[16] asthma,[30,36] and IBD.[34,35]

A shift of the cellular immune function is one of the major mechanisms responsible for early and late mortality in SAP. Recent researches suggested that immune dysregulation plays an important role in the development of SAP.[45,46] In this study, the decreased proportion of $CD4^+$ T cells is observed in SAP and it returns toward normal at the resolving stage. HLA-DR expression on peripheral monocytes is correlated with the clinical course and it is dependent on

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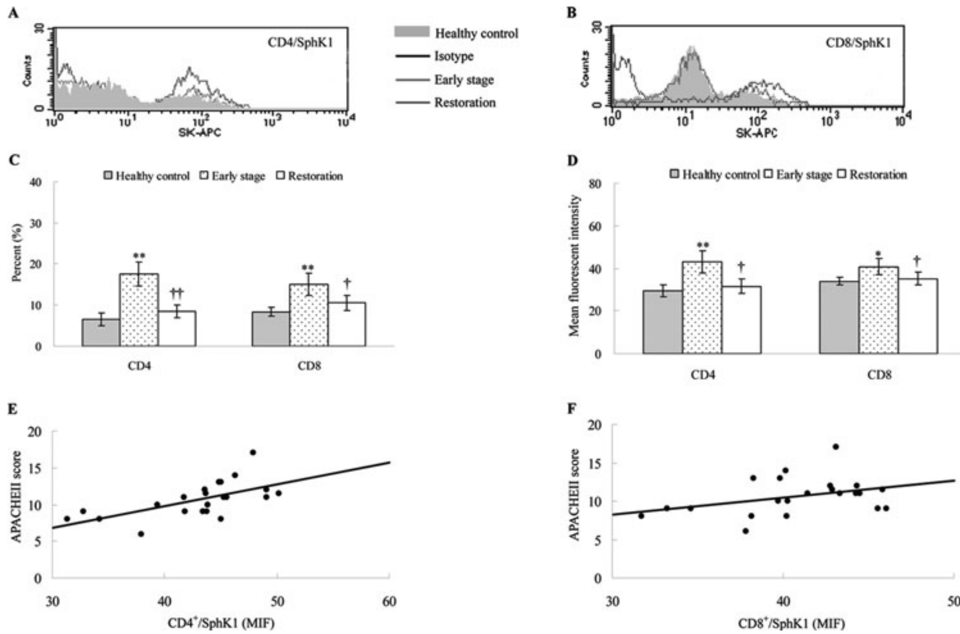

FIGURE 6. SphK1 protein expression in lymphocytes during SAP. A, B, Representative image of flow cytometric analysis. C, Proportion of SphK1-positive lymphocytes. D, Mean fluorescent intensity of SphK1 positive cells. $^{**}P < 0.01$, $^{*}P < 0.05$ (early stage vs control) and ‡‡$P < 0.01$, ‡$P < 0.05$ (restoration vs early stage). E, F, Pearson test showed that SphK1 expression in $CD4^{+}$ T lymphocytes was positively correlated with APACHE II scores in SAP patients ($P < 0.01$) (E). There was not correlation between SphK1 expression in $CD8^{+}$ T lymphocytes and APACHE II scores in these patients ($P > 0.05$) (F).

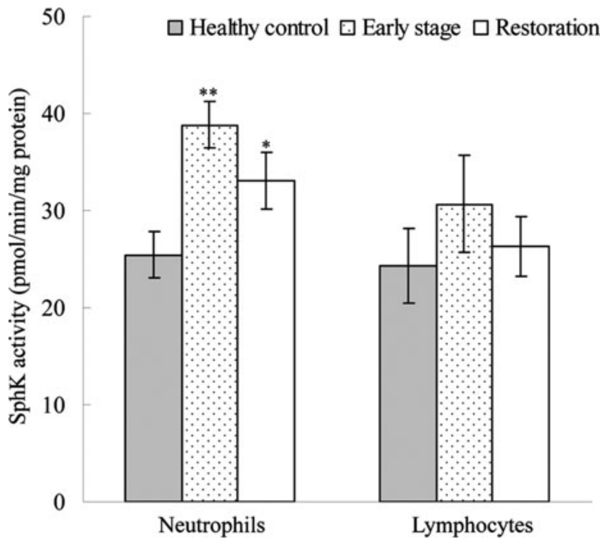

FIGURE 7. SphK enzymatic activity in neutrophils and lymphocytes. Results shown are the mean ± SD of triplicate measurements. Asterisks indicate significant difference as compared with healthy controls. $^{**}P < 0.01$, $^{*}P < 0.05$ (early stage vs control).

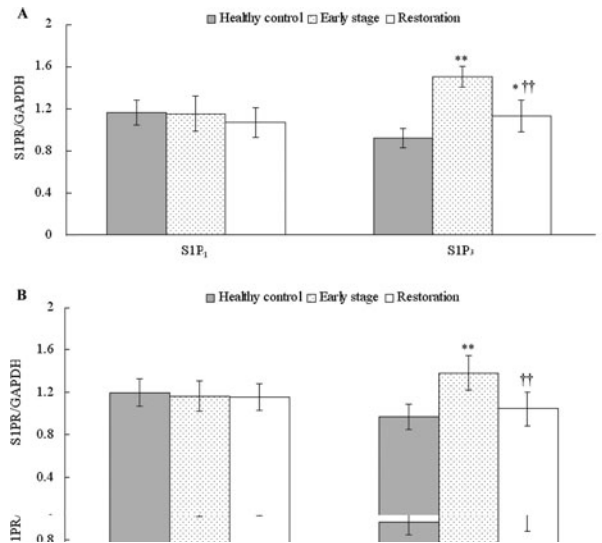

FIGURE 8. S1P receptors mRNA expression level in neutrophils (A) and lymphocytes (B) of SAP patients and healthy subjects. $S1P_3$ mRNA expression level was marked increased in SAP patients, whereas the levels of $S1P_1$ mRNA had no significant difference as compared with the healthy controls in both neutrophils and lymphocytes. $^{**}P < 0.01$, $^{*}P < 0.05$ (early stage vs control) and ‡‡$P < 0.01$, ‡$P < 0.05$ (restoration vs early stage).

the severity of acute pancreatitis.[47] We show that HLA-DR expression on monocytes is profoundly altered during the course of SAP. In the early stage of SAP, the expression of HLA-DR on monocytes is strikingly depressed and it is elevated at the restoration period in these patients. We identify a significant inversed correlation between the level of HLA-DR expression on monocytes and APACHE II scores in SAP patients. SAP is associated with a SIRS, which is manifested by elevated proinflammatory cytokines including TNF-α, IL-1β, and IL-6 in the serum of SAP patients. The severity and the clinical course of SAP seem to be correlated with the levels of proinflammatory

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cytokines.[48–50] In the present study, the levels of serum cytokines such as TNF-α, IL-1β, and IL-6 are significantly elevated in SAP, which is consistent with previous studies.[11]

In summary, we present evidence that activated SphK1/S1P signaling may be implicated in inflammatory responses in SAP. We further show a potential link between SphK1 expression on peripheral immune cells and the severity of SAP. These findings suggest that SphK1/S1P signaling may play an important role in modulating inflammatory response in SAP, and it may be considered as future therapeutic targets for the treatment of SAP.

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REFERENCES

1. Mofidi R, Duff MD, Wigmore SJ, et al. Association between early systemic inflammatory response, severity of multiorgan dysfunction and death in acute pancreatitis. *Br J Surg.* 2006;93:738–744.
2. Bradley EL, III, Allen K. A prospective longitudinal study of observation versus surgical intervention in the management of necrotizing pancreatitis. *Am J Surg.* 1991;161:19–24.
3. Gloor B, Muller CA, Worni M, et al. Late mortality in patients with severe acute pancreatitis. *Br J Surg.* 2001;88:975–979.
4. Gloor B, Muller CA, Worni M, et al. Pancreatic infection in severe pancreatitis: the role of fungus and multiresistant organisms. *Arch Surg.* 2001;136:592–596.
5. Widdison AL, Karanjia ND. Pancreatic infection complicating acute pancreatitis. *Br J Surg.* 1993;80:148–154.
6. Company L, Saez J, Martinez J, et al. Factors predicting mortality in severe acute pancreatitis. *Pancreatology.* 2003;3:144–148.
7. Lankisch PG, Pflichthofer D, Lehnick D. Acute pancreatitis: which patient is most at risk? *Pancreas.* 1999;19:321–324.
8. Banks PA, Freeman ML. Practice guidelines in acute pancreatitis.*Am J Gastroenterol.* 2006;101:2379–2400.
9. Mentula P, Kylanpaa ML, Kemppainen E, et al. Early prediction of organ failure by combined markers in patients with acute pancreatitis. *Br J Surg.* 2005;92:68–75.
10. Formela LG, Galloway SW, Kingsnorth AN. Inflammatory mediators in human acute pancreatitis. *Br J Surg.* 1995;82:6–13.
11. Mayer JM, Rau B, Gansauge F, et al. Inflammatory mediators in human acute pancreatitis: clinical and pathophysiological implications. *Gut.* 2000;47:546–552.
12. Norman JG, Fink GW, Messina J, et al. Timing of tumor necrosis factor antagonism is critical in determining outcome in murine lethal acute pancreatitis. *Surgery*. 1996;120:515–521.
13. Norman JG, Fink GW, Franz MG. Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. *Arch Surg.* 1995;130:966–970.
14. Pandol SJ, Saluja AK, Imrie CW, et al. Acute pancreatitis: bench to the bedside. *Gastroenterology.* 2007;132:1127–1151.
15. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4:397–407.
16. Puneet P, Yap CT, Wong LK, et al. SphK1 regulates proinflammatory responses associated with endotoxin and polymicrobial sepsis. *Science.* 2010;328:1290–1294.
17. Xia P, Gamble JR, Rye KA, et al. Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. *Proc Natl Acad Sci USA*. 1998;95:14196–14201.
18. Billich A, Bornancin F, Mechtcheriakova D, et al. Basal and induced sphingosine kinase 1 activity in A549 carcinoma cells: function in cell survival and IL-1beta and TNF-alpha induced production of inflammatory mediators. *Cell Signal.* 2005;17:1203–1217.
19. Alvarez SE, Milstien S, Spiegel S. Autocrine and paracrine roles of sphingosine-1-phosphate. *Trends Endocrinol Metab.* 2007;18:300–307.
20. Melendez AJ. Sphingosine kinase signalling in immune cells: potential as novel therapeutic targets. *Biochim Biophys Acta.* 2008;1784:66–75.
21. Egger M, Beer AG, Theurl M, et al. Monocyte migration: a novel effect and signaling pathways of catestatin. *Eur J Pharmacol.* 2008;598:104–111.
22. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* 2004;427:355–360.
23. Allende ML, Sasaki T, Kawai H, et al. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem.* 2004;279:52487–52492.
24. MacKinnon AC, Buckley A, Chilvers ER, et al. Sphingosine kinase: a point of convergence in the action of diverse neutrophil priming agents. *J Immunol.* 2002;169:6394–6400.
25. Wu W, Mosteller RD, Broek D. Sphingosine kinase protects lipopolysaccharide-activated macrophages from apoptosis. *Mol Cell Biol.* 2004;24:7359–7369.
26. Yadav M, Clark L, Schorey JS. Macrophage's pro-inflammatory response to a mycobacterial infection is dependent on Sphingosine kinase mediated activation of PI-PLC, PKC, ERK1/2 and PI-3 kinase. *J Immunol*. 2006;176:5494–5503.
27. Ibrahim FB, Pang SJ, Melendez AJ. Anaphylatoxin signaling in human neutrophils: a key role for sphingosine kinase. *J Biol Chem*. 2004;279:44802–44811.
28. Zhi L, Leung BP, Melendez AJ. Sphingosine kinase 1 regulates proinflammatory responses triggered by TNF-α in primary human monocytes. *J Cell Physiol*. 2006;208:109–115.
29. Vlasenko LP, Melendez AJ. A critical role for sphingosine kinase in anaphylatoxin-induced neutropenia, peritonitis, and cytokine production in vivo. *J Immunol*. 2005;174:6456–6461.
30. Lai WQ, Goh HH, Bao Z, et al. The role of sphingosine kinase in a murine model of allergic asthma. *J Immunol*. 2008;180:4323–4329.
31. Pancretology group of Chinese Surgical Society Chinese Medical Association. Preliminary regimen for diagnosis and treatment of severe acute pancreatitis. *Chinese J Surg*. 2001;16:699–700.
32. Billich A, Bornancin F, Dévay P, et al. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem*. 2003;278:47408–47415.
33. Kitano M, Hla T, Sekiguchi M, et al. Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium: regulation of synovial proliferation and inflammatory gene expression. *Arthritis Rheum*. 2006;54:742–753.
34. Deguchi Y, Andoh A, Yagi Y, et al. The S1P receptor modulator FTY720 prevents the development of experimental colitis in mice. *Oncol Rep*. 2006;16:699–703.
35. Snider AJ, Kawamori T, Bradshaw SG, et al. A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis. *FASEB J*. 2009;23:143–152.
36. Nishiuma T, Nishimura Y, Okada T, et al. Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol*. 2008;294:L1085–L1093.
37. Masini E, Giannini L, Nistri S, et al. Ceramide: a key signaling molecule in a Guinea pig model of allergic asthmatic response and airway inflammation. *J Pharm Exp Therap*. 2008;324:548–557.
38. Lai WQ, Irwan AW, Goh HH, et al. Anti-inflammatory effects of sphingosine kinase modulation in inflammatory arthritis. *J Immunol*. 2008;181:8010–8017.
39. Tauseef M, Kini V, Knezevic N, et al. Activation of sphingosine kinase-1 reverses the increase in lung vascular permeability through sphingosine-1-phosphate receptor signaling in endothelial cells. *Circ Res*. 2008;103:1164–1172.
40. Camerer E, Regard JB, Cornelissen I, et al. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J Clin Invest*. 2009;119:1871–1879.
41. Pappu R, Schwab SR, Cornelissen I, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science*. 2007;316:295–298.
42. Kee TH, Vit P, Melendez AJ. Sphingosine kinase signalling in immune cells. *Clin Exp Pharmacol Physiol*. 2005;32:153–161.
43. Melendez A, Floto RA, Gillooly DJ, et al. FcgRI-coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J Biol Chem*. 1998;273:9393–9402.
44. Niwa M, Kozawa O, Matsuno H, et al. Tumor necrosis factor-α-mediated signal transduction in human neutrophils: involvement of sphingomyelin metabolites in the priming effect of TNF-α on the fMLP-stimulated superoxide production. *Life Sci*. 2000;66:245–256.
45. DiMagno MJ, DiMagno EP. New advances in acute pancreatitis. *Curr Opin Gastroenterol*. 2007;23:494–501.
46. Frossard JL, Steer ML, Pastor CM. Acute pancreatitis. *Lancet*. 2008;371:143–152.

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47. Götzinger P, Sautner T, Spittler A, et al. Severe acute pancreatitis causes alterations in HLA-DR and CD14 expression on peripheral blood monocytes independently of surgical treatment. *Eur J Surg*. 2000;166:628–632.
48. Grewal HP, Kotb M, el Din AM, et al. Induction of tumor necrosis factor in severe acute pancreatitis and its subsequent reduction after hepatic passage. *Surgery*. 1994;115:213–222.
49. McKay CJ, Gallagher G, Brooks B, et al. Increased monocyte cytokine production in association with systemic complications in acute pancreatitis. *Br J Surg*. 1996;83:919–923.
50. de Beaux AC, Goldie AS, Ross JA, et al. Serum concentrations of inflammatory mediators related to organ failure in patients with acute pancreatitis. *Br J Surg*. 1996;83:349–353.

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