Supplemental Digital Content 1

Materials and Methods

Bronchoalveolar lavage fluid (BALF)

Lavage procedure of the mouse lung was performed as previously described.[1] Briefly, mice were anesthetized with ketamine/xylazine cocktail and euthanized by exsanguination. The trachea was surgically exposed and intubated with a syringe catheter. The lungs underwent lavage with 1 ml pre-warmed PBS for 5 times. Totally 5 ml BALF was obtained from each mouse and cells in BALF were pelleted by centrifugation (500 g for 10 minutes at 4°C). Cells were then resuspended in cold PBS and counted and immediately applied to subsequent analyses. The supernatants were stored at -80°C for later measurements.

Lung morphology and lung injury scoring

Paraffin-embedded lung sections were stained with hematoxilin and eosin and scored from 1 (normal) to 5 (severe) as described previously.[2]

Assessment pulmonary oedema and pulmonary vascular permeability

To assess pulmonary oedemas, the lung wet/dry weight ratios were calculated. In brief, after the blood was drained from the excised lungs, measurements of the lung wet weight were made. Lungs were then heated to 65°C in a gravity convection oven for 72 hrs and weighed to determine baseline lung dry mass levels. Pulmonary vascular permeability was assessed by measuring the BALF protein accumulation. Protein concentrations in BALF were measured using an BCA Protein Assay Reagent (Fisher Thermo Scientific, Rockford, IL) according to the manufacturer's recommendations.

Optical density readings of samples were converted to milligrams/milliliters, using values obtained from a standard curve generated with serial dilutions of BSA (0.1-1.5 mg/ml). [3]

Flow cytometry analysis

The fluorescence-conjugated antibodies against F4/80 (Alexa Fluor 488), Gr1 (APC), CD11b (FITC), CD45.1 (PE) and CD45.2 (PerCP-Cy5.5), all from eBioscience (eBioscience, San Diego, CA), were used for FACS analysis. Cells were incubated with Fc block prior to staining with these antibodies for 20 minutes at 4°C in the dark. In the case of blood samples, red blood cells were lysed with FACS lysing solution. Cells were then washed and resuspended in 300 μl of FACS buffer (PBS containing 2% FBS) before applied to the BD LSR2 flow cytometer (BD Biosciences, San Jose, CA). The raw data were collected and analyzed with the FlowJo software.

S1P measurement

S1P in supernatants from BALF or cultured bone marrow derived macrophages (BMDMs) were quantified with HPLC-ESI-MS/MS. Calibration curve for S1P (C-18) measurement was prepared using seven different concentrations ranging from 0-200 pmol/ml. FTY720 was used as an internal reference for each sample. The BALF (200 μl) or the supernatant (400 μl) was diluted to 1 ml volume, including 50 μl of 1 nM FTY720. The mixture was extracted by adding 1 ml of isopropanol/ethyl acetate (v:v=15:85) and centrifuged at 4000 rpm for 10 minutes. The upper organic phase was transferred to a new tube. The lower aqueous phase was acidified with 50 μl of formic acid and re-extracted. The organic phases was combined and dried under a

steady nitrogen stream at 40°C. The dried lipids were then reconstituted in 100 μl of mobile phase (1mM ammonium formate in methanol containing 0.2% formic acid), vortexed and centrifuged at 4000 rpm for 5 minutes. The supernatant was transferred to an autosampler HPLC vial with 200 μl insert standard, 20 μl of which was then injected into the HPLC system for analysis (Agilent Technologies, Santa Clara, CA). Quantification of the S1P peak was calculated from the standard calibration curve.

Cell culture

Cells were maintained in a humidified incubator (Fisher Thermo Scientific, Rockford, IL) with 5% CO_2 at 37°C. BMDMs were prepared according to a published protocol with modifications.[4] Briefly, bone marrow cells were isolated from mouse hind legs in single cell suspension and were plated on P100 petri dishes with growth media, DMEM supplemented with 10% fetal calf serum and 20 ng/ml mouse granulocyte macrophage - colony stimulating factor (PeproTech, Rocky Hill, NJ). After 2 days, adherent cells were washed twice with warm PBS and exchanged for fresh growth media. These cells were cultured for another 7 days with replacement of fresh growth media in every 3 days. Maturation of BMDMs was confirmed with double staining of CD11b and F4/80. Then BMDMs were harvest for further plating or analysis.

To isolate AMs, cell pellets from BALF were resuspended in warm RPMI 1640 and plated on P60 petri dishes for 1h at 37°C. Then adherent cells were harvested with a rubber scraper. Cell viability (>90%) was determined by trypan blue exclusion. The purity (>95%) was analyzed with F4/80 staining by FACS analysis. About 3×10^5 cells were plated in each well of 24-well plates for further experiments.

Neutrophils were isolated from WT and *S1pr2*$^{-/-}$ mice as previously described.[5] The neutrophils were suspended in RMPI 1640 (10^6/ml) and ready for further treatment and analysis.

Confocal microscopy

Bacterial challenged or control BMDMs were fixed with formalin for 30 minutes at room temperature and permeabilized with 0.5% Triton X-100/PBS. Actin cytoskeleton was stained with Alexa Fluor 488-labeled phalloidin (Life Technologies, Grand Island, NY), according to manufacturer's instruction. For staining IQGAP1 and Rac1, cells were blocked and then incubated with primary antibodies against IQGAP1 (sc-10792; Santa Cruz Biotechnology, Santa Cruz, CA) and Rac1 (Thermo Fisher Scientific, Rockford, IL). After stained with fluorescent secondary antibodies (Millipore, Billerica, MA), cells were counterstained with DAPI. Fluorescent images of BMDMs or *E. coli* were visualized using a LSM 710 confocal microscope (Zeiss, Oberkochen, Germany) with a NeoFluor 63×/1.4 NA oil-immersion objective at room temperature.

Western blot

Samples were lysed in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and 30 μg of total lysate was resolved with 6-12% SDS-PAGE. Proteins were then transferred to PDVF membranes (Millipore, Billerica, MA). The membrane was blocked with 2% BSA/PBS and blotted with antibodies against IQGAP1, Rac1 or GAPDH (Cell Signaling, Boston, MA), followed by HRP-conjugated second antibodies (Millipore, Billerica, MA). The membranes were developed with enhanced

chemical luminescence (Biological Industries, Kibbutz Beit-Haemek, Israel) and exposed to X-ray films. The resulting protein bands were analyzed with Image J software.

Pull-Down assay

Pull-down assays were performed to determine active GTP-bound forms of RhoA and Rac1 by GST pull-down Kits of Rho GTPases (Thermo Fisher Scientific, Rockford, IL), according to manufacturer's instruction. Briefly, 200 μg of cell lysates were prepared and incubated with GST-Rhotekin (binding with RhoA-GTP) or GST-Pak1 (binding with Rac1-GTP), which were immobilized agarose beads. The pull-down proteins were analyzed with Western blot using antibodies against RhoA and Rac1.

Isolation of F-actin

F-actin fractions from BMDMs were prepared as previously described.[6] Briefly, cells incubated with live *E. coli* for 90 minutes were lysed in a cytoskeletal fraction buffer (50 mM Tris HCl, pH 7.5, 100 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, and 1% Nonidet P-40) and were kept on ice for 10 minutes followed by incubation at room temperature for 30 minutes. One milligram of total protein was taken from each sample and pelleted at 10,000 g for 5 minutes. The pellets were washed once with cytoskeletal fraction buffer and resuspended in Laemmeli buffer. F-actin was extracted with boiling for 5 minutes and analyzed with Western blot.

Co-Immunoprecipitation

Cells were stimulated with live *E. coli* for 60 minutes and then solubilized in lysis

buffer (PBS containing 1% NP-40 and protease inhibitor cocktail). The cell lysates (0.5 mg in 1 ml lysis buffer) were incubated with protein G magnetic beads (Millipore, Billerica, MA) coated with anti-IQGAP1 antibody overnight at 4°C. After eluted from the magnetic beads, the protein complex was resolved by SDS-PAGE. The resultant PVDF membranes were blotted with antibodies against IQGAP1, phosphotyrosine (clone 4G10; Millipore, Billerica, MA) or Rac1 (Thermo Fisher Scientific, Rockford, IL).

Small interfering RNA

Small interfering RNA for mouse IQGAP1 gene and related negative control were purchased from Santa Cruz Biotechnology (sc-35701 and sc-37007). BMDMs were transfected with 90 nM siRNA duplexes using Lipofectamine RNAiMax (Life Technologies, Grand Island, NY) in serum free DMEM, according to the manufacturer's instructions. After incubation for 72 hours, the cells were collected and knockdown of IQGAP1 was evaluated with Western blot.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY) and first strand cDNA was synthesized with Reverse Transcription System (Promega, Madison, WI) as previously described.[7] PCR reaction was performed in triplicate using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative expression level was determined using the comparative threshold method. β-actin was used as an internal control. Primer sequences were described in Supplemental Digital Content 2.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

PCR was performed by using Platinum Taq DNA polymerase (Life Technologies, Grand Island, NY) in a PCR Thermal Cycler Dice (Fisher Thermo Scientific, Rockford, IL) under the following conditions: 95°C for 10 min and then the appropriate number of cycles at 95°C for 10 s; appropriate annealing temperature for 30 s; 72°C for 10 s], and finally 72°C for 7 min.

Human subjects

Patients who met with the clinical criteria of sepsis within the first 24 hours after they were admitted to Intensive Care Unit were screened for eligibility.[8] Totally 28 septic patients stayed in the Intensive Care Unit of the First Affiliated Hospitals of Zhejiang University between August 2013 and January 2015 were enrolled. And 9 non-septic patients but in critical conditions were selected as controls. The clinical data, including Acute Physiology and Chronic Health Evaluation II scores and Sequential Organ Failure Assessment scores, causes of sepsis, microbial culture results, lengths of Intensive Care Unit stay, and mortalities during 28-day study period, were recorded. The study protocol was approved by the local institutional review board (Hangzhou, China), and informed consents were obtained from all patients or their surrogates.

Peripheral blood mononuclear cells of each patient were isolated by density gradient centrifugation as previously described.[9] Expression of S1PR2 was assessed at the level of mRNA by quantitative RT-PCR.

References

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Table 1: Primers for Polymerase Chain Reaction

Gene	Sequence (from 5' to 3')
human S1PR2	Forward primer-GGCCTAGCCAGTTCTGAAAGC
	Reverse primer-GCGTTTCCAGCGTCTCCTT
human β-actin	Forward primer-AGAAAATCTGGCACCACACC
	Reverse primer-AGAGGCGTACAGGGATAGCA
mouse S1PR2	Forward primer- CACTGCTCAATCCTGTCA
	Reverse primer- GAAATGTCGGTGATGTAGG
mouse β-actin	Forward primer- CTACAATGAGCTGCGTGTG
	Reverse primer- GCGTGAGGGAGAGCATAG

Table 2: Patients characteristics

Characteristics	Sepsis (n=25)	Control (n=9)	P value
Age (yr)	57.92±15.28	52.89±15.19	0.79
Sex, male (%)	17 (68%)	6 (66.67%)	1
APACHE II score	18.04±6.89	10.33±1.87	0.025
SOFA score	7.72±3.93	5.89±2.85	0.182
Sepsis due to		NA	
Peritonitis	7 (28%)		
Pneumonia	8 (32%)		
Urinary tract infections	4 (16%)		
Multiple injuries	6 (24%)		
Microb species	12 (48%)	NA	
Escherichia coli	3 (12%)		
Staphylococcus aureus	5 (20%)		
Klebsiella pneumoniae	1 (4%)		
Pseudomonas aeruginosa	1 (4%)		
Candida albicans	2 (8%)		
Length of ICU stay	11.56±6.36	7.22±3.99	0.225
28-day mortality	3 (12%)	0 (0)	0.29

Data are expressed as the mean ± SD or number (%) where applicable.

APACHE II=Acute Physiology and Chronic Health Evaluation II; ICU=intensive care unit; NA=not applicable; SOFA=Sequential Organ Failure Assessment.

Table 3: Patients characteristics

Characteristics	Non-septic control	Septic patient 1	Septic patient 2	Septic patient 3
Age (yr)	70	56	65	74
Sex, male (%)	female	male	female	male
APACHE II score	10	12	18	25
SOFA score	2	10	13	17
Sepsis due to	NA	Pneumonia	Peritonitis	Peritonitis
Length of ICU stay	2	3	8	17

APACHE II=Acute Physiology and Chronic Health Evaluation II; ICU=intensive care unit; NA=not applicable; SOFA=Sequential Organ Failure Assessment.

Figure 1

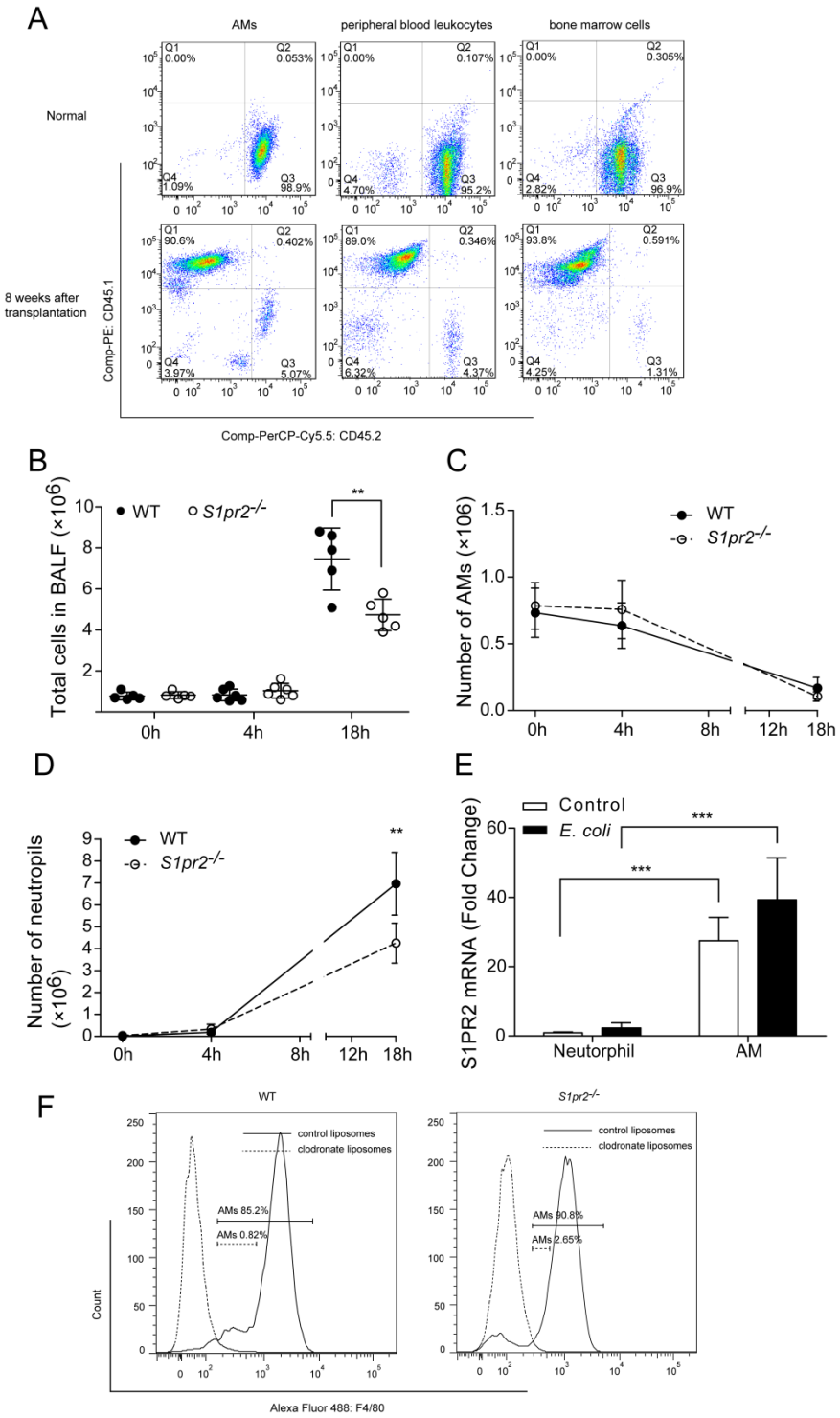

Figure 1. (A) Flow cytometry analysis confirms successful generation of bone marrow chimeras. Bone marrow chimeras were prepared by reciprocal transfer of bone marrow cells between CD45 isotype-mismatched animals with the indicated

genotypes (WT: CD45.1; *S1pr2*$^{-/-}$: CD45.2). Eight weeks after hematopoietic reconstitution, peripheral blood leukocytes, bronchoalveolar lavage fluid (BALF) cells and bone marrow cells were analyzed by flow cytometer using PE-conjugated antibody against CD45.1 and PerCP-Cy5.5- conjugated antibody against CD45.2. (B-D) Numbers of total cells (B), alveolar macrophages (AMs, C) and neutrophils (D) in BALF obtained from wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*$^{-/-}$ mice at time points as indicated. n=5 mice per group at each time point. Data are presented as the mean ± SD. $^{**}P<0.01$; Student's t test between *S1pr2*$^{-/-}$ and WT mice within each time point. (E) Relative mRNA levels of S1PR2 in isolated mouse AMs and neutrophils with or without *E. coli* challenge. Values were normalized to that in the quiescent neutrophils and β-actin was used as an internal reference gene. Data are presented as the mean ± SD. $^{***}P<0.001$; Two-way ANOVA with Bonferroni corrections for multiple comparisons. (F) Flow cytometry analysis confirms depletion of AMs in vivo. Forty-eight hours after intratracheal instillation of clodronate liposomes or control liposomes into mice, cells were collected from BALF, and analyzed by flow cytometer using F4/80 antibody.

Figure 2

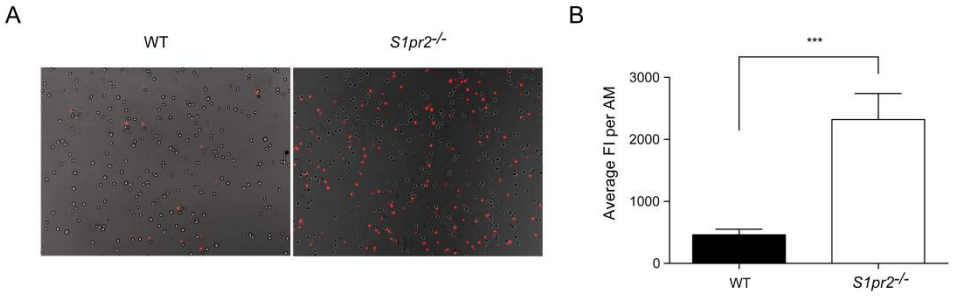

Figure 2. (**A**) Increased phagocytosis of Texas Red-labelled microspheres by the *S1pr2*$^{-/-}$ alveolar macrophages (AMs). Original magnification, ×200. (**B**) Quantification of phagocytosis according to fluorescent microscopic images in panel A. In each microscopic field, total AMs and fluorescent 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 each groups. WT, wild-type. Data are presented as mean ± SD and were analyzed by Mann-Whitney test. ****P*< 0.001.

Figure 3

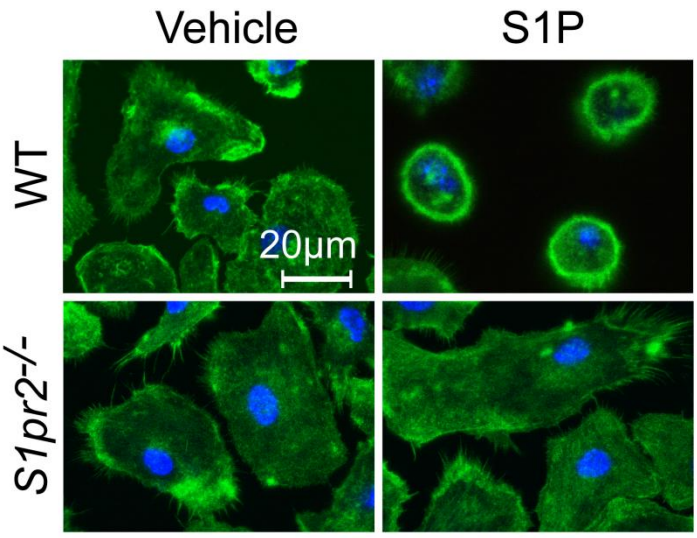

Figure 3. Incubation with S1P induces cytoskeletal rearrangement and cell contraction in wild-type (WT, *S1pr2*$^{+/+}$) bone marrow-derived macrophages (BMDMs) but not in *S1pr2*$^{-/-}$ BMDMs. F-actin (green) and nuclei (blue) were fluorescently stained. The staining was observed under LSM 710 confocal microscope with a NeoFluor 63×/1.4 NA oil-immersion objective at room temperature.

Figure 4

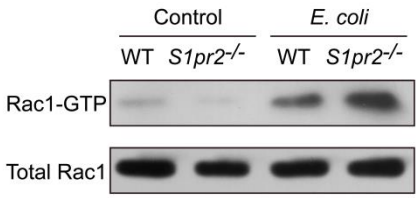

Figure 4. Rac1-GTP levels in bronchoalveolar lavage fluid (BALF) cells isolated from normal wild-type (WT, *S1pr2*$^{+/+}$) and *S1pr2*$^{-/-}$ mice or 4 hours after mice challenged with *E. coli*. GTP-bound Rac1 levels were determined with Glutathione-S-transferase pull-down assays and normalized to the total Rac1.

Figure 5

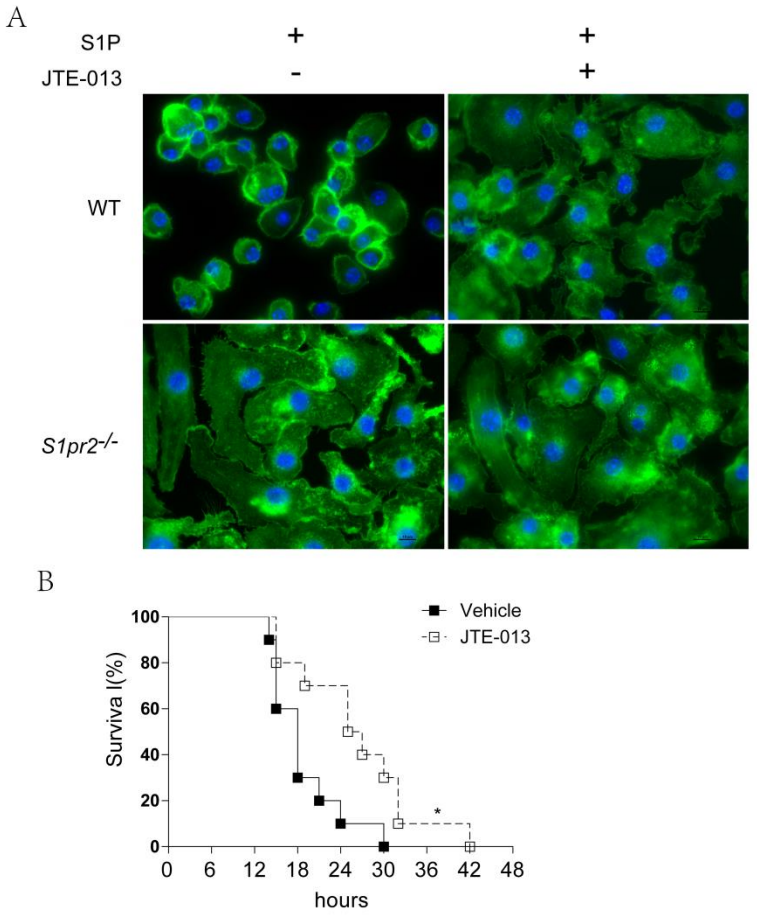

Figure 5. (**A**) Antagonism of S1PR2 with JTE-013 protects against S1P induced macrophage contraction. Bone marrow-derived macrophages were pretreated with 5 μM JTE-013 or vehicle for 30 minutes before stimulation with 100 nM S1P for 30 minutes. Cytoskeleton was stained with Alexa Fluor 488-labeled phalloidin and the staining was observed under fluorescence microscopy. Original magnification, ×1000, oil. (**B**) JTE-013 treatment protects wild-type (WT, *S1pr2*$^{+/+}$) mice from lethal infection caused by *E. coli* infection. A single dose of JTE-013 (4 mg/kg) or vehicle was intratracheally administered immediately after i.t. *E. coli*. n=10 per group from 2 independent experiments. The Mantel-Cox test.