Supporting Information

Kannan et al. 10.1073/pnas.1309393110

SI Experimental Procedures

Mice. Mice were obtained from The Jackson Laboratory and were housed and maintained in a temperature-controlled room on a 12 h light/dark cycle and fed with the standard food and water ad libitum at Beth Israel Deaconess Medical Center (BIDMC) Animal Research Facility. Mice underwent a 7-d period of acclimatization before experimentation. All experimental protocols were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals and were approved by the BIDMC Institutional Animal Care and Use Committee.

Mesenteric Model of Ischemia/Reperfusion. At the end of 30 min R-spondin3 (R-spo3) pretreatment, mice were anesthetized with 72 mg/kg of nembutal (Butler Schein) given intraperitoneally. In addition, 36 mg/kg of nembutal was given intramuscularly during the experiment when necessary. All procedures were performed on anesthetized, spontaneously breathing animals with body temperature maintained at 37 °C with a controlled heating pad. Briefly, a midline laparotomy was performed before a 30-min equilibration period. The superior mesenteric artery was identified and isolated, and a small nontraumatic vascular clamp (Roboz Surgical Instruments) delivering ~85 g of pressure was applied for 30 min. After this ischemic phase, the clamp was removed and the intestine was allowed to reperfuse for 3 h. Sham and sham + R-spo3 groups underwent an identical surgical intervention except for arterial clamping. The laparotomy incision was sutured with 4-0 Sofsilk (Synture), and mice were resuscitated with 1.0 mL of prewarmed sterile phosphate-buffered saline (PBS) s.c. and monitored during the reperfusion period. At the end of 3 h reperfusion, the mice were euthanized and the organs were harvested for histopathological, immunohistochemical, mRNA, and protein analysis. A time course reperfusion experiment including 30 min ischemia followed by 15, 30, 60, 120, and 180 min reperfusion and a short ischemia/reperfusion (I/R) of 30/30 min in the presence and absence of R-spo3 were performed in the same manner.

Histology and Tissue Injury Scoring. The harvested intestinal and lung tissues were rinsed and fixed overnight in 10% (vol/vol) phosphate-buffered formalin. Formalin-fixed tissues were then embedded in paraffin, sectioned transversely (5 μm), and stained with hematoxylin and eosin (H&E). In each intestinal tissue, 100 villi were graded on a six-tiered scale, as previously described (1). Normal villus was assigned a score of 0; villi with tip distortion received a score of 1; villi lacking goblet cells and containing Guggenheims' spaces were scored as 2; villi with patchy disruption of the epithelial cells were scored 3; villi with exposed but intact lamina propria and epithelial cell sloughing were scored 4; villi in which the lamina propria was exuding were scored as 5; and the highest score 6 was assigned to the villi displaying hemorrhage or denudation. To determine the lung tissue damage, three parameters were used: (*i*) periluminal infiltrates (airways/vessels): 0, when no infiltrates were observed; 1, when infiltrates were formed by 1–3 cell layers; 2, when infiltrates were 4–10 cell layers thick; 3, when they were >10 cell layers thick; (*ii*) pneumonitis (alveolar/interstitial): 0, when no infiltrates were observed; 1, when infiltrating cells were evident only at high magnification (400×); 2, when cell infiltrates were easily observed; 3, when lung consolidation by inflammatory cells was evident; and (*iii*) percentage of affected lung tissue: 0, 0%; 1, 5–25%; 2, 26–50%; 3, >50%. All histology analyses were performed in a third-party blinded manner. The images of the sections were captured using Nikon eclipse 80i microscope and analyzed using Nikon NIS-Elements software.

mRNA Analysis. Total RNA from cell cultures and tissues were isolated using the RNeasy Mini kit (Qiagen) and TRIZOL reagent (Invitrogen), respectively. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer protocol. Quantitative real-time PCR were performed (Light Cycler 480; Roche) for TNFα, IL-6, Cox-2, R-spo1, R-spo2, R-spo3, R-spo4, VCAM1, ICAM1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with 40 cycles at 94 °C for 12 s and 60 °C for 60 s using appropriate murine and human TaqMan assays (Applied Biosystems). The averaged cycle threshold values of each reaction derived from the target genes were normalized to GAPDH levels. All PCR reactions were run in triplicates. Cycle threshold values were used to calculate relative mRNA expression by the ΔΔCt relative quantification method.

ELISA. After euthanizing the mice, whole blood was drawn using cardiac puncture and was allowed to clot at room temperature for 60 min. Serum that separated on top of the clot was collected and spun down at 4,800 × *g* for 10 min to remove any insoluble material. Serum levels of IL-6 and TNFα were quantified using an ELISA kit specific for mouse IL-6 and TNFα (eBiosciences). The assay was carried out according to the manufacturer's recommendations.

Immunohistochemistry. Endogenous peroxidase activity in the tissue sections was quenched with 3% (vol/vol) H_2O_2 and antigen retrieval was performed using Retrievagen A (BD Pharmingen) according to the manufacturer's directions. The sections were then blocked for 60 min at room temperature with 10% (vol/vol) bovine serum albumin (BSA)/PBS containing the serum from host species of secondary antibody. A total of 10% BSA/PBS was used as antibody diluent. Primary antibodies were applied overnight at 4 °C. The following day, the slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 60 min at room temperature, developed with NovaRED (Vector Laboratories), counterstained with hematoxylin, and dehydrated. The images of the sections were captured using Nikon eclipse 80i microscope and analyzed using Nikon NIS-Elements software. For immunohistochemistry (IHC) studies, the following reagents were used: anti-IgM (Abcam), anti-complement 3 (C3) (Santa Cruz), horseradish peroxidase-conjugated anti-mouse IgG, and anti-goat IgG (Santa Cruz). Appropriate isotype controls were used.

Western Blotting. For Western blotting, samples were homogenized and lysed in freshly prepared RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) (Boston Biolaboratories) containing a complete protease and phosphatase inhibitor mixture (Roche). The lysates were clarified by centrifugation at 10,000 × *g* and supernatants were used to determine protein concentration by Bradford assay. Protein extracts were then separated by 4–12% gradient SDS/PAGE gels (Invitrogen) under reducing conditions and subjected to standard Western blot analysis. Bands were visualized with an enhanced chemiluminescence kit (Pierce), and images were obtained using a Fujifilm LAS-4000 luminescent image analyzer (Fujifilm). The density of each band was calculated with Multi Gauge software (Fujifilm) and normalized to actin levels. The following antibodies were used: anti-IgM

(Abcam), anti-COX2 (Abcam), anti–β-catenin (BD Biosciences), anti-Rho kinase (Cell Signaling), anti-actin (Sigma), horseradish peroxidase-conjugated anti-rabbit IgG, and anti-mouse IgG (Santa Cruz).

Esterase Staining. Paraffin sections of the intestinal tissues were dewaxed at 56 °C for 30 min followed by xylene, 100%, 95%, and 80% ethanol for 5 min. After four washes in PBS, the slides were incubated with freshly prepared chloroacetate esterase solution for 2 h in the dark at room temperature. They were washed in running tap water for 5 min followed by a rinse in distilled water. The slides were counterstained with nuclear fast red and mounted using aqueous medium. The images of the sections were captured using Nikon eclipse 80i microscope and analyzed using Nikon NIS-Elements software. Neutrophil cell number was obtaining by manually counting cells from 10 different fields under the light microscope for each group.

Evaluation of Vascular Leakage in the Gut. As illustrated in Fig. S6, for mice subjected to 30/180 min I/R and 30/30 I/R, Evans blue (20 mg/kg) was i.v. administered 15 min before the end of reperfusion and at the beginning of reperfusion, respectively. At the end of the indicated reperfusion time points, small intestines were harvested and the extravasation of dye was measured as described previously (2). The amount of Evans blue in the tissue (milligrams of Evans blue per 100 mg of tissue) was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in an ELISA plate reader.

Cell Culture. HEK293T cells were maintained in DMEM containing 10% FCS (vol/vol) at standard conditions. Different cell types from human peripheral blood were isolated by different procedures. Monocytes were isolated by positive selection using anti–CD14-coated magnetic beads (Miltenyi Biotech) and were differentiated into macrophages and dendritic cells, granulocytes using Polymorphprep (Fisher), T cells were purified using rosette T-cell purification kit (StemCell Technologies), and B cells using anti–CD19-coated magnetic beads (Miltenyi Biotech). The cells were cultured in RPMI 1640 (Mediatech) containing 10% FCS and maintained in a humidified incubator (37 °C, 5% CO_2). Human epithelial cell line caco-2 was obtained from ATCC. Murine primary epithelial cells were obtained from small intestine using the collagenase/dispase (Worthington) enzyme method, granulocytes from mice bone marrow using the Percoll (Fisher) gradient centrifugation method, and macrophages, dendritic cells, and T and B cells from mice spleen.

Measurement of Transendothelial Electrical Resistance. Transendothelial electrical resistance (TEER) across the endothelial cell monolayer was measured using an impedance sensor system (Applied Biophysics). Cells were exposed to BSA (1 μg/mL), histamine (negative control, 10 μM), recombinant human (h) R-spo3 (1 μg/mL; R&D Systems), and R-spo3 + histamine (same concentration as mentioned above). In R-spo3 + histamine group, human umbilical vein endothelial cells (HUVECs) were preconditioned with hR-spo3 15 min before histamine stimulation.

Measurement of Fluorescein Isothiocyanate-Dextran Leakage. Confluent HUVECs were treated with and without hR-spo3 (1 μg/mL) for 15 min before the addition of histamine (10 μM) and thrombin (1 units/mL). Permeability across the monolayers was measured by adding 0.1 mg fluorescein isothiocyanate (FITC)-conjugated dextran (molecular weight, ~500,000; Sigma) to the upper chamber. After 15 min of stimulation, the fluorescence in the lower chamber was determined using a microplate reader at an excitation wavelength of 490 nm and an emission wavelength of 515 nm.

Hypoxia/Reoxygenation Injury Condition. At 24 h before induction of hypoxia, HUVECs were grown under normal conditions and at the time of hypoxia, the culture medium was replaced with deoxygenated culture medium. Subsequently, the cells were placed in an anaerobic chamber system (Biospherix), which can catalytically reduce oxygen levels to 0.1% and cells were subjected to hypoxia for 2 h. HUVECs were then returned to normoxic conditions, supplied with fresh medium, and incubated for reoxygenation treatment for 30 min. These cells were used for FITC-dextran permeability assays and immunofluorescence staining.

Immunofluorescence and Localization of F-Actin. HUVECs pretreated with and without recombinant hR-spo3 (1 μg/mL) were subjected to hypoxy/reoxygenation (H/R) at the indicated time period and then were fixed with 4% paraformaldehyde for 15 min at room temperature, for detection of VE-cadherin. Then they were permeabilized for 5 min with PBS containing 0.3% Triton X-100, blocked with PBS containing 5% BSA for 60 min, and incubated with anti–VE-cadherin (Cell Signaling) overnight at 4 °C followed by Alexa 488-conjugated secondary antibody (Invitrogen) for 60 min at room temperature. The cells were then incubated with 1 μg/mL rhodamine-labeled phalloidin (Invitrogen) for 20 min to visualize F-actin. The nuclei were stained with di aminido phenyl indol (1 μg/mL in PBS) and mounted using a Prolong Gold antifade-mounting medium (Invitrogen). Single plain images of the cells were obtained by confocal laser scanning microscopy with an LSM 510 (Zeiss) using a 40× oil immersion objective.

Quantification of F-Actin. For F-actin quantification, confocal images from cells stained for rhodamine-labeled phalloidin were exported in LSM formats compatible with Volocity image analysis software (Perkin-Elmer) to obtain the mean fluorescent intensities. In each group, 10 images were captured and analyzed to quantify the F-actin content.

1. Yoshiya K, et al. (2011) Depletion of gut commensal bacteria attenuates intestinal ischemia/reperfusion injury. *Am J Physiol Gastrointest Liver Physiol* 301(6):G1020–G1030.

2. Souza DG, et al. (2004) The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J Immunol* 173(6):4137–4146.

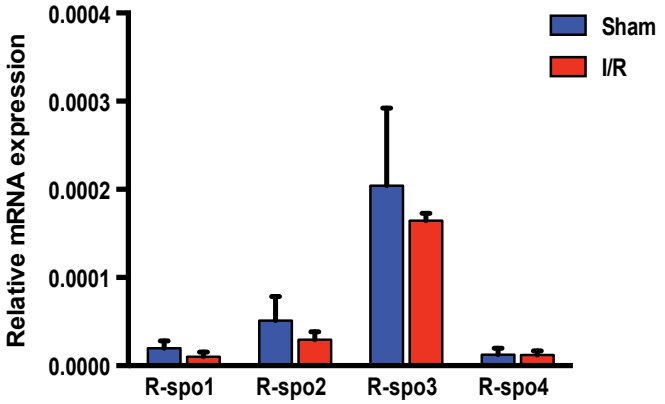

Fig. S1. Gene expression pattern of R-spondins in the small intestine of 10-wk-old C57B6/J mice. Total RNA samples prepared from small intestinal tissues of sham and I/R (30/180 min)-treated mice were subjected to qPCR analysis using mouse R-spo1-, R-spo2-, R-spo3-, and R-spo4-specific Taqman assays. Murine glyceraldehyde dehydrogenase (GAPDH) was used as a control for relative quantification. The endogenous mRNA levels of R-spo1, R-spo2, and R-spo4 in the sham are almost negligible except for R-spo3, which was weakly expressed but relatively high compared with the other family members. The expression pattern of all of the four R-spondins in the I/R group remained consistent with that of sham. $n = 6$ per group. Error bars are presented as SD.

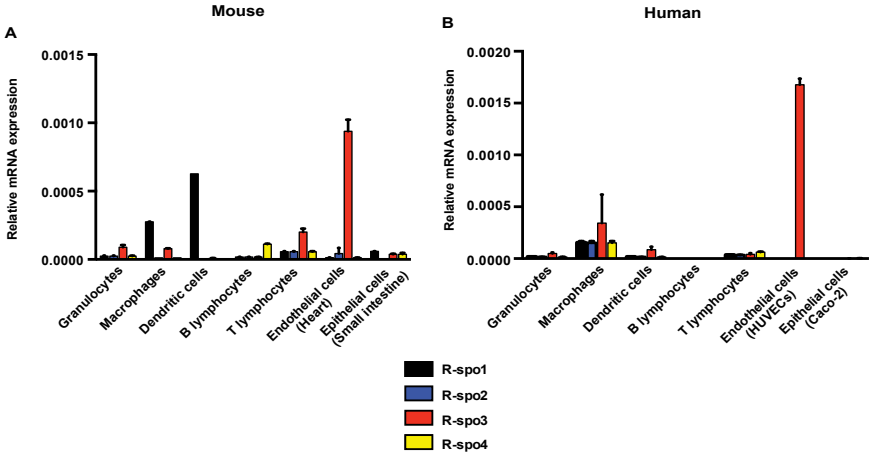

Fig. S2. R-spo3 is predominantly expressed in both murine and human endothelial cells. (*A*) mRNA expression levels of R-spondins in different murine cell types: bone marrow granulocytes, macrophages, dendritic cells, and lymphocytes from spleen, small intestinal epithelial cells, and heart endothelial cells ($n = 3$). (*B*) mRNA expression levels of R-spondins in different cell types: granulocytes, monocytes, dendritic cells, lymphocytes (from human peripheral blood), human umbilical vein endothelial cells (HUVECs), and Caco-2, a continuous epithelial cell line derived from human colon carcinoma ($n = 3$). Total RNA samples prepared from these cells were subjected to qPCR analysis using mouse and human R-spo1-, R-spo2-, R-spo3-, and R-spo4-specific Taqman assays. The expression level was normalized with GAPDH expression. Error bars are presented as SD.

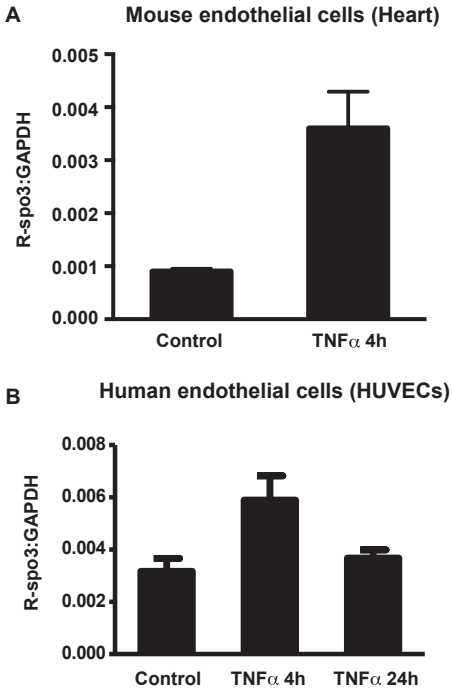

Fig. S3. TNFα stimulation increased the endogenous mRNA levels of R-spo3 in endothelial cells. Mouse heart endothelial cells (*A*) or human endothelial cells-HUVECs (*B*) were stimulated with TNFα of 20 ng/mL for 4 and/or 24 h and the cells were harvested for RNA isolation at the indicated time points. The expression of R-spo3 was analyzed by qPCR, which showed a significant increase in the expression of R-spo3 at 4 h, indicating that R-spo3 may have an autocrine protective effect under inflammatory conditions. All of the samples were prepared in triplicates. Error bars are presented as SD.

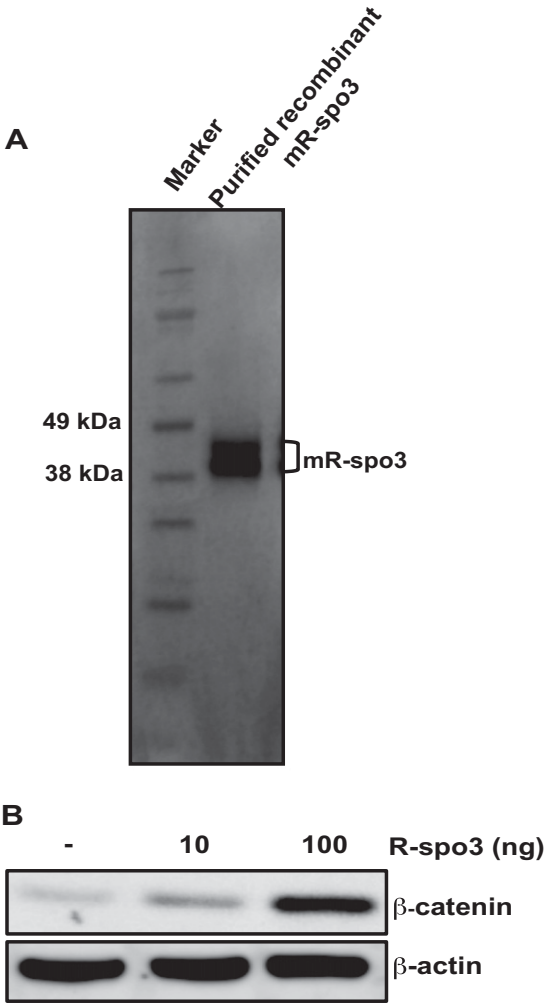

Fig. S4. Characterization of recombinant mouse R-spo3. (*A*) Silver staining of purified mouse R-spo3 obtained from R&D Systems. The two bands represent R-spo3 in its glycosylated forms. (*B*) Recombinant mouse R-spo3 stabilizes β-catenin in HEK293T cells. HEK293T cells were treated for 3 h with and without recombinant mouse R-spo3 of 10 or 100 ng as indicated. Cytosolic extracts were used to blot for β-catenin. β-Actin was used as a loading control. The experiment was repeated three times.

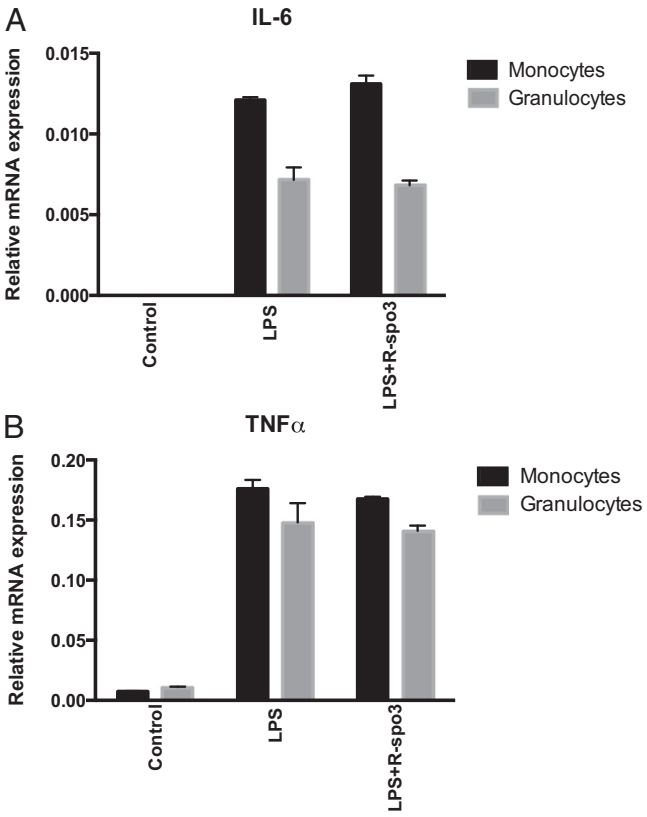

Fig. S5. R-spo3 does not alter LPS-induced mRNA levels of IL-6 (*A*) and TNFα (*B*) in monocyte and granulocyte cultures isolated from human peripheral blood. Monocytes and granulocytes (~1 × 10^6 cells per well) were first pretreated with R-spo3 (1 μg/mL) for 30 min and then stimulated with LPS (100 ng/mL) for 6 h. At the end of 6 h, cells were harvested for RNA isolation and were subjected to qPCR analysis using human IL-6 and TNFα-specific Taqman assays. The expression level was normalized with GAPDH expression. Error bars are presented as SD.

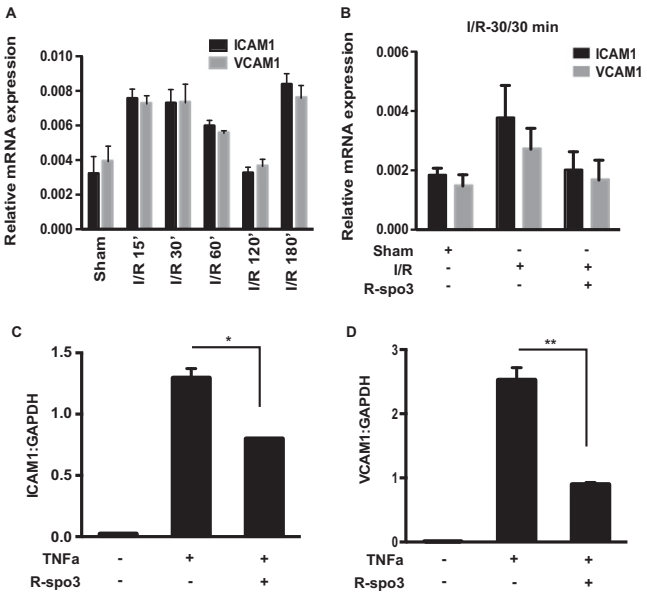

Fig. S6. (*A*) ICAM1 and VCAM1 transcript expression in the intestinal tissues of mice subjected to different groups, sham, 30/15 min I/R, 30/30 min I/R, 30/60 min I/R, 30/120 min I/R, and 30/180 min I/R ($n = 6$ per group). The expression levels were normalized with GAPDH expression. (*B*) R-spo3 reduces I/R-enhanced expression of ICAM1 and VCAM1 in the intestinal tissues of mice subjected to 30/30 min I/R ($n = 6$ per group). The expression levels were normalized with GAPDH expression. R-spo3 down-regulates TNFα-induced expression of ICAM1 (*C*) and VCAM1 (*D*) in in vitro HUVEC cultures. Primary HUVECs (passage 4) were treated for 4 h with TNFα of 20 ng/mL with or without human R-spo3 of 100 ng. At the indicated time periods, the cells were harvested for RNA isolation and the expression of ICAM1 and VCAM1 were analyzed by qPCR. $n = 3$, $^*P < 0.05$ and $^{**}P < 0.005$. Error bars are presented as SD.

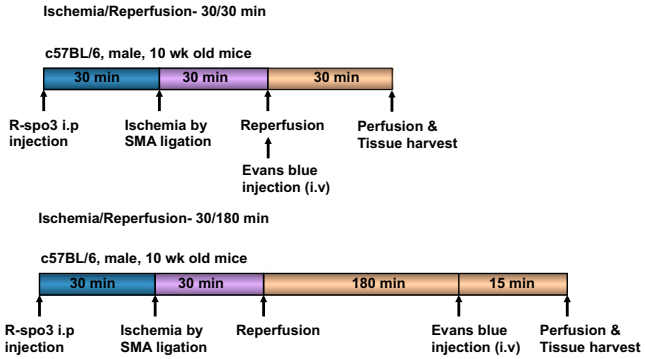

Fig. S7. Experimental approach to measure intestinal vascular leakage in an in vivo mesenteric model of 30/30 min and 30/180 min ischemia/reperfusion using Evans blue dye.

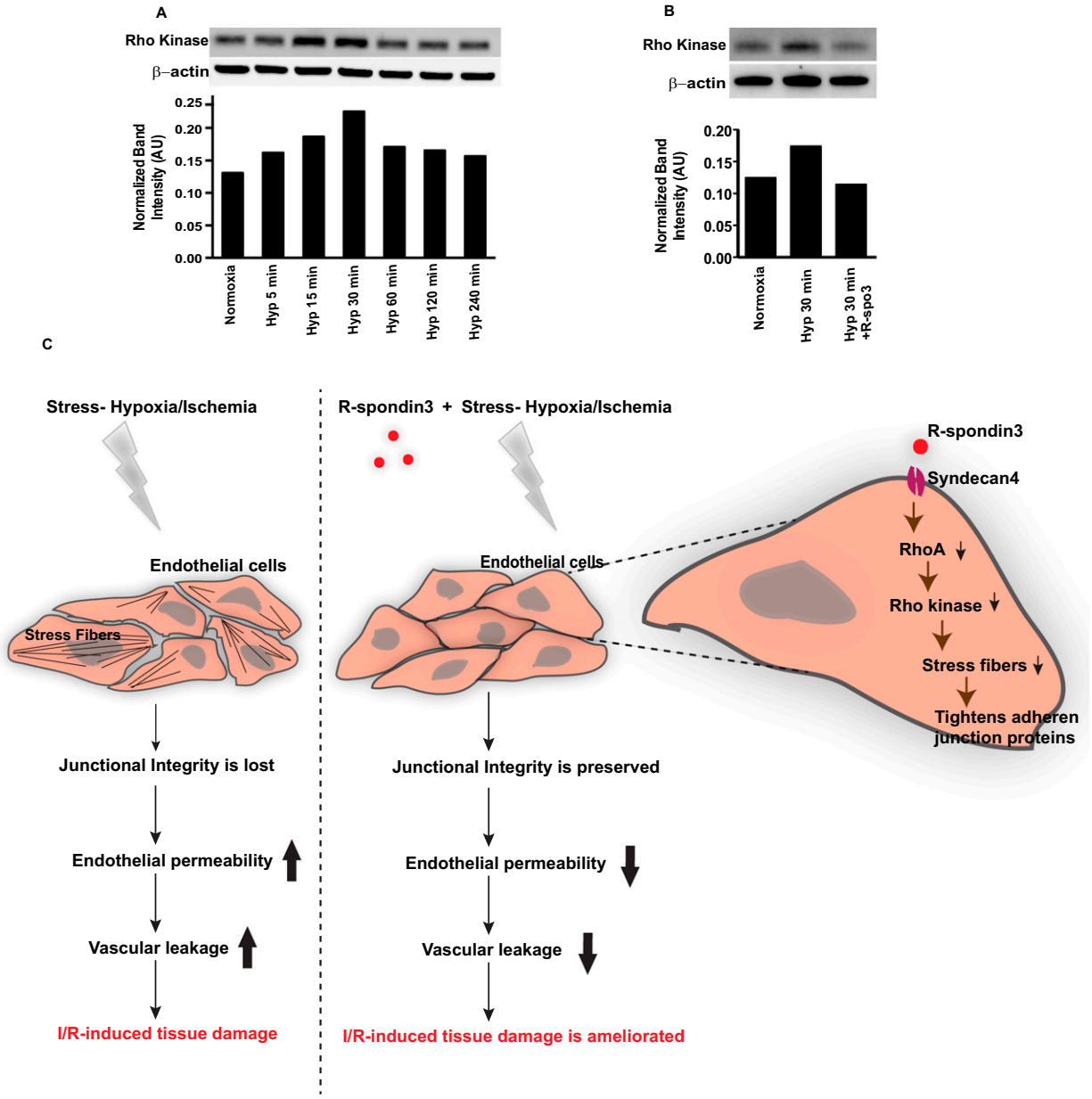

Fig. S8. (*A*) Hypoxia-induced changes in Rho kinase expression in HUVECs. HUVECs seeded on 35-mm dishes (5×10^5 cells per dish) were grown under normoxic conditions for 24 h. Before hypoxic conditions, the culture media was changed to the deoxygenated culture medium and cells were placed into an anaerobic chamber. After subjecting cells to hypoxia for the indicated time periods: 5, 15, 30, 60, 120, and 240 min, cells were lysed and used for immunoblotting with anti-Rho kinase and anti–β-actin antibodies. Data are representative of three independent experiments. Data were quantified by densitometric analysis and the Rho kinase level is presented relative to β-actin expression. (*B*) R-spo3 suppresses hypoxia-enhanced expression of Rho kinase in HUVECs. HUVECs grown in normoxic conditions were subjected to hypoxia in the same manner as in *A*. Cells were lysed and used for immunoblotting with anti-Rho kinase and anti–β-actin antibodies. Data are representative of three independent experiments. Data were quantified by densitometric analysis and the Rho kinase level is presented relative to β-actin expression. (*C*) Proposed schema illustrates the protective role of R-spondin3 in I/R-related conditions and a mechanism by which R-spo3 may attenuate barrier dysfunction in endothelial cells.