Sphingosine-1-phosphate Receptor 2 Signaling Promotes Caspase-11–dependent Macrophage Pyroptosis and Worsens *Escherichia coli* Sepsis Outcome

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**ABSTRACT**

**Background:** Pyroptosis, a type of proinflammatory programmed cell death, drives cytokine storm. Caspase-11–dependent macrophage pyroptosis contributes to mortality during sepsis. Sphingosine-1-phosphate receptor 2 (S1PR2) signaling can amplify interleukin-1β secretion in endotoxin-induced inflammation. Here, we hypothesized that S1PR2 signaling increases caspase-11–dependent macrophage pyroptosis and worsens Gram-negative sepsis outcome.

**Methods:** A Gram-negative sepsis model was induced through intraperitoneal injection of *Escherichia coli*. Primary peritoneal macrophages isolated from wild-type, *S1pr2*-deficient (*S1pr2*−/−), or nucleotide-binding oligomerization domain-like receptor protein-3–deficient mice were treated with *E. coli*. Caspase-11 activation, macrophage pyroptosis, and Ras homolog gene family, member A-guanosine triphosphate levels were assessed in these cells. Additionally, monocyte caspase-4 (an analog of caspase-11) expression and its correlation with S1PR2 expression were determined in patients with Gram-negative sepsis (*n* = 11).

**Results:** Genetic deficiency of S1PR2 significantly improved survival rate (2/10 [20%] in wild-type vs. 7/10 [70%] in *S1pr2*−/−, *P* = 0.004) and decreased peritoneal macrophage pyroptosis (pyroptosis rate: 35 ± 3% in wild-type vs. 10 ± 3% in *S1pr2*−/−, *P* < 0.001). Decreased caspase-11 activation in S1PR2 deficiency contributed to the reduced macrophage pyroptosis. In addition, RhoA inhibitor abrogated the amplified caspase-11 activation in wild-type or S1PR2–overexpressing cells. In patients with Gram-negative sepsis, caspase-4 increased significantly in monocytes compared to nonseptic controls and was positively correlated with S1PR2 (*r* = 0.636, *P* = 0.035).

**Conclusions:** S1PR2 deficiency decreased macrophage pyroptosis and improved survival in *E. coli* sepsis. These beneficial effects were attributed to the decreased caspase-11 activation of S1PR2-deficient macrophages. S1PR2 and caspase-11 may be promising new targets for treatment of sepsis. *(Anesthesiology 2018; 129:311-20)*

**What We Already Know about This Topic**

- No molecular-targeted treatments for sepsis have proved successful in humans. The role of sphingosine-1-phosphate receptor 2 (S1PR2) signaling in sepsis is uncertain.

**What This Article Tells Us That Is New**

- In an *in vivo* mouse model of Gram-negative sepsis, deletion of the gene for sphingosine-1-phosphate receptor 2 (S1PR2) reduced pyroptosis, possibly by decreased activation of caspase-11, and increased survival. S1PR2 and caspase-11 may be testable targets in sepsis.

Sepsis is a heterogeneous syndrome that is caused by infectious disease. This clinical syndrome is characterized by inappropriate and excessive host inflammatory responses to infection. Macrophages are the resident sentinel cells within the body and play a key role in processing cytokine storm. A better understanding of the inflammatory pathways that contribute to the pathogenesis of sepsis is crucial for the development of more effective diagnostic and therapeutic strategies for sepsis.

Pyroptosis is a proinflammatory programmed cell death. Initiation of pyroptosis requires at least one type of inflammasome, such as the canonical or noncanonical inflammasome. Nucleotide-binding oligomerization domain-like receptor 3, the best studied canonical inflammasome, is a cytosolic protein complex composed of nucleotide-binding oligomerization domain-like receptor 3, apoptosis-associated speck-like protein containing a caspase recruitment domain, and caspase-1 and assembled in response to both microbial infection and endogenous "danger signals." The caspase-11 inflammasome, also known as the noncanonical inflammasome, is formed by cytosolic exposure to endotoxin, a Gram-negative bacterial component that binds and directly activates mouse caspase-11, as well as the human paralogue caspase-4. Recent studies have shown that inflammasome-dependent macrophage pyroptosis contributes to mortality during sepsis. However, the upstream signaling that induces inflammasome activation remains unclear.
Sphingosine 1-phosphate (S1P) is a biologically active lipid that is generated from metabolites of membrane sphingolipids. S1P exerts its various biologic effects by binding to its five specific receptors (S1PR1-5), and S1PR2 is the predominant S1PR in macrophages. S1PR2 signaling was shown to promote cell death in diffuse large B-cell lymphoma and play a proinflammatory role in atherosclerosis, vascular inflammation, and sepsis.18–22 S1PR2 deficiency alleviated lipopolysaccharide-induced proinflammatory cytokine interleukin-1β levels in serum.22 Our previous study demonstrated that S1PR2 signaling decreased the survival rate of polymicrobial sepsis by inhibiting bacteria phagocytosis.19 However, the role of S1PR2 signaling in regulating *Escherichia coli*–induced sepsis and the host inflammatory response has not been addressed. This study aimed to determine (1) whether S1PR2 signaling promotes macrophage pyroptosis and worsens *E. coli*–induced sepsis, and (2) the mechanisms underlying S1PR2 signaling-induced macrophage pyroptosis.

**Materials and Methods**

**Animals**

All animal experiments were authorized by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China). Adult male C57BL/6 mice aged 8 to 10 weeks and weighing 20 to 25 g were used in our experiments. S1PR2 knockout (*S1pr2*−/−) and nucleotide-binding oligomerization domain-like receptor 3 knockout (*Nlrp3*−/−) mice were purchased from the Jackson Laboratory (USA). All gene knockout mice were backcrossed to a C57BL/6 background for 10 generations. Wild-type and knockout animals used in the experiments were age- and weight-matched littermates. The animals used in this study were randomized to experimental conditions. Blinding of the experimenters was used in flow cytometry analysis and human monocye S1PR2 expression detection.

**Peritoneal Sepsis Model**

*E. coli* (ATCC25922) was processed as previously reported.19 Mice were injected intraperitoneally with 3.5 × 10⁶ colony-forming units of *E. coli*. In experiments involving S1PR2 inhibitor, 4 mg/kg JTE-013 (Tocris Bioscience, USA) or vehicle control was administered intraperitoneally 30 min after bacterial injection. The survival rate was monitored hourly. Peritoneal lavage fluid and serum were obtained at 0 h and 6 h after infection.

**Peritoneal Lavage Fluid**

At 0 and 6 h after *E. coli* peritoneal model, peritoneal lavage fluid was lavaged and centrifuged at 350 g for 5 min at 4°C. The supernatants were stored at −80°C for enzyme-linked immunosorbent assay. The pellets were resuspended in Dulbecco’s modified eagle medium (Gibco, USA) containing 100 U/ml penicillin and 100 μg/ml streptomycin in 12-well tissue culture plates for attachment. After 30 min, the adherent macrophages were washed twice and collected for Western blots.

**Flow Cytometry**

Peritoneal lavage fluid cells were incubated with Fc block before staining with fluorescently labeled active caspase FAM-YVAD-FMK (ImmunoChemistry Technology, USA), propidium iodide (ImmunoChemistry Technology), annexin V (BD Biosciences, USA) and F4/80 (eBioscience, USA) according to the manufacturer’s instructions. Flow cytometry analysis was conducted with a BD LSR2 flow cytometer (BD Biosciences). Raw data were analyzed using CXP software (Beckman Coulter Inc., USA) or FlowJo software (TreeStar Corporation, USA). F4/80−, fluorescently labeled active caspase−, and propidium iodide−positive cells indicated macrophage pyroptosis. F4/80− and annexin V−positive and propidium iodide−negative cells referred to macrophage apoptosis.

**Cell Culture and Stimulation**

Mouse peritoneal macrophages were elicited by intraperitoneal injection 2 ml of 3% thioglycollate in phosphate buffered saline. Three days after the injection, mice were euthanized, and the abdominal wall was exposed. The abdominal cavity was lavaged with 15 ml phosphate buffered saline, and peritoneal cells were centrifuged at 350 g for 5 min at 4°C. The pellets were resuspended in Dulbecco’s modified eagle medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin and plated on 12-well tissue culture plates at a density of 1 × 10⁶ cells per well. After overnight incubation in 5% CO₂ at 37°C, adherent cells were washed twice with warm phosphate buffered saline and exchanged for fresh growth media. When indicated, cells were infected with *E. coli* at a multiplicity of infection of 25 for 1 h. The cells were then washed twice followed by 100 μg/ml gentamycin treatment for another 15 h. Control groups were incubated with phosphate buffered saline for the same duration. For the S1PR2 inhibitor experiments, 5 μM JTE-013 (Tocris Bioscience) or an equivalent volume of vehicle control (dimethyl sulfoxide) was incubated 30 min before infection. For the Ras homolog gene family, member A inhibitor experiments, Y27632 (Sigma-Aldrich, Germany) or an equivalent volume of vehicle control (phosphate buffered saline) was added to the medium after *E. coli* was removed. For S1PR2-overexpressing macrophages plated on glass-coated culture plates, either adenovirus-GFP-S1PR2 or adenovirus-GFP-vector control was added to the culture medium at a multiplicity of infection of 100 for 48 h. Transfection efficiency was evaluated by Western blot analysis of S1PR2 protein expression.

**Cell Cytotoxicity Assay**

Cell cytotoxicity was measured by the release of lactate dehydrogenase in the culture supernatant. Lactate dehydrogenase release was assessed by the LDH Cytotoxicity Assay Kit (Promega, USA) according to the manufacturer’s instructions and was calculated as the percentage of total lactate dehydrogenase.

**Enzyme-linked Immunosorbent Assay**

Cells and animals were treated as described above. Cell supernatants, serum, and peritoneal lavage fluid were harvested,
and interleukin-β secretion was assayed by enzyme-linked immunosorbent assay (eBioscience).

**Western Blotting**

Cell lysates and supernatant were processed as described previously. Proteins were transferred to nitrocellulose filter membranes (Bio-Rad, USA) after electrophoresis. The membranes were blocked with 5% nonfat dry milk for 1 h and incubated with primary antibodies, including anti-caspase-11 antibody (1:500, Sigma-Aldrich), anti-S1PR2 (1:500, OriGene Technologies, USA), anti-Ras homolog gene family, member A, antibody (1:1000, Thermo Pierce, USA) and anti-β-actin antibody (1:1000, Santa Cruz Biotechnology, USA), overnight on a shaker at 4°C. After three washes with Tris-buffered saline with 0.05% Tween-20, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (Lianke Technology, China) for 1 h at room temperature on a shaker. Signals were measured with an enhanced chemical luminescence kit (Thermo Pierce) and quantified by ImageJ software (Rawak Software Inc., Germany).

**Real-time Quantitative Polymerase Chain Reaction**

Total RNA was extracted using TRIzol (Life Technologies, USA) as described previously.19 RNA was reverse transcribed with a Reverse Transcription kit (Takara, Japan) followed by DNA amplification with the Roche 480 PCR System (Roche, USA). Primer sets specific for human β-actin, caspase-4, and S1PR2 were as follows: forward primer, 5′-CTC CAT CCT GGC CTC GCT GT-3′, and reverse primer, 5′-GCT GTC ACC TTC ACC GTC CC-3′; forward primer, 5′-AAAG AAG CAA CGT ATG GCA-3′; and reverse primer, 5′-AGG CAG ATG TGC AAA CTC TGT A-3′; forward primer, 5′-CAT CGT CAT CCT CGT TTG CG-3′; and reverse primer, 5′-GCC TGC CAG TAG ATC GGA G-3′. Relative expression level was determined by the 2-ΔΔCT model. β-Actin was used as an internal reference.

**Ras Homolog Gene Family, Member A Activation Assay**

Activated Ras homolog gene family, member A was measured by Glutathione S-transferase pull-down assays according to the manuals (Thermo Fisher Scientific, USA). Briefly, cells were lysed with radio-immunoprecipitation assay buffer containing protease inhibitor cocktail. After centrifugation, the supernatants of lysates were incubated with Glutathione S-transferase-rhotekin immobilized on agarose beads at 4°C for 60 min. After the samples were washed, the pull-down guanosine triphosphate-bound Ras homolog gene family, member A was examined by Western blot analysis with an anti-Ras homolog gene family, member A antibody.

**Blood Sample Collection**

The local institutional review board (Hangzhou, China) approved this study. Informed consent was obtained from patients or their surrogates before peripheral blood collection. Patients who met the clinical criteria for sepsis-3 were screened for eligibility within the first 24 h after they were admitted to the Intensive Care Unit of Zhejiang University Hospital, China, between September 2016 and January 2017. Simultaneously, nonseptic patients in critical condition were enrolled as controls. A total of 11 sepsis patients with Gram-negative bacterial infection and 8 nonseptic controls were enrolled. Patients younger than 18 yr or with cancer, pregnancy, human immunodeficiency virus, viral hepatitis, and immunosuppressant treatment were excluded. Clinical characteristics of the patients were recorded, including the Acute Physiology and Chronic Health Evaluation II score, the Sequential Organ Failure Assessment score, microbial species, sites of infection, and 28-day mortality.

**Human Monocyte Isolation**

Human monocytes were isolated from Ficoll density gradient centrifugation as previously described. The cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco) containing 10% fetal bovine serum in 12-well plates for 2 h. After the cells were washed three times with phosphate buffered saline, the adherent cells were harvested. Caspase-4 and S1PR2 levels were measured by real-time quantitative polymerase chain reaction.

**Statistical Analysis**

Data are presented as mean ± SD unless otherwise stated. There were no data lost for final analysis. An unpaired two-tailed Student’s t test or Welch’s correction t test was applied to compare two independent groups. One-way ANOVA followed by a Bonferroni post hoc test was used to analyze multiple groups. To determine the difference between two groups with time, we used a two-way ANOVA followed by a Bonferroni post hoc test. Survival rates were determined with the Mantel–Cox test. The association between caspase-4 and S1PR2 in the monocytes was examined by Pearson correlation analysis. Statistical values were defined as significant at P < 0.05. No a priori statistical power calculation was conducted; sample sizes were based on our previous experience.19 The diagrams and statistical analysis were conducted using GraphPad 5 software (GraphPad Software, USA).

**Results**

**S1PR2 Deficiency Improves E. coli Sepsis Outcome and Decreases Macrophage Pyroptosis**

To explore the role of S1PR2 signaling in vivo, we first established an E. coli sepsis model using intraperitoneal injection of E. coli and observed the survival rates in wild-type and S1PR2 intervention mice. Compared to wild-type mice, S1pr2−/− mice exhibited a significantly improved survival rate (from 2/10 [20%] to 7/10 [70%]) in E. coli sepsis (fig. 1A). S1PR2 inhibition using the specific antagonist JTE-013 showed a similar protective effect (Supplemental fig. 1A, Supplemental Digital Content, http://links.lww.com/ALN/B690). Inflammasome-induced pyroptosis was reported to exacerbate mortality in endotoxemia.16,17 Consistent with the reduced mortality, the flow cytometry results showed that S1PR2 deficiency or inhibition significantly decreased macrophage pyroptosis, from 35% in wild-type mice to 10% in S1pr2−/− mice (fig. 1B; Supplemental fig. 1B, Supplemental Digital Content, http://links.lww.com/ALN/B690).
Important Role of S1PR2 in Macrophage Pyroptosis

Drug therapy: 19 ± 3% in vehicle vs. 12 ± 4% in S1PR2 antagonist, \( P = 0.023 \), while macrophage apoptosis assay showed no significant difference between wild-type and S1pr2−/− mice at 6 h after sepsis onset (Supplemental fig. 2, Supplemental Digital Content, http://links.lww.com/ALN/B690; \( P = 0.805 \)). Pyroptosis leads to cellular proinflammatory content release, especially inflammasome-activated interleukin-1 family cytokines.8,17 We further found that S1PR2 deficiency or inhibition decreased interleukin-1β levels in the serum and peritoneal lavage fluid 6 h after E. coli injection (fig. 1C; Supplemental fig. 1C, Supplemental Digital Content, http://links.lww.com/ALN/B690). By contrast, tumor necrosis factor–α levels in the serum and peritoneal lavage fluid were comparable in wild-type and S1PR2 intervention mice (fig. 1D; Supplemental fig. 1D, Supplemental Digital Content, http://links.lww.com/ALN/B690). This phenomenon was also verified in the female mice (Supplemental fig. 3, Supplemental Digital Content, http://links.lww.com/ALN/B690). Taken together, these results indicate that S1PR2 aggravates the outcome of E. coli sepsis and promotes macrophage pyroptosis.

S1PR2 Signaling Regulates E. coli–induced Pyroptosis in Cultured Peritoneal Macrophages

Genetic deficiency of S1PR2 reduced macrophage pyroptosis in vivo. We therefore investigated whether S1PR2 signaling regulates pyroptosis in cultured peritoneal macrophages. Peritoneal macrophages isolated from S1pr2−/− and wild-type mice were stimulated with E. coli for 16 h. Macrophage pyroptosis was detected by measuring lactate dehydrogenase in the cell culture supernatant. Consistent with the observations in vivo, macrophage pyroptosis was substantially reduced in S1pr2−/− macrophages compared with wild-type macrophages (fig. 2A). In addition,
a significant decrease in interleukin-1β secretion was observed in the \( S1pr2^- \) macrophages (fig. 2B). However, tumor necrosis factor-α levels were comparable in the supernatants of wild-type and \( S1pr2^- \) macrophages (fig. 2C). To determine whether upregulation of S1PR2 expression could enhance macrophage pyroptosis, we transfected adenovirus-GFP-S1PR2 or adenovirus-GFP-vector control into macrophages for 48h. Western blots showed S1PR2 upregulation in adenovirus-GFP-S1PR2–transfected macrophages (fig. 2D). As shown in figure 2, E and F, S1PR2 overexpression significantly increased \( E. coli \)-induced macrophage pyroptosis and interleukin-1β secretion. However, S1PR2 overexpression did not influence tumor necrosis factor-α secretion (fig. 2G). These results indicate that S1PR2 signaling plays a crucial role in \( E. coli \)-induced macrophage pyroptosis.

**S1PR2 Signaling-induced Macrophage Pyroptosis Is Dependent on Caspase-11 Activation**

Next, we investigated the mechanisms underlying the facilitative role of S1PR2 signaling in macrophage pyroptosis. The nucleotide-binding oligomerization domain-like receptor 3 canonical inflammasome and caspase-11 noncanonical inflammasome are the major inflammasomes that can be activated by \( E. coli \) in macrophages. To identify which inflammasome is involved in the S1PR2-induced macrophage pyroptosis, we first collected peritoneal macrophages from wild-type and \( Nlrp3^- \) mice and treated them with \( E. coli \) for 16h. As shown in figure 3, there was no difference in lactate dehydrogenase release between the \( Nlrp3^- \) group and the wild-type group (fig. 3A). Furthermore, the lactate dehydrogenase levels were also comparable in S1PR2-inhibited \( Nlrp3^- \) macrophages and S1PR2-inhibited wild-type macrophages, indicating that nucleotide-binding oligomerization domain-like receptor 3 was not involved in S1PR2 signaling–mediated macrophage pyroptosis (fig. 3A).

To determine whether caspase-11 was involved in S1PR2-induced pyroptosis, we measured caspase-11 activation in macrophages by detecting its p26 cleavage product (caspase-11 active form) using Western blots. S1PR2 deficiency significantly decreased the caspase-11 p26 subunit in macrophages (fig. 3, B and C). In contrast, wild-type cells with S1PR2 overexpression showed enhanced caspase-11 activation after \( E. coli \) challenge (fig. 3D). We further determined whether nucleotide-binding oligomerization domain-like receptor 3 is involved in S1PR2-mediated caspase-11 activation. As shown in figure 3E, the levels of caspase-11 activation were similar in \( Nlrp3^- \) macrophages and wild-type macrophages, and the S1PR2 antagonist JTE-013 decreased caspase-11 activation in both \( Nlrp3^- \) macrophages and wild-type macrophages (fig. 3E). These results indicated that the S1PR2-induced macrophage pyroptosis is not dependent on the nucleotide-binding oligomerization domain-like

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**Fig. 2.** Sphingosine-1-phosphate receptor 2 (S1PR2) signaling regulates Escherichia coli–induced pyroptosis in cultured peritoneal macrophages. (A–C) Wild-type (WT) and \( S1pr2^- \) peritoneal macrophages were stimulated with \( E. coli \) (multiplicity of infection, 25). (A) Pyroptosis was examined by lactate dehydrogenase (LDH) activity in cell supernatant and expressed as the percentage of total cellular LDH activity (n = 4). Data are presented as mean ± SD and were analyzed by Student’s t test. (B) Interleukin (IL)-1β and (C) tumor necrosis factor–α (TNF-α) secretion to supernatant were determined using enzyme-linked immunosorbent assay (n = 6). Data are presented as mean ± SD and were analyzed by Student’s t test. (D–G) WT peritoneal macrophages were transfected with adenovirus-GFP-S1PR2 (Ad-S1PR2; multiplicity of infection, 100) or adenovirus-GFP-vector control (Ad-null; multiplicity of infection, 100) for 48h and then stimulated with \( E. coli \) (multiplicity of infection, 25). (D) S1PR2 level was detected by Western blot, and β-actin was used as an internal control. Values are presented as mean ± SD from three independent experiments and were analyzed using Welch’s correction t test. (E) Pyroptosis was examined by LDH activity in cell supernatant and expressed as the percentage of total cellular LDH activity (n = 4). Data are presented as mean ± SD and were analyzed by Student’s t test. (F) IL-1β and (G) TNF-α release to supernatant were detected by enzyme-linked immunosorbent assay (n = 6). Data are presented as mean ± SD and were analyzed by Student’s t test. *P < 0.05, **P < 0.01. Cont = control.
Fig. 3. Sphingosine-1-phosphate receptor 2 (S1PR2) signaling–induced macrophage pyroptosis is dependent on caspase-11 activation. (A) Wild-type (WT) and Nlrp3−/− peritoneal macrophages were pretreated with 5 μM JTE-013 (a S1PR2 antagonist) or vehicle for 30 min before *Escherichia coli* (multiplicity of infection, 25) stimulation. Pyroptosis was determined by lactate dehydrogenase (LDH) activity in cell supernatant and expressed as the percentage of total cellular LDH activity (n = 4). Data are presented as mean ± SD and were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. (B) Caspase-11 activation of peritoneal macrophages from *E. coli* sepsis was measured by Western blot at the indicated time point. Data are presented as mean ± SD from three independent experiments and were analyzed by Welch’s correction t test. (C) WT and S1pr2−/− peritoneal macrophages were stimulated with *E. coli* (multiplicity of infection, 25). Caspase-11 activation was detected by Western blot. Data are presented as mean ± SD from three independent experiments and were analyzed by Welch’s correction t test. (D) WT peritoneal macrophages were transfected with adenovirus-GFP-S1PR2 (Ad-S1PR2; multiplicity of infection, 100) or adenovirus-GFP-vector control (Ad-null; multiplicity of infection, 100) for 48 h and then stimulated with *E. coli* (multiplicity of infection, 25). Caspase-11 activation was detected by Western blot. Data are presented as mean ± SD from three independent experiments and were analyzed by Welch’s correction t test. (E) WT and Nlrp3−/− peritoneal macrophages were pretreated with 5 μM JTE-013 or vehicle for 30 min before *E. coli* (multiplicity of infection, 25) stimulation. Caspase-11 activation was detected by Western blot. Data are presented as mean ± SD from three independent experiments and were analyzed using two-way ANOVA analysis followed by a Bonferroni post hoc test. Representative images are shown on the top, and band intensity quantifications are shown on the bottom. *P < 0.05, **P < 0.01, ***P < 0.001. Cont = control.
Ras homolog gene family, member A Regulates S1PR2 Signaling-mediated Caspase-11 Activation in Macrophages

Although the above results indicated that S1PR2 was required to regulate caspase-11 activation in response to *E. coli* stimulation, the detailed mechanism was still unclear. Ras homolog gene family, member A/Rho-associated protein kinase signaling is responsible for inflammatory responses, and endogenous S1P treatment increased Ras homolog gene family, member A-guanosine triphosphate levels in wild-type cells but not in *S1pr2*<sup>−/−</sup> cells.¹⁹⁻²¹ Thus, we hypothesized that S1PR2 signaling might promote caspase-11 activation through Ras homolog gene family, member A signaling. To test this hypothesis, we first measured guanosine triphosphate-bound Ras homolog gene family, member A using affinity pull-down assays to verify the Ras homolog gene family, member A activation in *S1pr2*<sup>−/−</sup> and S1PR2 overexpression macrophages at 16 h after *E. coli* stimulation. Figure 4A shows that Ras homolog gene family, member A-guanosine triphosphate protein was significantly reduced in *S1pr2*<sup>−/−</sup> macrophages compared to wild-type macrophages. S1PR2-overexpressing macrophages showed increased Ras homolog gene family, member A activation (fig. 4B). To investigate the role of Ras homolog gene family, member A in caspase-11 activation, we treated macrophages with the minimum effective concentration of Y27632 (Ras homolog gene family, member A inhibitor, 10 μM; Supplemental fig. 4, Supplemental Digital Content, http://links.
lww.com/ALN/B690, which shows the dose-dependent effect of the Ras homolog gene family, member A inhibitor on caspase-11 activation). We found that the \textit{E. coli}–induced caspase-11 activation was suppressed by Y27632 in wild-type and S1PR2 overexpression cells (fig. 4, C and D). Collectively, these results indicate that S1PR2 leads to Ras homolog gene family, member A activation, which in turn promotes caspase-11 activation.

**Monocyte Caspase-4 Correlates with S1PR2 Expression Levels in Patients with Gram-negative Sepsis**

Caspase-4 is the human orthologue of murine caspase-11 in human monocytes.\textsuperscript{12,24,25} To further characterize the role of S1PR2 and caspase-4 in Gram-negative sepsis, we determined the expression levels of caspase-4 and S1PR2 in monocytes isolated from Gram-negative septic patients (n = 11) and critical controls (n = 8). The characteristics of the study subjects are shown in table 1. Monocytes caspase-4 and S1PR2 messenger RNA (mRNA) levels were significantly higher in Gram-negative septic patients than nonseptic controls (P < 0.05; fig. 5, A and B). Furthermore, increased caspase-4 expression was positively correlated with the S1PR2 levels in septic patients (r = 0.636; P = 0.035; fig. 5C). These data suggest that S1PR2 and caspase-4 may be promising new targets for treatment of sepsis.

**Discussion**

The current study demonstrated that S1PR2 is an upstream signaling molecule of caspase-11 activation in \textit{E. coli} sepsis. S1PR2 deficiency decreased caspase-11 activation, macrophage pyroptosis, and interleukin-1\(\beta\) secretion both \textit{in vivo} and \textit{in vitro}. In contrast, S1PR2 overexpression promoted macrophage pyroptosis and subsequent interleukin-1\(\beta\) secretion after \textit{E. coli} stimulation. Ras homolog gene family, member A inhibitor could abrogate the enhanced caspase-11 activation in wild-type or S1PR2 overexpression macrophages. In addition, nucleotide-binding oligomerization domain-like receptor 3 was not involved in S1PR2 signaling–mediated macrophage pyroptosis. In patients with Gram-negative bacterial sepsis, caspase-4 mRNA levels increased significantly in blood monocytes compared to those in nonseptic controls and were positively correlated with S1PR2 expression.

A recent study showed that variations in pathogens and their characteristics interact with the host to produce variations in sepsis incidence and mortality.\textsuperscript{26} Furthermore, the site of infection is also associated with mortality when controlling for organisms, with the risk of death highest for intraabdominal infections.\textsuperscript{27} In this study, Gram-negative bacterial sepsis model was induced by intraperitoneal injection of \textit{E. coli}. The rationale for using this model was based on the finding that infections due to Gram-negative bacteria outnumbered those caused by Gram-positive bacteria.\textsuperscript{28} Moreover, Gram-negative infections were associated with an increased risk of mortality in sepsis.\textsuperscript{29} The \textit{E. coli}–induced sepsis was characterized by a hyperinflammatory response at the early stage. In addition, we observed that mouse death was concentrated within 24 h after sepsis onset. In this model, \textit{S1pr2}\textsuperscript{–/–} mice exhibited a significantly improved survival rate, which may be attributed to the decreased macrophage pyroptosis and interleukin-1\(\beta\) secretion. Combined with our previous study showing that S1PR2 deficiency protected against cecal ligation and puncture–induced sepsis,\textsuperscript{19} these findings indicated that S1PR2 is harmful during sepsis.

Macrophages are the resident sentinel cells within the body and play a key role in \textit{E. coli} internalization and clearance through phagocytosis. In our previous study, we found that S1PR2 signaling impairs phagocytosis and antimicrobial defense in the pathogenesis of polymicrobial sepsis.\textsuperscript{19} Intracellular \textit{E. coli} can be recognized by the cytoplasmic inflammasome and then triggers pyroptosis and proinflammatory cytokine interleukin-1\(\beta\) release.\textsuperscript{8,14,17} In this study, we found that genetic deficiency of S1PR2 significantly improved survival, along with decreased peritoneal macrophage pyroptosis and interleukin-1\(\beta\) secretion in \textit{E. coli} sepsis, indicating that S1PR2 signaling is critical in the regulation of macrophage pyroptosis.

Apoptosis is a type of noninflammatory programmed cell death. The contents of apoptotic cells are packed in “apoptotic bodies” and then targeted for phagocytosis, thereby avoiding inflammatory responses.\textsuperscript{30} In contrast, pyroptosis releases cellular contents to the extracellular space and thus generates a robust proinflammatory response.\textsuperscript{8,9} In our study, we used flow cytometry to detect apoptosis and pyroptosis and found there was no significant difference in apoptosis level between the wild-type and \textit{S1pr2}\textsuperscript{–/–} groups at 6 h after sepsis, while pyroptosis was substantially reduced in the \textit{S1pr2}\textsuperscript{–/–} group compared with the wild-type group.

### Table 1. Characteristics of Gram-negative Septic Patients and Nonseptic Controls

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<th>Characteristics</th>
<th>Sepsis (n = 11)</th>
<th>Control (n = 8)</th>
<th>P Value</th>
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<tr>
<td>Age (yr)</td>
<td>55 ± 15</td>
<td>53 ± 17</td>
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<td>Sex, male</td>
<td>11 (100)</td>
<td>5 (62.5)</td>
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<td>Days in ICU*</td>
<td>3 (2–10)</td>
<td>10 (5–15)</td>
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<td>APACHE II score</td>
<td>12 ± 7</td>
<td>13 ± 6</td>
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<td>SOFA score</td>
<td>7 ± 3</td>
<td>6 ± 2</td>
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<td>2 (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple injuries</td>
<td>2 (18)</td>
<td></td>
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</table>

*Data are shown as the mean (SD) or number (%) unless stated otherwise. *Data are shown as the median (interquartile range). †One patient has urogenital infection and multiple injuries.

APACHE II = Acute Physiology and Chronic Health Evaluation II; ICU = intensive care unit; NA = not applicable; SOFA = Sequential Organ Failure Assessment.
Therefore, we concluded that S1PR2-mediated macrophage pyroptosis worsens the outcome of *E. coli* sepsis.

Initiation of pyroptosis requires at least one type of inflammasome, canonical or noncanonical inflammasomes. We first investigated whether the canonical inflammasome nucleotide-binding oligomerization domain-like receptor 3 participated in S1PR2 signaling–mediated macrophage pyroptosis. Interestingly, nucleotide-binding oligomerization domain-like receptor 3 deficiency neither altered *E. coli*–induced pyroptosis nor influenced the inhibitory effect of S1PR2 antagonist on pyroptosis. However, noncanonical inflammasome caspase-11 activation was enhanced through S1PR2 signaling. These results are consistent with previous reports showing that the caspase-11–dependent noncanonical inflammasome promotes pyroptosis during *Gram*-negative bacterial infections.17,23 Above all, our study found that S1PR2 signaling drives caspase-11 activation and subsequent macrophage pyroptosis in *E. coli* sepsis.

Ras homolog gene family, member A belongs to the small guanosine triphosphate kinase of the Rho family and plays a role in inflammatory diseases.20,31 In our current study, we observed that Ras homolog gene family, member A activation was reduced in S1pr2−/− macrophages compared with wild-type macrophages after *E. coli* stimulation. S1PR2 overexpression could enhance Ras homolog gene family, member A activation. In addition, Ras homolog gene family, member A inhibitor reversed *E. coli*–induced caspase-11 activation in both wild-type and S1PR2-overexpression macrophages. Therefore, Ras homolog gene family, member A is a key regulator of S1PR2 signaling–mediated caspase-11 activation in macrophages.

To test the clinical significance of the results from our animal studies, we collected peripheral blood monocytes from patients with Gram-negative sepsis and nonseptic controls. S1PR2 and caspase-4 expression levels in the monocytes were measured by real-time quantitative polymerase chain reaction. We found that both S1PR2 and caspase-4 mRNA levels were significantly increased in monocytes from the sepsis patients. Furthermore, the increased caspase-4 expression was positively correlated with S1PR2 expression in septic patients. Combined with the knowledge that S1PR2 and caspase-4 are associated with severity of sepsis,12,19,20 our findings suggest that S1PR2 and caspase-4 may be promising new targets for treatment of sepsis.

In our clinical study, the septic patients were all males, whereas the control group included both sexes. Previous studies showed that sex was a crucial prognostic factor for sepsis.32–34 To detect whether sex impacted our findings, we conducted our animal experiments using both male and female mice. We observed similar results showing that S1PR2 deficiency improved the survival rate and reduced macrophage pyroptosis in animals of both sexes. Therefore, we concluded that the role of S1PR2 in the clinic is not affected by sex.

In summary, the current study showed that interfering with S1PR2 signaling decreased caspase-11 activation and subsequent macrophage pyroptosis. Interventions targeting S1PR2 signaling may offer a promising therapeutic approach for cytokine storm control during sepsis.

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**Competing Interests**

The authors declare no competing interests.

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**References**


Important Role of S1PR2 in Macrophage Pyroptosis


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