Sphingosine 1-phosphate Receptor 2 Signaling Suppresses Macrophage Phagocytosis and Impairs Host Defense against Sepsis

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ABSTRACT

Background: Sepsis is characterized by an inappropriate systemic inflammatory response and bacteremia that promote multiorgan failure and mortality. Sphingosine 1-phosphate receptor 2 (S1PR2) modulates endotoxin-induced inflammation in endothelium. However, as a highly expressed S1P receptor in macrophages, its role in regulating macrophage response to bacterial infection remains unclear.

Methods: Cecal ligation and puncture or intratracheal instillation of *Escherichia coli* was induced in wild-type or *S1pr2*-deficient mice. The antibacterial ability of cell-specific S1PR2 was tested in bone marrow reconstitution mice or mice with macrophage-specific deletion. Signaling molecules responsible for S1PR2-mediated phagocytosis were also measured in the bone marrow–derived macrophages. In addition, S1PR2 expression levels and its correlation with severity of sepsis were determined in critically ill patients (n = 25).

Results: Both genetic deletion and pharmaceutical inhibition of S1PR2 significantly limited bacterial burden, reduced lung damage, and improved survival (genetic deletion, 0% in $S1pr2^{+/+}$ *vs.* 78.6% in $S1pr2^{-/-}$, $P < 0.001$; pharmaceutical inhibition, 9.1% in vehicle *vs.* 22.2% in S1PR2 antagonist, $P < 0.05$). This protection was attributed to the enhanced phagocytic function of S1PR2-deficient macrophages (mean fluorescent intensity, 2035.2 ± 202.1 *vs.* 407.8 ± 71.6, $P < 0.001$). Absence of S1PR2 in macrophage inhibits RhoA-dependent cell contraction and promotes IQGAP1-Rac1-dependent lamellipodial protrusion, whose signaling pathways depend on extracellular stimulators. In septic patients, increased S1PR2 levels in peripheral blood mononuclear cells were positively correlated with the severity of sepsis ($r = 0.845$, $P < 0.001$).

Conclusions: This study implies that S1PR2, as a critical receptor in macrophage, impairs phagocytosis and antimicrobial defense in the pathogenesis of sepsis. Interventions targeting S1PR2 signaling may serve as promising therapeutic approaches for sepsis. **(Anesthesiology 2015; 123:409-22)**

SEPSIS is the leading cause of death among critically ill patients worldwide. This clinical syndrome is characterized by systemic inflammatory response and bacteremia that promote multiorgan failure and mortality.[1] The lung is usually the first organ to be affected by this process. Sepsis-induced acute respiratory failure has a mortality rate greater than 40%.[2,3] Despite advancements in life-support care, improvements in patient survival rates after sepsis have been limited because of the increasing resistance of some bacterial strains to antimicrobial agents.[1,3,4] Thus, approaches to enhance the host immune response are becoming particularly important for addressing such infectious threats.

Macrophages are the resident sentinel cells within the body and play a key role in innate immunity during the early phase

What We Already Know about This Topic

- Sphingosine 1-phosphate receptor 2 (S1PR2) modulates endotoxin-induced inflammation in endothelial cells. Although it is a highly expressed S1P receptor in macrophages, its role concerning antimicrobial defense in sepsis is unclear.

What This Article Tells Us That Is New

- Deficiency in S1PR2 enhanced bacterial clearance and improved survival in the mouse model of sepsis. These beneficial effects are attributed to an increase in the phagocytic activity of S1PR2-deficient macrophages. Interventions targeting S1PR2 signaling may thus offer a promising therapeutic approach for the prevention and/or treatment of sepsis.

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of infection. Effective internalization and clearance of invading pathogens through phagocytosis by alveolar macrophages (AMs) is a key defense mechanism in maintaining the sterility of the airway tract.[5,6] Phagocytosis is a dynamic and diverse process using various receptors and signaling pathways to remodel the actin cytoskeleton specifically to engulf particulate targets.[7] In addition to the well-studied opsonin-mediated mechanisms, recent studies have reported that oxidized phospholipids, which are generated through peroxidation of host membrane phospholipids during infection and inflammation, participate in the signaling events that drive actin reorganization.[8,9] These findings uncovered a vital role for endogenous lipid mediators in orchestrating phagocytosis.

Sphingosine-1-phosphate (S1P) is a natural bioactive metabolite of mammalian membrane sphingolipids and regulates biological functions of many cells.[10] The cell-extrinsic function of S1P is mediated by five different transmembrane G protein-coupled receptors: sphingosine 1-phosphate receptor 1 (S1PR1) to S1PR5, in which S1PR2 is one of the most studied. Activation of S1PR2 is critical for mast cell degranulation and follicular helper T-cell retention.[11–13] Endothelial S1PR2 is a key regulator of vascular inflammation during endotoxemia.[14,15] Studies have also shown that S1PR2 is the most abundant S1P receptor on macrophages.[16,17] The biological role of S1PR2 in antimicrobial host defense has not been well characterized. Recent studies have found that macrophages isolated from S1PR2-null mice were defective in opsonic phagocytosis of fungus and that in the absence of opsonization, S1PR2-silenced dendritic cells had enhanced endocytic function.[16,18] The inconsistency of these findings requires further clarification using more appropriate methodology and physiologically relevant mouse models. Herein, we hypothesized that S1PR2 signaling is important in macrophage phagocytosis in cecal ligation and puncture (CLP)–induced sepsis and bacterial infusion sepsis models. Therefore, we dissected the role of S1PR2 in sepsis and elaborated the signaling mechanisms involved in S1PR2-mediated phagocytosis *in vitro*.

Materials and Methods

Animals

CD45.2 mice (C57BL/6 × 129Sv mixed background) with targeted disruption of the *S1pr2* gene were obtained from the Mutant Mouse Regional Resource Centers (USA; MMRRC Strain ID, 12830). Mice were maintained in a specific pathogen-free mouse facility with food and water *ad libitum*. All mice used in this study, including wild-type (WT, $S1pr2^{+/+}$), heterozygote ($S1pr2^{+/-}$), and knockout ($S1pr2^{-/-}$), were sex- and age-matched littermates. CD45.1 WT mice (C57BL/6) were kindly provided by Prof. Zhenyu Ju, M.D. (Institute of Aging Research, School of Medicine, Hangzhou Normal University, Hangzhou, Zhejiang Province, China). All animal experiments were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China). Animals were randomized to experimental conditions. Blinding of the experimenters was used in any fashion.

Polymicrobial Sepsis and Microbiology Inspection

The polymicrobial septic mouse model was induced using CLP as described recently.[19] Mortality was assessed daily. To determine the bacterial burden, peritoneal lavage fluid, blood samples, and lung homogenate were obtained at 72 h after CLP challenge, serially diluted in 10-fold with sterile phosphate-buffered saline (PBS), and assayed as described previously.[19]

Pulmonary Bacterial Infection and Sample Harvest

Mice were treated by intratracheal instillation of 50 μl saline containing 2×10^6 colony-forming units of *Escherichia coli* (ATCC 25922) as described previously.[20] In some experiments, a single dose of JTE-013 (4 mg/kg; Tocris Bioscience, USA) or vehicle only was administered intratracheally 30 min before bacterial inoculation. Mortality was assessed hourly. Bronchoalveolar lavage fluid (BALF), blood, and lung tissue were obtained at 0, 4, and 18 h after infection and were assayed as described previously.[21]

Bone Marrow Transplantation

The recipients, namely WT CD45.2 or $S1pr2^{-/-}$ CD45.2 mice (8-week old), were lethally irradiated with a single dose of 8 Gy using the RS 2000 X-ray Biological Irradiator (Rad Source Technologies, USA). Two to three hours later, freshly isolated bone marrow (BM) cells (3 to 5×10^6) from young adult donors, namely WT CD45.1 or $S1pr2^{-/-}$ CD45.2, were administrated to the recipients through retro-orbital injection to create the following BM chimeras: WT→WT, $S1pr2^{-/-} \rightarrow S1pr2^{-/-}$, WT→ $S1pr2^{-/-}$, and $S1pr2^{-/-}$ →WT. The chimeras were allowed to recover under sterile conditions for 8 weeks. Hematopoietic reconstitution by the donor cells was verified with flow cytometry analysis of the CD45.1/CD45.2 expression ratio on the myeloid lineage cells in blood, BM, and BALF of the recipients.

Depletion of AMs In Vivo

Mouse AMs were depleted as described previously.[22] Briefly, 100 μl of either clodronate-conjugated liposomes or control liposomes (FormuMax Scientific, USA) were administered to the mouse through intratracheal instillation 48 h before inoculation with *E. coli*. AM depletion was confirmed *via* flow cytometry analysis of BALF.

Cell Culture

See text documents, Supplemental Digital Content 1, http://links.lww.com/ALN/B157, which lists the necessary methods used in this study.

Phagocytosis and Bactericidal Assay

AMs, BM-derived macrophages (BMDMs), or peripheral blood mononuclear cells (PBMCs) were cultured in Dulbecco's minimum essential medium containing 2%

charcoal-treated fetal calf serum for 24 h. On the following day, cells were starved in serum-free Dulbecco's minimum essential medium for another 2 h and then incubated with *E. coli* for 30 or 60 min at 37°C. Phagocytosis was stopped by washing three times with cold PBS.

To opsonize bacteria, 100 μl of the reconstituted opsonizing reagent (rabbit polyclonal IgGs, Life Technologies, USA) was mixed with either 100 μl Texas Red coupled *E. coli* (Life Technologies) or 100 μl live *E. coli* (1×10^8 colony forming units) and incubated at 37°C for 1 h. After three washes, the mixture was resuspended in cold PBS. The multiplicity of infection of viable bacteria was estimated as described.[23]

Macrophage bactericidal assays were performed as described previously.[24] AMs were incubated with either opsonized or unopsonized live *E. coli* (multiplicity of infection = 20) for 1 h. Gentamicin was then added to a final concentration of 1 μg/ml, and the mixture was incubated for an additional 30 min to kill extracellular bacteria. Cells were washed twice with warm PBS and cultured in fresh growth media. At 0, 4, and 12 h after incubation, AMs were lysed in 0.1% Triton X-100/PBS. Surviving bacteria were quantified as described.[19]

S1P Measurement

See text documents, Supplemental Digital Content 1, http://links.lww.com/ALN/B157, which lists the necessary methods used in this study.

Confocal Microscopy

See text documents, Supplemental Digital Content 1, http://links.lww.com/ALN/B157, which lists the necessary methods used in this study.

Western Blot, Pull Down Assay, and Coimmunoprecipitation

See text documents, Supplemental Digital Content 1, http://links.lww.com/ALN/B157, which lists the necessary methods used in this study.

Small Interfering RNA, Semiquantitative Reverse Transcription–Polymerase Chain Reaction and Quantitative Reverse Transcription–Polymerase Chain Reaction

See text documents, Supplemental Digital Content 1, http://links.lww.com/ALN/B157, which lists the necessary methods used in this study, and see Supplemental Digital Content 1, table 1, http://links.lww.com/ALN/B157, which lists all the primers used in this study

Human Subjects

See Supplemental Digital Content 1, tables 2 and 3, http://links.lww.com/ALN/B157, which lists all the patients characteristics studied in this study.

Statistical Analysis

Data are presented as the means ± SD unless stated otherwise. A two-tailed Student *t* test or a Mann–Whitney test was used to compare difference between two independent groups. Multigroup comparisons were assessed using ANOVA followed by Bonferroni *post hoc* tests. Among which, the two-way ANOVA was conducted to examine the effects of two independent variables (cell types and stimulus) on *E. coli* phagocytosis and RhoA or Rac1 activity separately. Survival rates were analyzed with the Mantel–Cox test. The relationship between S1PR2 expression levels in PBMCs and Acute Physiology and Chronic Health Evaluation II score of the septic patients were assessed using the Pearson correlation analysis. Sample sizes were based on our previous experience. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., USA) or Prism 6.0 (GraphPad software Inc., USA), and *P* value less than 0.05 was considered statistically significant.

Results

S1PR2 Signaling Negatively Regulates Host Response to Infection

We first assessed the overall effect of S1PR2 during polymicrobial sepsis. In a sublethal CLP model, the survival rate of the *S1pr2*$^{-/-}$ mice was 66.7%, whereas only 28.6% of WT mice survived more than 10 days (fig. 1A). Next, we evaluated the bacterial burden in the mice. S1PR2 deficiency led to a significant decrease in bacterial counts within the peritoneal lavage fluid and peripheral blood at 72 h after CLP (fig. 1, B and C). Furthermore, a decreased pulmonary bacterial burden was also observed in the *S1pr2*$^{-/-}$ mice (fig. 1D), which was concomitant with alleviated lung injury in these mice (fig. 1E). This finding suggests that S1PR2 deficiency plays a protective role in polymicrobial sepsis and its related lung injury.

To further investigate whether S1PR2 participates in lung immune defense against bacterial infection, mice with different genotypes were challenged with *E. coli* intratracheal inoculation. There was no survival of WT and heterozygote mice beyond 32 h. Remarkably, 80% of *S1pr2*$^{-/-}$ mice were still alive 48 h after *E. coli* challenge (fig. 2A). Four hours after inoculation of *E. coli*, bacterial counts in the blood collected from the WT mice were higher than those from the *S1pr2*$^{-/-}$ mice by 10-folds. Bacterial counts continued to increase at 18 h after *E. coli* inoculation in the WT mice (fig. 2B). In contrast, bacterial counts in the blood samples taken from the *S1pr2*$^{-/-}$ mice remained very low from 4 to 18 h (fig. 2B). Consistent with the serologic findings, bacterial counts in BALF from the WT mice were significantly higher than those from the *S1pr2*$^{-/-}$ mice at both time points (fig. 2C). Along with reduced pulmonary bacterial load, diminished lung injury and permeability were observed in the *S1pr2*$^{-/-}$ group (fig. 2, D–F). Taken together, our results indicate that the absence of S1PR2 enhances lung immune defense to eliminate invading *E. coli* and prevents the pathogen from spreading, ultimately improving the outcome.

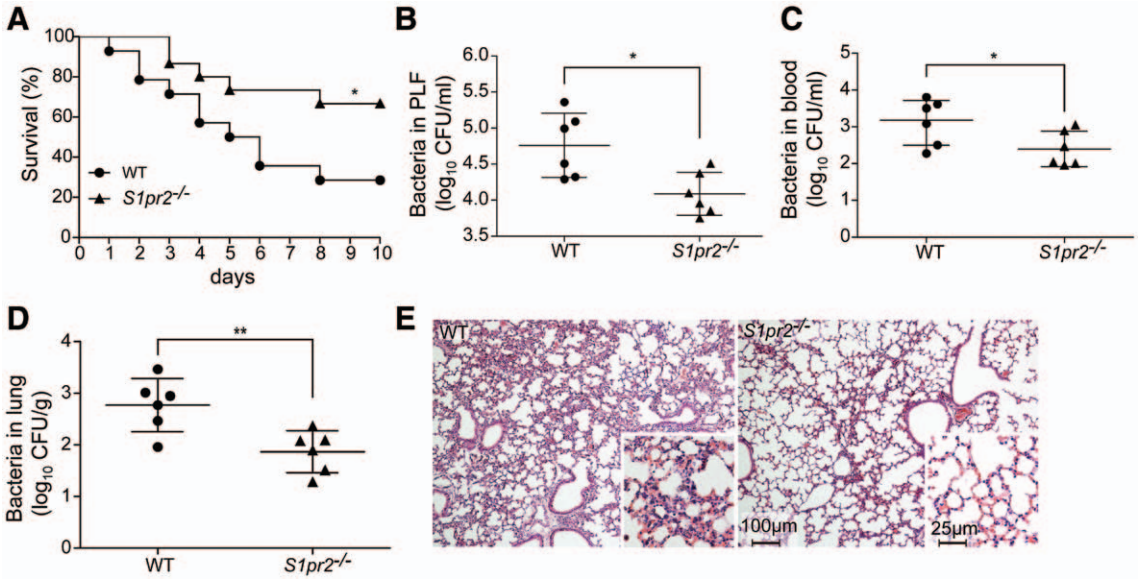

Fig. 1. S1PR2 signaling negatively regulates host response to cecal ligation and puncture (CLP) sepsis. (*A*) Survival curves of wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*-deficient (*S1pr2*$^{-/-}$) littermates after performance of CLP. Survival rates were monitored for 10 days. Data consist of two independent experiments (n = 15 for WT group, n = 14 for *S1pr2*$^{-/-}$ group) and were analyzed by the Mantel–Cox test. (*B–D*) Peritoneal lavage fluid (PLF, *B*), blood (*C*), and lung tissue (*D*) were collected at 72 h after performance of CLP. Bacterial burden was determined as colony-forming unit (CFU). *Horizontal bars* represent median values, and *dots* represent individual mice. n = 6 per group. Data are presented as mean ± SD. Student *t* test was used to compare difference between two independent groups. (*E*) Representative images of hematoxylin and eosin staining of mouse lung tissue sections taken at 72 h after performance of CLP. Magnification is ×100 and that of the inset is ×400. S1PR2 = sphingosine 1-phosphate receptor 2. **P* < 0.05 and ***P* < 0.01.

Deletion of S1PR2 in Macrophage Is Responsible for Its Protective Function against Bacterial Infection

To identify which cells were responsible for the different rates of bacterial clearance observed earlier (see S1PR2 Signaling Negatively Regulates Host Response to Infection; fig. 2, B and C), we created reciprocal BM transplantation between WT and *S1pr2*$^{-/-}$ mice (see Supplemental Digital Content 1, fig. 1A, http://links.lww.com/ALN/B157, which confirms the successful generation of BM chimeras). The chimeras were then subjected to intratracheal *E. coli* challenge. Replacement of WT mouse BM with that from *S1pr2*$^{-/-}$ mice mirrored the phenotype observed in the *S1pr2*$^{-/-}$ mice, but engraftment of WT BM into the *S1pr2*$^{-/-}$ recipients restored the WT phenotype (fig. 3A). The *S1pr2*$^{-/-}$→WT chimeric mice survived from bacterial infection better than the WT→WT chimeras (fig. 3B). These data indicate that deletion of S1PR2 in the BM-derived cells leads to increased bacterial clearance activity in the lung.

The primary BM-derived cells involved in acute pulmonary infections are AMs and neutrophils. We found that AMs were dominant in the BALF samples at 0 and 4 h after infection. The recruitment of neutrophils dramatically increased 18 h after infection. However, far fewer neutrophils were present in *S1pr2*$^{-/-}$ mice BALF samples, indicating that less pulmonary injury was present in the *S1pr2*$^{-/-}$ mouse cohort (see Supplemental Digital Content 1, fig. 1, B–D, http://links.lww.com/ALN/B157, which shows the immune cell numbers). Interestingly, the mRNA level of S1PR2 was approximately 25-fold higher in AMs than in neutrophils, regardless of *E. coli* stimulation (see Supplemental Digital Content 1, fig. 1E, http://links.lww.com/ALN/B157, which shows the expression levels of S1PR2 in immune cells). On the basis of the findings detailed earlier (Supplemental Digital Content 1, fig. 1, B–E, http://links.lww.com/ALN/B157), we hypothesized that the deletion of S1PR2 in AMs is responsible for the increased host defense observed in *S1pr2*$^{-/-}$ mice. To test this, we depleted AM populations through intratracheal administration of liposome-encapsulated clodronate before *E. coli* challenge (see Supplemental Digital Content 1, fig. 1F, http://links.lww.com/ALN/B157, which confirms the successful depletion of AMs *in vivo*). Clodronate treatment abrogated the difference of bacterial loads and survival between the *S1pr2*$^{-/-}$ and WT cohorts (fig. 3, C and D). Together, these findings demonstrate that deletion of S1PR2 in AMs improves pulmonary bacterial clearance and leads to increased survival rates after bacterial infection.

S1PR2 Signaling Suppresses Phagocytic Function

The functional enhancement present in *S1pr2*$^{-/-}$ AMs was further confirmed. *S1pr2*$^{-/-}$ AMs exhibited increased rates of bacterial engulfment (fig. 4, A and B). Opsonizing *E. coli* using rabbit IgG increased ingestion of the bacteria by approximatrely threefold in WT AMs but did not increase phagocytosis in *S1pr2*$^{-/-}$ AMs (fig. 4, A and B). Significantly increased phagocytosis of Texas Red–labeled microspheres

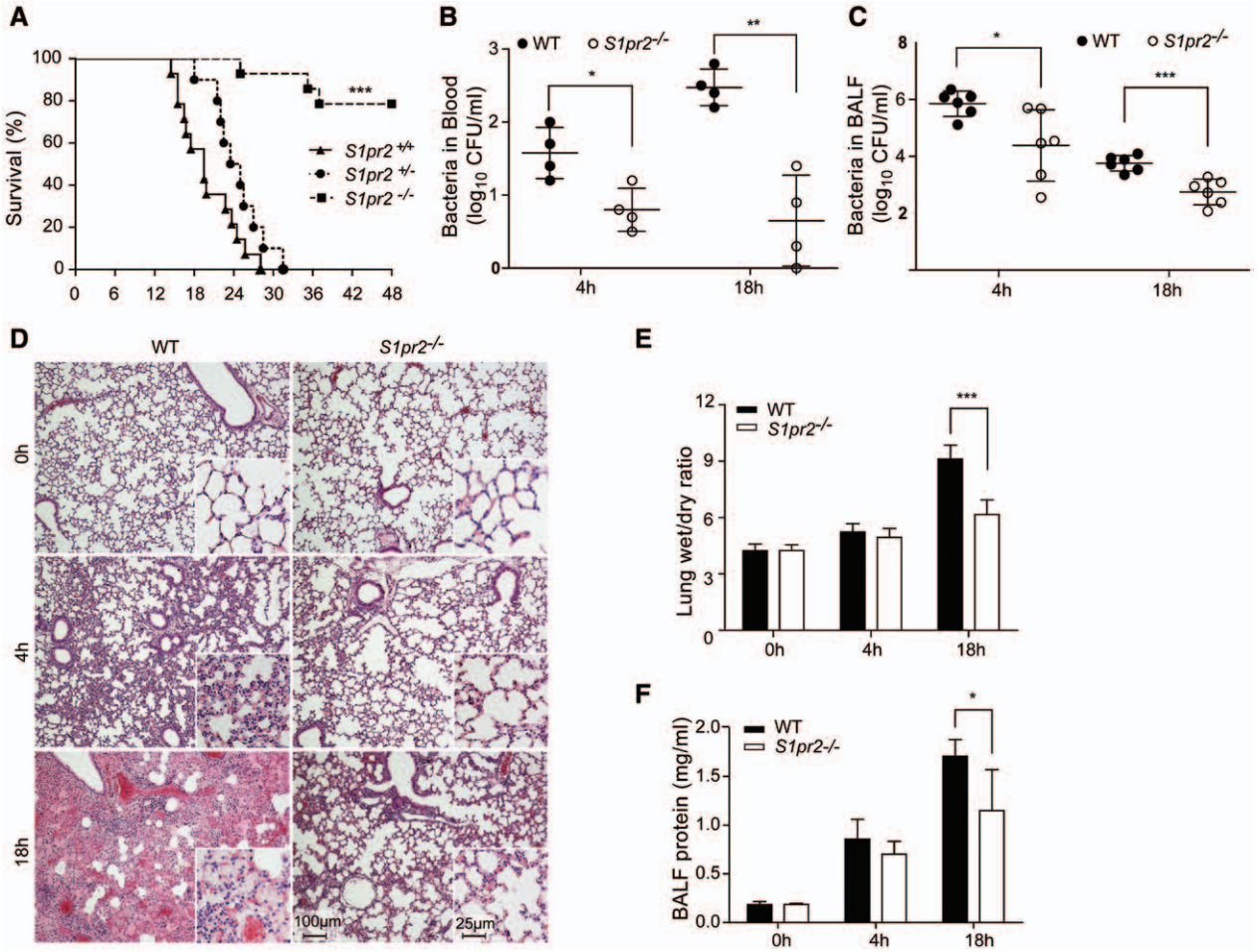

Fig. 2. S1PR2 signaling negatively regulates host response to bacterial infection caused by intratracheal inoculation with *Escherichia coli*. (*A*) Survival curves of *S1pr2*$^{+/+}$, *S1pr2*$^{+/-}$, and *S1pr2*$^{-/-}$ littermates after inoculation with *E. coli* (2×10^6 colony-forming units [CFU]). Data consist of two independent experiments (n = 14 for *S1pr2*$^{+/+}$ and *S1pr2*$^{-/-}$ group, n = 10 for *S1pr2*$^{+/-}$ group) and were analyzed by the Mantel–Cox test. (*B*, *C*) Number of *E. coli* bacteria recovered from blood (*B*) and lung tissues (*C*) from the infected *S1pr2*$^{-/-}$ and wild-type (WT, *S1pr2*$^{+/+}$) mice at indicated time points. n = 4 for blood detection, n = 6 for lung tissue detection. *Significant difference was compared with respective WT control mice. Data were analyzed by Student *t* test. (*D*) Representative images of hematoxylin and eosin staining of mouse lung tissue sections taken at indicated time points. Magnification is ×100 and that of the inset is ×400. (*E*, *F*) The pulmonary wet-to-dry weight ratio (*E*) and bronchoalveolar lavage fluid (BALF) total protein levels (*F*) were assayed before and after *E. coli* administration. *Significant difference was compared with respective WT control mice. Data are presented as mean ± SD and were analyzed by the Student *t* test. S1PR2 = sphingosine 1-phosphate receptor 2. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

was also found in *S1pr2*$^{-/-}$ AMs (see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/B157, which shows the Texas Red–labeled microspheres phagocytosis by AMs). These data indicate that S1PR2 deficiency enhances AMs phagocytic function in a substrate-independent manner.

We next used bactericidal assays to determine whether S1PR2 plays a role in killing *E. coli* internalized by AMs. Bactericidal events took place mainly within the first 4 h after ingestion and very few intracellular bacteria survived more than 12 h (fig. 4, C and D). These data indicate that the bactericidal rate of AMs is varied in the short term depending on the quantity of ingested bacteria, but presence of S1PR2 does not affect bactericidal capacity in the long run. In agreement with these conclusions, 50% of WT mice survived at 33 h postchallenge with opsonized *E. coli* (fig. 4E), which is 15 h longer than when they were challenged with unopsonized *E. coli* (fig. 2A). As expected, opsonization of *E. coli* did not affect the survival rate of *S1pr2*$^{-/-}$ mice (fig. 4E). These data demonstrate that S1PR2 deficiency enhances the phagocytic function of AMs, thereby improving host defense and survival rates after bacterial infection.

RhoA and Rac1 Differently Mediate S1PR2 Signaling–induced Negative Regulation of Macrophage Phagocytosis

Opsonization-induced bacterial phagocytosis of cultured WT AMs was within very close range to that measured for *S1pr2*$^{-/-}$ AMs. However, survival rate of WT mice challenged

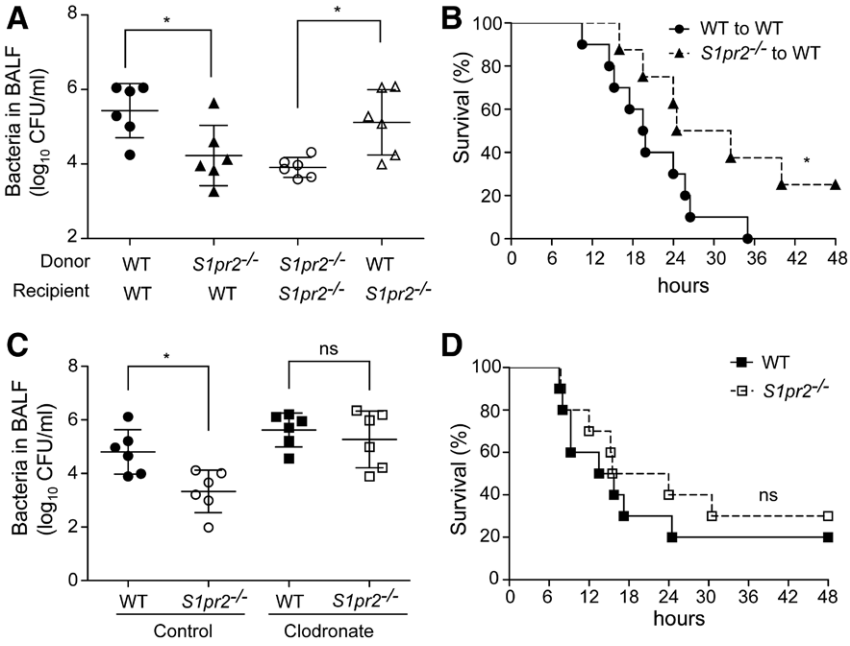

Fig. 3. Deletion of S1PR2 in macrophages improves host defense against intratracheal bacterial infection. (*A*) Numbers of bacteria recovered in bronchoalveolar lavage fluid (BALF) from various bone marrow (BM) chimeric mice at 4 h after *Escherichia coli* infection. Data were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons and represented by mean ± SD. (*B*) Survival of BM chimeras as indicated. n = 10 for wild-type (WT, *S1pr2*$^{+/+}$) →WT chimeras, and n = 8 for *S1pr2*$^{-/-}$→WT chimeras. Data were analyzed by the Mantel–Cox test. (*C*, *D*) WT and *S1pr2*$^{-/-}$ mice were intratracheally treated with control or clondronate liposomes before *E. coli* challenge. Bacteria in BALF were quantified at 4 h (*C*), and survival rates were evaluated (*D*). Data are presented as mean ± SD and were analyzed using one-way ANOVA with Bonferroni corrections (*C*) or Mantel–Cox test (*D*). CFU = colony forming units; S1PR2 = sphingosine 1-phosphate receptor 2. *$P < 0.05$.

with opsonized *E. coli* was not improved as much as expected compared with those measured for *S1pr2*$^{-/-}$ mice, suggesting other host factors may also contribute to the survival of bacterial infection. During acute lung infection, S1P concentration within collected BALFs increased from 0 to 18 h after bacterial infection (fig. 5A). In contrast, secretion of endogenous S1P from WT and *S1pr2*$^{-/-}$ BMDMs before and after *E. coli* infection alone was very limited (fig. 5A). We further found that incubation with exogenous S1P (from 100 nM to 5 μM) reduced phagocytosis by approximately 40% in WT BMDMs. However, phagocytosis by *S1pr2*$^{-/-}$ BMDMs was not inhibited (fig. 5B). This could explain why opsonization of *E. coli* did not increase survival rates *in vivo* as efficiently as it enhanced phagocytosis *in vitro* where very low levels of S1P are present in the growth media.

Pseudopodia are formed by microtubule and filament structures including lamellipodia and serve a locomotive function by sensing and capturing "prey" for phagocytosis.[25] Exogenous S1P treatment stimulated rapid redistribution of actin in WT BMDMs leading to the cellular adoption of a round-shaped morphology within 10 min of S1P application; however, no such morphological changes were observed in *S1pr2*$^{-/-}$ cells (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/B157, which shows the morphology of cells). When incubating cells with *E. coli* alone, autocrine signaling of S1PR2 through the macrophage-derived S1P ligand failed to induce cellular contraction; instead, it was found that S1PR2 signaling decreased the presence of prey-stimulated formation of lamellipodia in WT cells (fig. 5C, F-actin). However, the presence of prey did not induce lamellipodiual formation in WT cells pretreated with S1P.

Contraction of actin filaments requires RhoA.[26] Exogenous S1P treatment increased RhoA-GTP levels in WT cells but not in *S1pr2*$^{-/-}$ cells (fig. 5D). Incubation with *E. coli* alone did not significantly affect the levels of RhoA-GTP (fig. 5D), suggesting that *E. coli*–stimulated formation of lamellipodia is independent of RhoA. Therefore, these findings suggest that activation of the S1P-S1PR2-RhoA pathway impairs the phagocytic function of WT macrophages and that a RhoA-independent mechanism enhances phagocytosis in *S1pr2*$^{-/-}$ macrophages.

Translocation of Rac1 from the cytosol to the site of particle attachment is a prerequisite for rearrangement of the actin cytoskeleton and formation of lamellipodial protrusions or pseudopodia. Immunofluorescent staining revealed that Rac1 was primarily localized within the perinuclear area of the cytoplasm in both WT and *S1pr2*$^{-/-}$ BMDMs in the absence of bacterial stimulation (fig. 6A). However, when *S1pr2*$^{-/-}$ cells were challenged with *E. coli,* Rac1 almost completely relocated from the perinuclear area to the cell periphery. In contrast, Rac1 localization

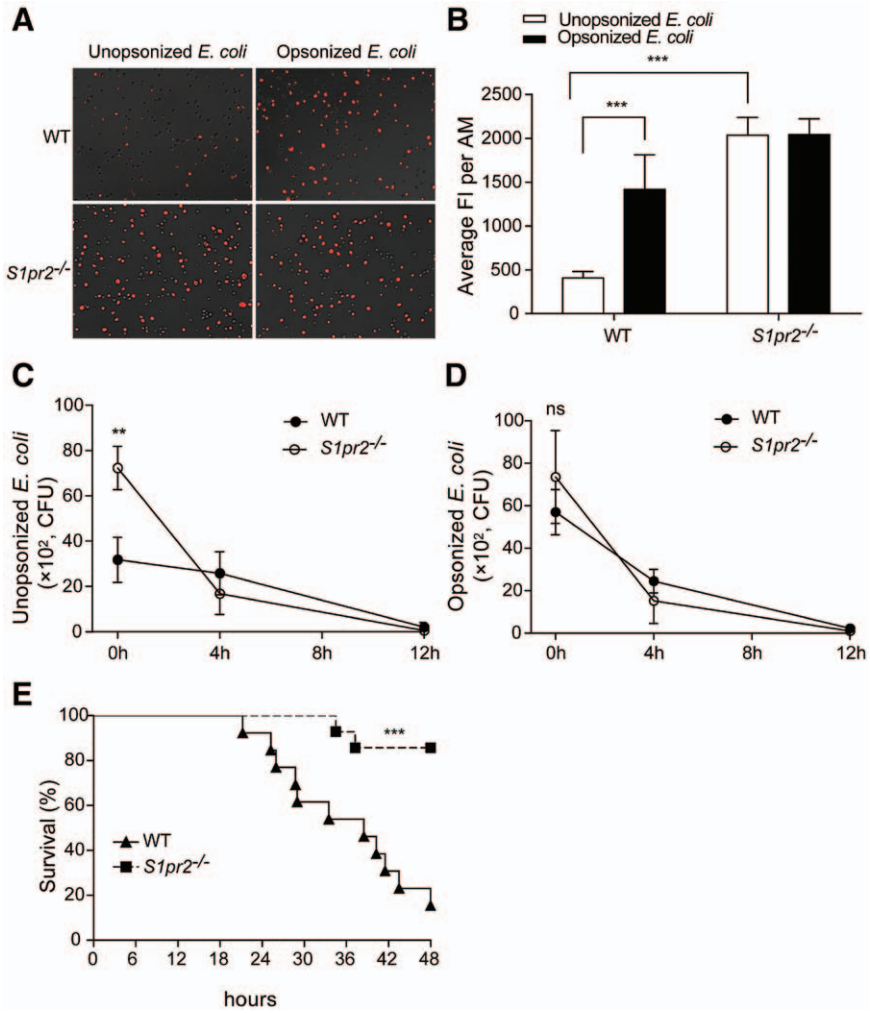

Fig. 4. S1PR2-deficient alveolar macrophages (AMs) show enhanced phagocytic function. (*A*) Representative microscopic images of red fluorescent–labeled *Escherichia coli* engulfed by isolated wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*$^{-/-}$ AMs. The bacteria were also pretreated with or without opsonin. Magnification is ×200. (*B*) Quantification of phagocytosis according to fluorescent microscopic images in *A*. In each microscopic field, total AMs and fluorescent intensity (FI) inside these cells were quantified. The average FI per AM was calculated as total FI per field/total number of AMs per field. Three fields per sample were analyzed. n = 4 for WT AMs groups, and n = 6 for *S1pr2*$^{-/-}$ AMs groups. Data are presented as mean ± SD and were analyzed by two-way ANOVA with Bonferroni corrections. (*C*, *D*) Killing curves of internalized *E. coli* in WT and *S1pr2*$^{-/-}$ AMs. n = 4 per group. Data are presented as mean ± SD and were analyzed by Mann–Whitney test. (*E*) Survival curve in *S1pr2*$^{-/-}$ and WT mice with opsonized *E. coli* (2×10^6 colony forming units [CFU]) induced lung injury. Data consist of two independent experiments (n = 13 or WT group, n = 14 for *S1pr2*$^{-/-}$ group) and were analyzed by Mantel-Cox test. S1PR2 = sphingosine 1-phosphate receptor 2. $^{**}P < 0.01$ and $^{***}P < 0.001$.

did not substantially change in WT BMDMs after cellular exposure to *E. coli* (fig. 6A). As expected, incubation with *E. coli* stimulated a dramatic increase of Rac1-GTP levels in *S1pr2*$^{-/-}$ BMDMs, but only a mild increase of Rac1 activity in WT cells (fig. 6B). Consistent with the findings in cultured BMDMs, Rac1-GTP levels were greatly increased in both WT and *S1pr2*$^{-/-}$ AMs isolated 4 h after intratracheal inoculation of *E. coli*, with far more robust Rac1 activation in *S1pr2*$^{-/-}$ AMs versus WT AMs (see Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/B157, which shows the Rac1-GTP levels in BALF cells isolated from normal mice or 4 h after mice challenged with *E. coli*).

Lamellipodial formation results from Rac1-activated reorganization of actin structures.[27] The leading edges of lamellipodia contain detergent-insoluble polymerized actin filaments (F-actin).[28] Consistent with Rac1-GTP levels in these cells, bacterial incubation increased the presence of F-actin slightly in WT BMDMs and greatly in the *S1pr2*$^{-/-}$ BMDMs (fig. 6C). Together, these results demonstrate that the absence of S1PR2 increased both Rac1 activation and the formation of phagocytic pseudopodia in macrophages after bacterial stimulation.

IQGAP1 is a widely expressed Rac1-binding protein.[29] Binding of IQGAP1 to Rac1 inhibits intrinsic Rac1 GTPase activity and stabilizes its GTP-bound form. Similar to Rac1,

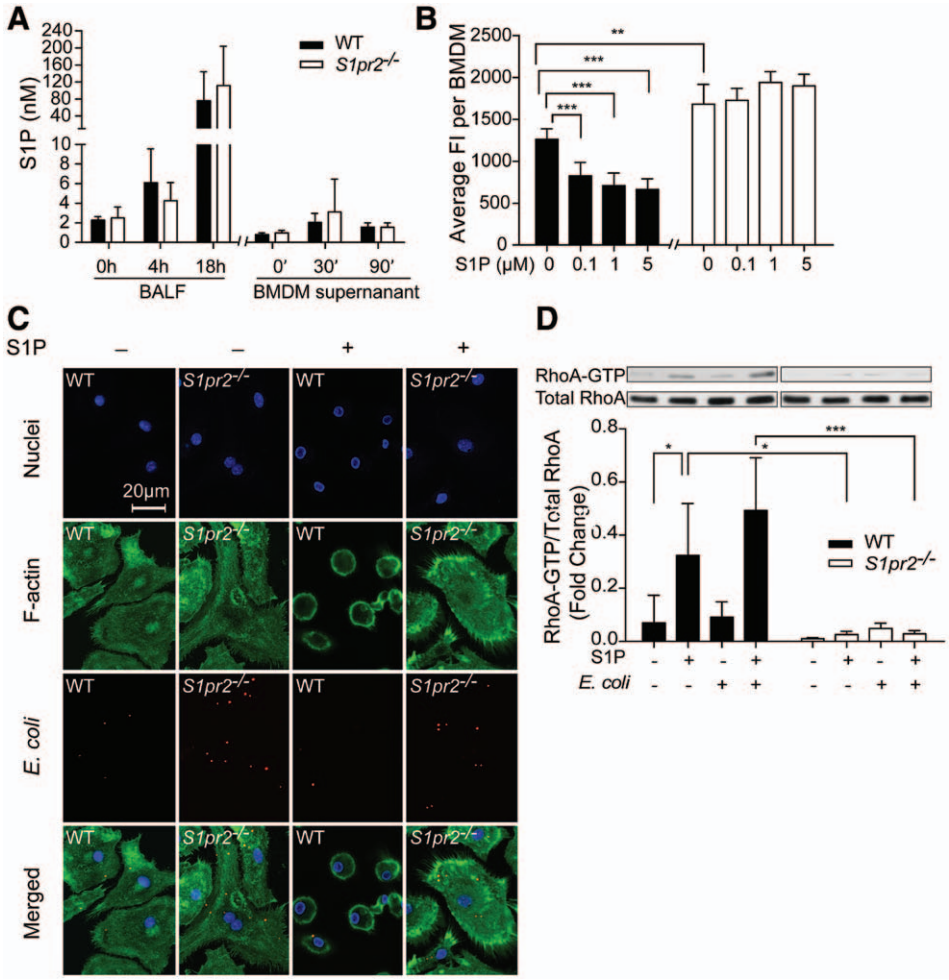

Fig. 5. Engagement of S1PR2 with exogenous S1P leads to RhoA activation and inhibition of phagocytosis. (*A*) S1P levels in bronchoalveolar lavage fluid (BALF) from wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*$^{-/-}$ mice, as well as in the supernatants of cultured bone marrow–derived macrophages (BMDMs), at different time points (as indicated) after challenge with live *Escherichia coli*. Data are presented as the mean ± SD and were analyzed by Student *t* test. n = 6 per group. (*B*) Phagocytosis of fluorescent *E. coli* by BMDMs challenged with various doses of S1P. Data are presented as mean ± SD from three independent experiments and analyzed by two-way ANOVA with Bonferroni corrections. FI = fluorescent intensity. (*C*) Activation of S1PR2 with S1P prevents phagocytosis of fluorescent *E. coli* (*red*) in BMDMs. WT and *S1pr2*$^{-/-}$ BMDMs were starved and pretreated with or without S1P (100 nM) for 30 min and then incubated with fluorescent *E. coli* for 30 min. F-actin (*green*) and nuclei (*blue*) were fluorescently stained. (*D*) S1P activates small RhoA GTPase in BMDMs through S1PR2. RhoA-GTP level was detected in *E. coli*-stimulated WT and *S1pr2*$^{-/-}$ BMDMs using glutathione-S-transferase pull-down assay. Total RhoA protein was used a loading control. Data are presented as mean ± SD from three independent experiments and were analyzed by two-way ANOVA with Bonferroni corrections. S1PR2 = sphingosine 1-phosphate receptor 2. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

IQGAP1 was found to be located in the cytosol of both WT and *S1pr2*$^{-/-}$ BMDMs. *E. coli* incubation efficiently induced membrane localization of IQGAP1 in *S1pr2*$^{-/-}$ BMDMs but not in WT cells (fig. 7A). Furthermore, there was a greater degree of colocalization between IQGAP1 and Rac1 in *S1pr2*$^{-/-}$ cells after bacterial stimulation, consistent with the coimmunoprecipitation result that bacterial stimulation led to a greater quantity of Rac1 pulled down with IQGAP1 in *S1pr2*$^{-/-}$ cells (fig. 7B). Interestingly, a greater degree of IQGAP1 tyrosine phosphorylation was also observed in *S1pr2*$^{-/-}$ cells (fig. 7B). To determine the degree to which IQGAP1 is required for enhanced phagocytosis in *S1pr2*$^{-/-}$ macrophages, IQGAP1 was silenced with siRNA (fig. 7C). Knockdown of IQGAP1 resulted in negligible levels of Rac1-GTP in both WT and *S1pr2*$^{-/-}$ BMDMs after *E. coli* stimulation and reduced bacterial phagocytosis to a similar degree in both cell types (fig. 7, D and E). Thus, these findings identify IQGAP1 as a downstream effector of S1PR2 that is required for the enhancement of phagocytosis in S1PR2-deficient macrophages.

Pharmacologic Inhibition of S1PR2 Demonstrates a Protective Effect on Host Defense against Bacterial Infection

JTE-013 is a well-characterized S1PR2 antagonist.[10] In the current study, WT BMDMs pretreated with JTE-013 not only retained their morphology after stimulation with

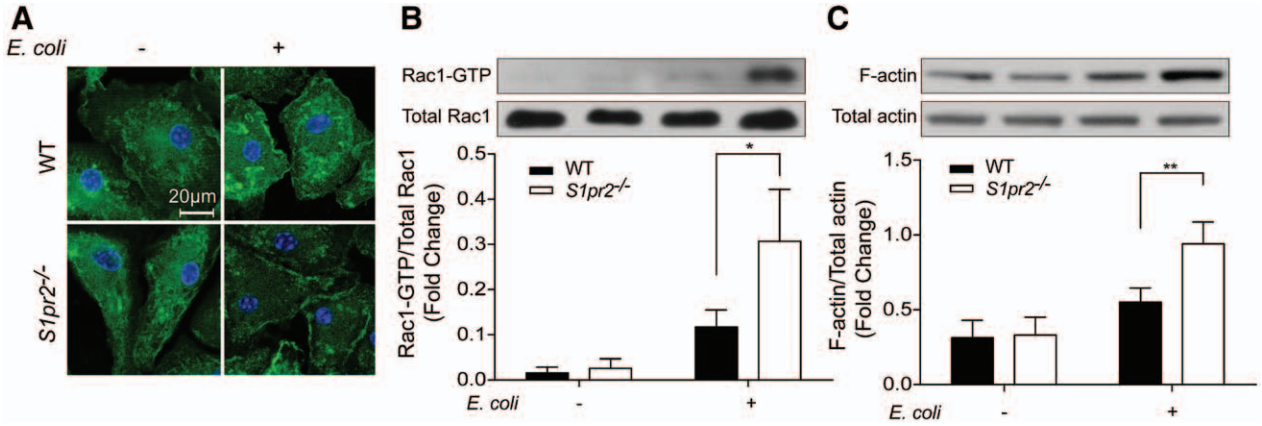

Fig. 6. Engagement of S1PR2 with *Escherichia coli* alone prevents Rac1 activation and F-actin polymerization. (*A*) Translocation of Rac1 in bone marrow–derived macrophages (BMDMs) on *E. coli* stimulation. Wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*$^{-/-}$ BMDMs were starved and incubated with live *E. coli* for 30 min. Rac1 (*green*) and nuclei (*blue*) were fluorescently stained. (*B*) Deletion of S1PR2 increases *E. coli*–induced Rac1-GTP levels in BMDMs. GTP-bound Rac1 levels were determined with glutathione-S-transferase pull-down assays and normalized to the total Rac1. Data are presented as mean ± SD from three independent experiments and were analyzed by Student *t* test. *Significant difference was compared with respective WT control cells. (*C*) An increased actin polymerization in *S1pr2*$^{-/-}$ BMDMs. Data are presented as mean ± SD from three independent experiments and were analyzed by Student *t* test. **Significant difference was compared with respective WT control cells. S1PR2 = sphingosine 1-phosphate receptor 2. *P < 0.05 and **P < 0.01.

100 nM S1P (see Supplemental Digital Content 1, fig. 5A, http://links.lww.com/ALN/B157, which shows the function of JTE-013 in protecting against S1P-induced macrophage contraction) but also showed significantly increased phagocytosis of *E. coli*, regardless of the presence or absence of S1P (fig. 8A). However, treatment with any combination of S1P and JTE-013 did not affect the phagocytic function in *S1pr2*$^{-/-}$ BMDMs (fig. 8A), indicating that any inhibitory effects caused by treatment with JTE-013 are S1PR2 specific. After JTE-013 pretreatment, 50% of the mice survived for at least 36 h after bacterial infection, but only 10% of the mice treated with vehicle survived for this length of time (fig. 8B). Moreover, the bacterial burdens measured within the blood and BALF of these mice were also significantly reduced after JTE-013 treatment (fig. 8, C and D). In addition, a single dose of JTE-013 (4 mg/kg) or vehicle was also intratracheally administered immediately after administration of *E. coli* intratracheally. Notably, the survival time of WT mice received JTE-013 was prolonged by 1.5 times compared with that of the vehicle-treated mice, although all the mice died in both of the groups (see Supplemental Digital Content 1, fig. 5B, http://links.lww.com/ALN/B157, which shows the function of JTE-013 in protecting against *E. coli* infection). These findings are consistent with our studies in *S1pr2*$^{-/-}$ mice, suggesting that JTE-013 may be a promising therapeutic for enhancing host defense against bacterial infection.

S1PR2 Expression in Monocytes Was Increased in Septic Patients

We also determined the expression level of S1PR2 in PBMCs obtained from 25 septic patients and 9 nonseptic controls. As shown in figure 9A, S1PR2 mRNA levels were significantly higher in septic patients compared with those in nonseptic controls. Furthermore, an increased S1PR2 expression was positively correlated with the severity of sepsis, evaluated by Acute Physiologic and Chronic Health Evaluation II scores (r = 0.845, fig. 9B). The relationship between S1PR2 expression level and phagocytic function of PBMCs was also assayed in one nonseptic control and newly recruited three septic patients (see Supplemental Digital Content 1, table 3, http://links.lww.com/ALN/B157, which lists the four patients characteristics studied in this study). Significantly decreased phagocytosis of red fluorescent–labeled *E. coli* was found in PBMCs collected from septic patients (fig. 9, C). Moreover, PBMCs with higher expression levels of S1PR2 showed lower phagocytic ability, which further supports the positive relationship between S1PR2 expression and severity of sepsis (fig. 9, B and D).

Discussion

In the current study, we discovered a previously unrecognized role of S1PR2 in sepsis. Knockout of S1PR2 alleviated lung injury and improved survival rates in mouse models of polymicrobial sepsis and intratracheal inoculation with *E. coli*. This protective effect was attributed to the absence of S1PR2 on macrophages, which altered their phagocytic function and increased bacterial clearance. Importantly, we elaborated an extracellular stimulator-dependent mechanism for S1PR2 in activating Rho family proteins and regulating actin rearrangement during phagocytosis.

The role of S1PR2 in host antibacterial defense is unclear. McQuiston's *in vitro* study suggested that extracellular S1P increased opsonin-mediated phagocytosis of *Cryptococcus*

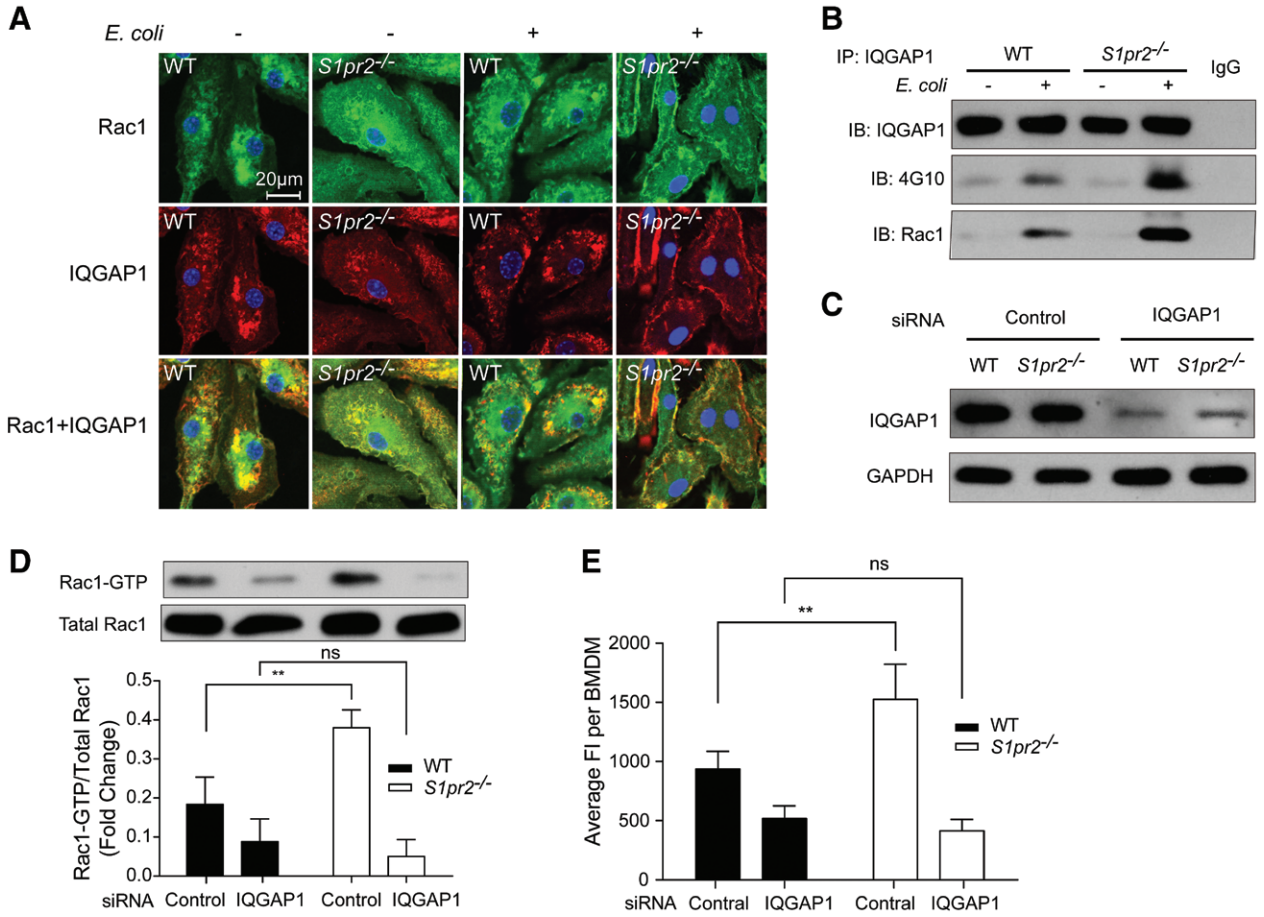

Fig. 7. S1PR2 inhibits Rac1 activation in macrophages through IQGAP1. (*A*) Localization of IQGAP1 and Rac1 in bone marrow–derived macrophages (BMDMs) on *Escherichia coli* challenge. IQGAP1 (*red*) and Rac1 (*green*) were detected with immunofluorescent staining, and nuclei were stained with DAPI (*blue*). (*B*) *E. coli* induced phosphorylation of IQGAP1 and association between IQGAP1 and Rac1. IQGAP1 was immunoprecipitated, and Rac1 and IQGAP1 levels were examined using Western blot. (*C*) Knockdown of IQGAP1 with siRNA in wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*$^{-/-}$ BMDMs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (*D*) Knockdown of IQGAP1 diminishes *E. coli*–induced Rac1-GTP levels in both *S1pr2*$^{-/-}$ and WT BMDMs. Data are presented as mean ± SD from three independent experiments and were analyzed by two-way ANOVA with Bonferroni corrections. (*E*) Knockdown of IQGAP1 expression abolishes enhanced phagocytosis caused by S1PR2 deficiency. Data are presented as mean ± SD from three independent experiments and were analyzed by two-way ANOVA with Bonferroni corrections. ns = not significant; S1PR2 = sphingosine 1-phosphate receptor 2. $^{**}P < 0.01$.

neoformans through S1PR2 by up-regulating the expression of the phagocytic Fcγ receptors (FcγRI, FcγRII, and FcγRIII). In our *in vitro* study, *S1pr2*$^{-/-}$ AMs exhibited increased rates of *E. coli* engulfment in an opsonin-independent manner. Mechanistically, absence of S1PR2 in macrophages inhibits RhoA-dependent cell contraction and promotes IQGAP1-Rac1–dependent lamellipodial protrusion, both of which promoted *E. coli* phagocytosis. This different responses observed by the two studies are probably because of the different functions of S1PR2 in bacterial and fungal defense. In McQuiston's *in vivo* study, they found *S1pr2*$^{+/+}$ mice survived significantly shorter than *S1pr2*$^{-/-}$ mice and demonstrated that the deficiency in S1PR2 provides protection against cryptococcosis. They speculated that the conflicting results might be due to decreased ability of *C. neoformans* to pass through the endothelial lining of the lungs to enter into bloodstream and then cause disseminated disease in *S1pr2*-deficient mice. However, the assessments of lung bacterial burden and lung edema were ignored in their study. Therefore, it is difficult to attribute the observed protective effects in this model only to the effects of S1PR2 signaling on vascular permeability. In our *in vivo* study, we found that both genetic deletion and pharmaceutical inhibition of S1PR2 significantly limited bacterial burden, alleviated lung damage, and improved survival in experimental sepsis. The observation that the survival of *S1pr2*$^{-/-}$→WT chimeric mice was better than the WT→WT chimeras but still worse than *S1pr2*$^{-/-}$ mice suggests that the effect of S1PR2 signaling on endothelial cells may also be relevant. However, the pulmonary edema (wet-to-dry weight ratio and BALF protein levels) did not show differences at the early time point of this model, *i.e.*, at 4 h, when clear differences were already noted

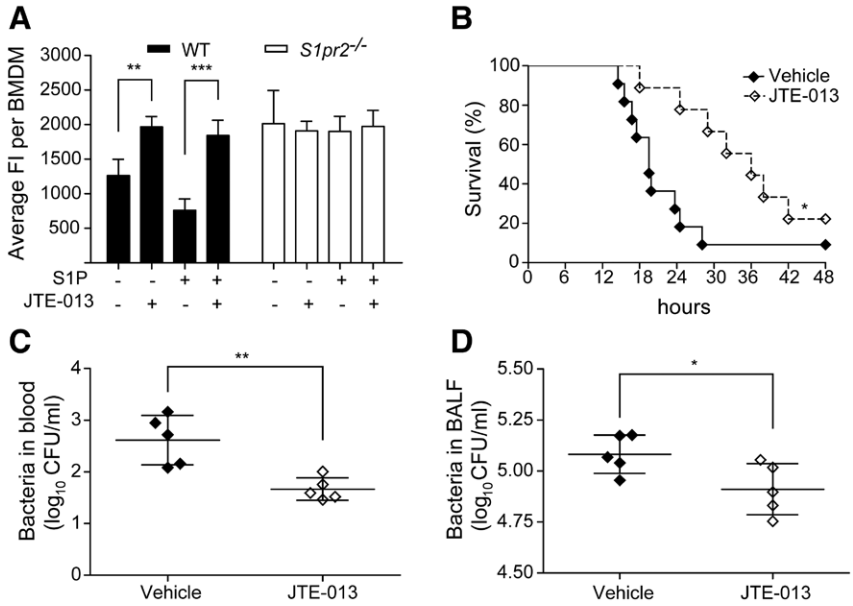

Fig. 8. Pharmacologic inhibition of S1PR2 increases bacterial phagocytosis of macrophages and improved survival of wild-type (WT, *S1pr2*$^{+/+}$) mice challenged with a lethal dose of *Escherichia coli*. (*A*) Inhibition of S1PR2 enhances phagocytosis of WT bone marrow–derived macrophages (BMDMs). WT and *S1pr2*$^{-/-}$ BMDMs were starved and pretreated with 5 μM JTE-013 (an S1PR2 antagonist) or vehicle for 30 min before stimulation with 100 nM S1P for another 30 min. Phagocytosis was assessed as described in fig. 3B. Data were analyzed by two-way ANOVA with Bonferroni corrections. (*B*) JTE-013 treatment protects WT mice from lethal infection caused by *E. coli* infection. WT mice were intratracheally instilled with JTE-013 (4 mg/kg) or vehicle 30 min before *E. coli* infection. The survival rates were assessed. n = 11 for the vehicle control group, and n = 9 for JTE-013 group from two independent experiments. Data were analyzed by Mantel–Cox test. (*C*, *D*) Number of *E. coli* bacteria recovered from blood (*C*) and lung tissue (*D*) from the JTE-013 treatment group or control group at 4 h after bacterial infection. *n* = 5 at each time point. Data are presented as mean ± SD and were analyzed by Student *t* test. BALF = bronchoalveolar lavage fluid. S1PR2 = sphingosine 1-phosphate receptor 2. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

in bacterial burden and histology. Depletion of AMs further demonstrated the pivotal role of AMs S1PR2 expression in eliminating invading bacteria, which is consistent with our *in vitro* study. Taken together, both *in vitro* and *in vivo* findings support the hypothesis that S1PR2-mediated effects on pulmonary immune defense, rather than S1PR2-mediated direct modulation of endothelial permeability, plays a major role during *E. coli* infection.

S1PR2 signaling functions in response to receptor activation by S1P. S1P is synthesized in most cells by the phosphorylation of sphingosine *via* sphingosine kinase 1 (Sphk1) and 2 (Sphk2). Although the activation of Sphk1/2 was not measured in this study, it is worth considering that the increased levels of S1P that were observed following *E. coli* challenge may result from an increase in Sphk1/2 activity within contaminated pulmonary cells at early stages of infection (less than 4 h). As infection progresses, S1P could leak out of blood vessels and into alveolar space due to increased permeability of alveolar capillary barriers resulting from inflammation. Previous studies have found that the degree to which S1P mediates biological responses produced by S1PRs is largely dependent on S1P concentration.[30,31] As *E. coli* infection of BMDMs induced only low levels of S1P production *in vitro*, during our mechanistic studies, we used this condition to represent early stage bacterial infection and then further applied exogenous S1P (100 nM) to mimic an *in vivo* environment representative of later stages of the disease (18 h).

Small GTPases of the Rho family—primarily Rho, Rac, and Cdc42—have a central role in actin reorganization and cell shaping during particle internalization. These Rho GTPases produce distinct effects on the actin cytoskeleton: RhoA-GTP induces the formation of contractile actomyosin filaments, whereas Rac1-GTP and Cdc42-GTP direct peripheral actin assemblies into the formation of lamellipodia and filopodia.[32] By using confocal microscopy, we observed that WT cells adopted a contracted round morphology on stimulation with 100 nM S1P and produced fewer membrane protrusions in response to *E. coli* challenge relative to *S1pr2*$^{-/-}$ BMDMs. In agreement with the morphological changes, an increased amount of RhoA-GTP was observed in WT cells on stimulation with exogenous S1P and a decreased level of Rac1-GTP was observed in response to *E. coli* challenge. These findings are consistent with those demonstrating that S1P-S1PR2 signaling activates RhoA-GTP and inhibits Rac1-GTP through different intracellular G-proteins.[32,33] Furthermore, we also found that activated IQGAP1 participated in S1PR2-mediated actin translocation and phagocytosis *via* binding to Rac1 in macrophages. Taken together, our data demonstrate a ligand dose-dependent S1PR2 signaling mechanism for regulating actin rearrangement and phagocytosis in macrophages that can

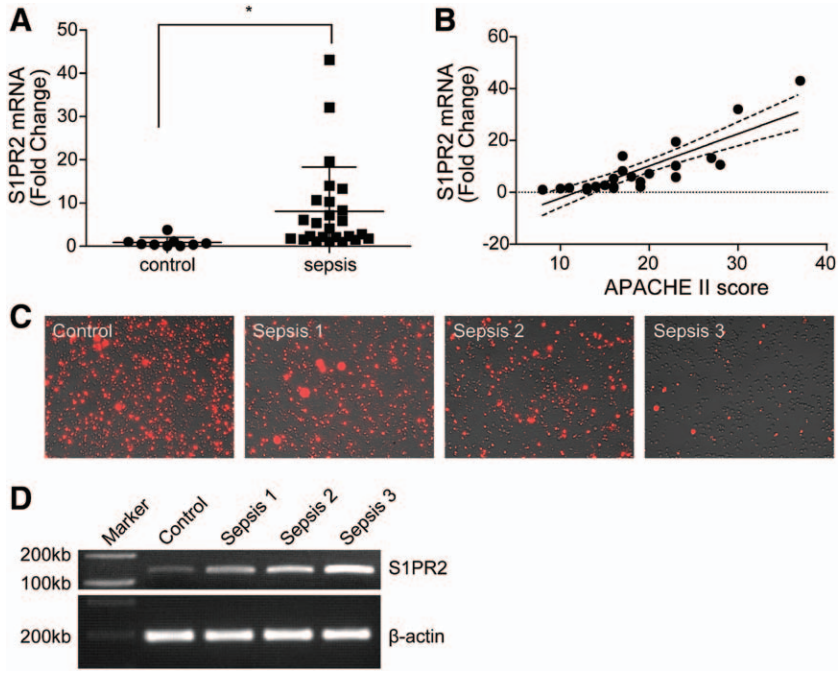

Fig. 9. Increased S1PR2 levels in peripheral blood mononuclear cells were positively correlated with the severity of sepsis. (*A*) S1PR2 mRNA levels in peripheral blood mononuclear cells isolated from human subjects. Expression level of S1PR2 was evaluated using quantitative reverse transcription–polymerase chain reaction. β-Actin was used as an internal control. n = 9 for controls, and n = 25 for septic patients. *Dots* represent individual subjects, and data are presented as mean ± SD. Data were analyzed by Student *t* test. (*B*) Correlations of S1PR2 expression with Acute Physiologic and Chronic Health Evaluation II scores in the septic patients ($r = 0.845$, $P < 0.001$). Data were analyzed by Spearman correlation test. (*C*) Representative microscopic images of red fluorescent–labeled *Escherichia coli* engulfed by monocytes collected from peripheral blood of septic patients or controls. n = 1 for nonseptic patient group, and n = 3 for septic patients groups. Magnification is ×200. (*D*) Peripheral blood mononuclear cells of each patient (*C*) were isolated by density gradient centrifugation. Gene transcripts of S1PR2 were detected by semiquantitative reverse transcription–polymerase chain reaction and analyzed on an agarose gel by electrophoresis. β-Actin was served as an internal control. S1PR2 = sphingosine 1-phosphate receptor 2. *$P < 0.05$.

account for the severity of pulmonary infection observed in WT mice during different stages of *E. coli* challenge (fig. 10).

Given the clinical success of sphingosine analog FTY720 in treating multiple sclerosis, interventions targeting S1PR2 for the treatment of bacterial infection were expected. JTE-013 has been considered a S1PR2 antagonist and is widely used to characterize S1PR2-dependent effects. Although many studies have shown the usefulness of JTE-013 in targeting S1PR2, this compound also antagonizes S1PR4 and has off-target effects.[10,34,35] Therefore, the results obtained with JTE-013 should be validated by genetic knockout studies. Indeed, inhibition of endogenous S1PR2 with JTE-013 could reverse the suppressed phagocytic function of macrophages and rescue mice from fatalities caused by *E. coli* infection, consistent with the results from the *S1pr2*$^{-/-}$ mice. The survival of JTE-013 inhibited mice was certainly better than that of vehicle controls, but still much worse than that of *S1pr2*$^{-/-}$ mice. This is probably due to the solubility and distribution of JTE-013 in the lung. Combined with the reciprocal correlation of S1PR2 expression levels with the severity of septic patients, it may be promising to develop more selective and potent S1PR2 inhibitors and to translate these antagonists into clinical pharmaceutical therapy for sepsis.

The current study has uncovered a number of implications that should be considered in future research examining the role of S1PR2 in human disease states. First, a number of Gram-negative pathogens possess virulence factors that can escape from clearance by phagocytes.[36] It would be interesting to determine whether modulation of the S1PR2 signaling has the potential to augment phagocytosis of these pathogens. Furthermore, this work primarily aimed at assessing the role of S1PR2 signaling in different stages of bacterial infection. An increased risk of bacterial infection is usually a complicating factor of chronic diseases, such as type 2 diabetes and end-stage renal failure.[37,38] The reasons behind this enhanced susceptibility to bacterial infection are not well understood, suggesting it would be prudent to explore the role of S1PR2 under these conditions.

In summary, the current study finds that interfering with S1PR2 enhances bacterial clearance and improves survival in the mouse model of sepsis. These beneficial effects are attributed to increased phagocytic activity of S1PR2-deficient macrophages. Interventions targeting S1PR2 signaling may offer a promising therapeutic approach for the prevention and/or treatment of sepsis.

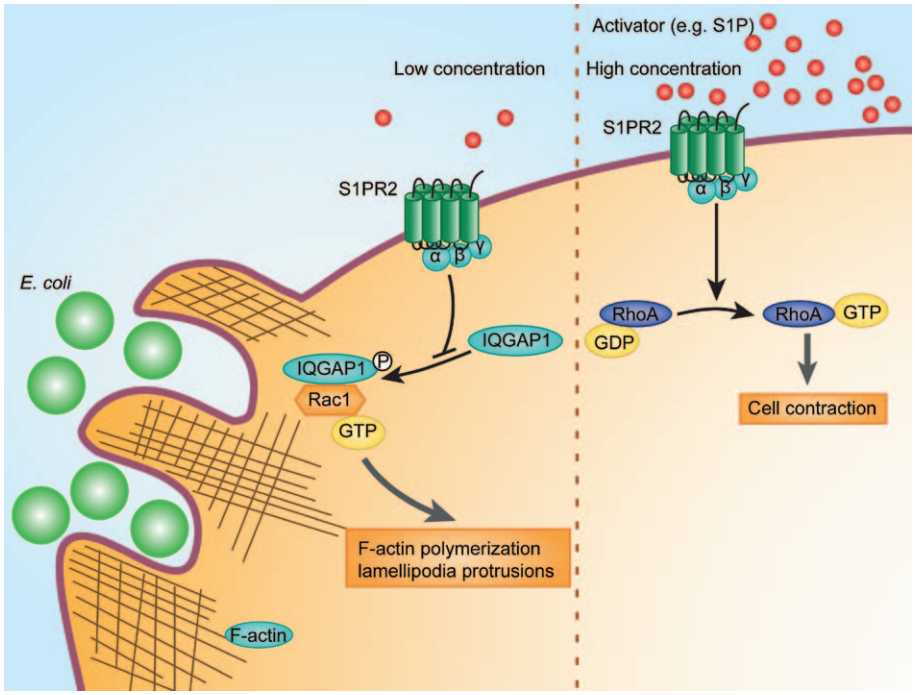

Fig. 10. Proposed mechanism involved in impaired bacterial clearance mediated by S1PR2 signaling in macrophage. S1PR2 = sphingosine 1-phosphate receptor 2.

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Competing Interests

The authors declare no competing interests.

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