Bone Marrow Progenitor Cells Induce Endothelial Adherens Junction Integrity by Sphingosine-1-Phosphate–Mediated Rac1 and Cdc42 Signaling

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Rationale: Little is known about the contribution of bone marrow–derived progenitor cells (BMPCs) in the regulation endothelial barrier function as defined by microvascular permeability alterations at the level of adherens junctions (AJs).

Objective: We investigated the role of BMPCs in annealing AJs and thereby in preventing lung edema formation induced by endotoxin (LPS).

Methods and Results: We observed that BMPCs enhanced basal endothelial barrier function and prevented the increase in pulmonary microvascular permeability and edema formation in mice after LPS challenge. Coculture of BMPCs with endothelial cells induced Rac1 and Cdc42 activation and AJ assembly in endothelial cells. However, transplantation of BMPCs isolated from sphingosine kinase-1–null mice (*SPHK1*$^{-/-}$), having impaired S1P production, failed to activate Rac1 and Cdc42 or protect the endothelial barrier.

Conclusions: These results demonstrate that BMPCs have the ability to reanneal endothelial AJs by paracrine S1P release in the inflammatory milieu and the consequent activation of Rac-1 and Cdc42 in endothelial cells. (*Circ Res*. 2009;105:696-704.)

Key Words: progenitor cells ■ endothelial permeability ■ S1P signaling ■ RhoGTPases

The endothelial cell monolayer lining blood vessels forms a semipermeable barrier that performs the vital tasks of regulating tissue fluid homeostasis and transmigration of blood cells. Bone marrow–derived endothelial progenitor cells induce angiogenesis and have been touted for therapeutic angiogenesis in the treatment of myocardial ischemia and other diseases requiring revascularization.[1] Recent studies showed that transplantation of bone marrow–derived mesenchymal cells can also limit lung inflammation in experimental models.[2,3] As disruption of the endothelial barrier in vascular inflammation leads to tissue edema and injury and there are no current means to mitigate vascular leakage, we addressed the possible role of bone marrow–derived progenitor cells (BMPCs) in regulating endothelial barrier function.

The phospholipid sphingosine-1-phosphatase (S1P), generated by platelets, red blood cells (RBCs), and endothelial cells, has an endothelial barrier protective function.[4,5] Sphingosine kinases phosphorylate sphingosine leading to formation of S1P.[6] Platelets lack the S1P degradation enzyme sphingosine-1-lyase and thus release S1P in the circulation.[7] In addition, RBCs and endothelial cells produce abundant amounts of S1P.[8–10] The endothelial barrier promoting effect of S1P is concentration-dependent and characterized by a rapid increase in transendothelial electric resistance.[4,11] S1P signaling on the basis of its effects on immune and endothelial cells is also a modulator of immune and inflammatory responses.[12] In the murine model of LPS-mediated acute lung injury, S1P was shown to attenuate pulmonary vascular leakage and inflammation.[12,13] The S1P effects depend on ligation of Gi-coupled Edg-1 and Edg-3 receptors.[14] In the present study, we surmised that BMPCs are an important source of S1P and thereby contribute to BMPC-mediated effects on the endothelium.

We previously showed that reestablishing endothelial contact and junction integrity after injury requires the activation of the RhoGTPases Cdc42 and Rac1 in endothelial cells.[15] Thus, we also investigated their involvement in mediating the underlying effects of BMPCs on endothelial junctions. We demonstrate here that homing of BMPCs to lungs profoundly enhanced endothelial barrier function at the level of adherens junctions (AJs) and prevented the LPS-induced increase in endothelial permeability by the generation of S1P and consequent activation of Rac1 and Cdc42 signaling.

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Methods

Mice

All mice were bred and maintained according to NIH guidelines, and experiments were approved by the Animal Care and Use Committee.

Isolation of Mouse BMPCs

Mouse BMPCs were isolated using modifications of methods based on published markers and methods used for human and rat BMPC isolation (see online Material and Methods, available online at http://circres.ahajournals.org).[16–18] The phenotype of confluent cell population was assessed by determining expression of protein markers by FACS analysis.[17]

Mouse Lung Vascular Endothelial Cells

Mouse lung vascular endothelial cells were isolated as described.[19]

Pulmonary Uptake of BMPCs

BMPCs labeled with rhodamine fluorophore chloromethyl tetramethyl rhodamine (CMTMR, Molecular Probes) were injected into mice (3×10^5 cells per mouse) through the external jugular vein. The animals were then euthanized at different times to determine the location of BMPCs within mouse lung microvasculature by confocal microscopy. In other studies, we labeled 3×10^5 BMPCs with ^{111}Indium oxine[20] to determine organ uptake of BMPCs after i.v. injection.

Flow Cytometry

Cells were detached using 1 mmol/L EDTA in PBS, and incubated with the following antibodies for 30 minutes before FACS analysis: phycoerythrin-labeled antimurine Sca1 (BD PharMingen), and antimurine CD31 (Becton-Dickinson); allophycocyanin-labeled antimurine c-Kit and CD34 antibodies (BD PharMingen); mouse antihuman CD133 (BD PharMingen), rabbit anti-human VE-cadherin (Santa Cruz), and rat anti-mouse CD45 (BD PharMingen). Rabbit anti-mouse or goat anti-mouse FITC (Vector) or Alexa Fluor-labeled anti-goat 594 was used as the secondary antibody (Invitrogen).

BMPC Injection

Mice in all cases were injected with 3×10^5 BMPCs (in 200 μL of EBM-2MV medium) through the external jugular vein.[17]

LPS Challenge

All mice (except those used for mortality studies) received a single sublethal dose of 10 mg/kg bw LPS (*E coli* 0111:B4, Sigma L-2630) i.p. Animals were euthanized at 6 hours after LPS administration for microvessel filtration coefficient ($K_{f,c}$) determinations (see below) and at 12 hours for final lung extravascular lung water measurements.[21]

Rho GTPase Activity Assays

Rac1, Cdc42, and RhoA activities were measured using the GSTrhotekin-Rho binding domain that precipitates activated Rac1 and Cdc42.[22,23] Mouse endothelial cell monolayers were cocultured with BMPCs for different times. Cell lysates were clarified by centrifugation at 4°C and 1000*g* for 5 minutes, and equal volumes of lysates were incubated with PAK-GST protein beads (Cytoskeleton Inc) at 4°C for 2 hours. Beads were washed 3×, and bound Rac1 or Cdc42 was eluted by boiling each sample in Laemmli sample buffer to elute samples. Total cell lysates were electrophoresed on 12.5% SDS-polyacrylamide gels and immunoblotted with either mouse anti-Rac1 or rabbit anti-Cdc42 antibody.

siRNA Transfection

siRNA constructs were transduced in cells by electroporation. Mouse endothelial cells, 60% confluent, were trypsinized, mixed with 2.5 μg siRNA for Rac1, 10 μg for Cdc42, or 3.0 μg for nonsilencing siRNA along with 100 μL of transfection solution (Thermo Scientific Dharmacon siGENOME). Cells were electroporated using a nucleofector device with manufacturer's protocol (VPI-1001, Amaxa). Cells were removed, mixed in EBM-MV2, and plated on either 100-mm dishes, or Transwell chamber filters, or gold-plated 10-well electrodes for functional analyses.

Non-standard Abbreviations and Acronyms

BMPC	bone marrow–derived progenitor cell
S1P	sphingosine-1-phosphatase
AJs	adherens junctions
CMTMR	chloromethyl tetramethyl rhodamine
$K_{f,c}$	microvessel filtration coefficient
TER	transendothelial electrical resistance

Statistical Analysis

Data are presented as mean±SEM. Differences were tested using analysis of variance (ANOVA with posthoc comparisons using unpaired *t* test or Mann–Whitney tests as appropriate). Survival data were analyzed using Peto-Peto-Wilcoxon test. $P<0.05$ denotes significant difference.

Results

FACS Analysis

Cultured wild-type (*wt*) BMPCs were positive for various stem/progenitor cell markers CD133 (92%), Sca1 (83%), CD34 (75%; Figure 1A). BMPCs based on their high expression of these markers are therefore referred to as progenitor cells. Only a small number of BPMCs expressed the mature endothelial markers CD31 (3%) and VE-cadherin (12%), and generic myeloid cell marker CD45 (0.3%; Figure 1A). Quantitative real-time RT-PCR analysis also showed minimal expression of CD45 in BMPCs (Online Figure IC).

In comparing *wt* and *SPHK1* KO BMPCs, we found similar CD133 expression (92% for *wt* and 90% for *SPHK1* KO BMPCs). Although Sca1 and CD34 expression was elevated in both cell types, their expression was greater in *wt* BMPCs (for Sca1, 83% in *wt* BMPCs and 40% in *SPHK1* KO BMPCs; for CD34, 75% in *wt* BMPCs and 40% for *SPHK1* KO BMPCs). There were also differences in CD31, VE-cadherin, and CD45 expression in *wt* and *SPHK1* KO BMPCs (for CD31, 2.6% in *wt* BMPCs and 66% in *SPHK1* KO BMPCs; for VE-cadherin, 12% in *wt* BMPCs and 40% for *SPHK1* KO BMPCs; for CD45, 0.3% in *wt* BMPCs and 15% for *SPHK1* KO BMPCs; Online Figure IA).

Localization of BMPCs in Lungs

We next determined the uptake of transplanted BMPCs in lungs. At day 3 after i.v. injection of ^{111}Indium oxine–labeled BMPCs, lungs contained 80% of the injected BMPCs compared to other organs (Online Figure IB). To visualize BMPCs, they were incubated with the fluorophore CMTMR (25 μmol/L) for 40 minutes, then washed and injected into the external jugular vein. We found similar 60% of the *wt* and *SPHK1*$^{-/-}$ BMPCs were retained at 20 minutes (with most in lung microvessels) after injection (Figure 1B and 1C). By 24 hours, total number of fluorescent cells was decreased in both groups with a greater decrease in *SPHK1*$^{-/-}$ BMPCs (24% in *SPHK1*$^{-/-}$ BMPCs and 47% in *wt* BMPCs ($P<0.05$). The location of cells in both groups was the same, with most cells detected in the pulmonary parenchyma or lodged extramu-

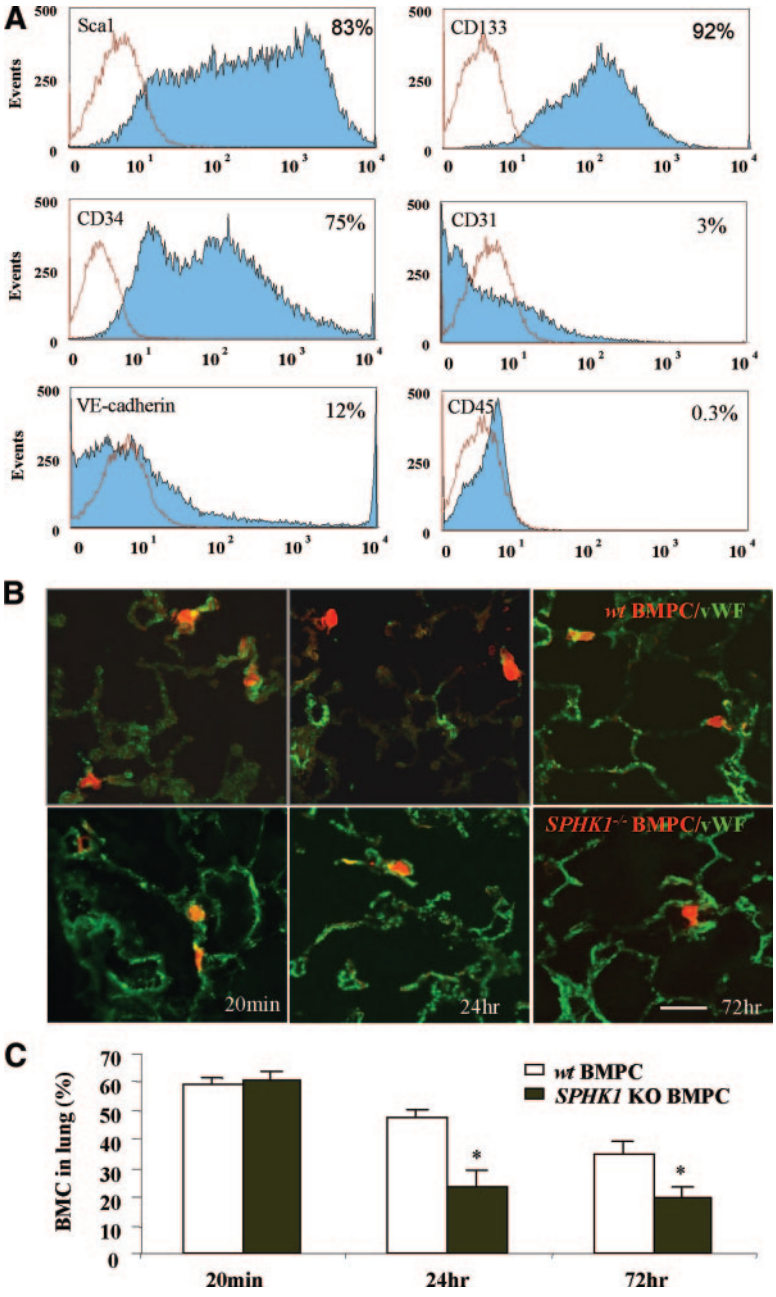

Figure 1. FACS analysis and lung uptake of mouse BMPCs. A, FACS analysis was performed using cultured BMPCs on day 21 for hematopoietic progenitor/stem cell markers (Sca-1, CD133, and CD34), endothelial cell markers (CD31 and VE-cadherin), and myeloid markers (CD45). Histogram plots for these markers are shown in blue and the control are shown in red. Results are representative of 3 experiments. B, Morphological assessment of BMPC sequestration in lungs. Rhodamine-labeled BMPCs were localized within lung microvessels as evident by BMPCs surrounded by lung vascular endothelial cells stained with FITC-conjugated von Willebrand Factor (vWF) at 20 minutes postinjection (left) and in lung parenchyma distinct from vessel lumen at 24 hours and 72 hours after injection (right, bar=30 μm). C, Quantification of total number of CMTMR-labeled *wt* BMPCs and *SPHK1*$^{-/-}$ BMPCs at 20 minutes, 24 hours, and 72 hours after cell injection.

rally in vessels (Figure 1B). At 3 days after injection, there was a further decrease in accumulation of both *wt* and *SPHK1*$^{-/-}$ BMPCs (34% and 20%, respectively, $P<0.05$ between 2 groups), with most cells localized in the parenchyma (Figure 1B).

BMPCs Prevent Increase in Lung Vascular Permeability

We quantified alterations in pulmonary microvascular permeability by determining the $K_{f,c}$ in mice receiving either BMPCs or mouse lung microvessel endothelial cells (ECs; in each case 3×10^5 cells were injected i.v.). The mice were challenged at day 3 after BMPC injection with a sublethal LPS dose (10 mg/kg i.p.). BMPC transplantation significantly prevented the increase in $K_{f,c}$ induced by LPS whereas injection of ECs afforded no barrier protection (Figure 2A). Lung extravascular water content increased in control mice (7.0 ± 1.8g/g) and EC-injected group (5.9 ± 1.1) in response to LPS, whereas it was normal in LPS-challenged mice transplanted with BMPCs (4.3 ± 1.0; Figure 2B).

We also determined the effects of BMPC transplantation on survival post-LPS insult (Figure 2C). Mice in this case were challenged with a lethal dose of LPS (22 mg/kg, i.p.) at day 3 after BMPC injection or either EC injection or PBS injection as controls. In PBS-injected mice, only 11% of mice survived 3 days post-LPS challenge. However, mice receiving 3×10^5 BMPCs i.v. exhibited 83% survival. In contrast, LPS-challenged mice receiving 3×10^5 ECs (Figure 2C) or 3×10^5 blood monocytes (not shown) showed no reductions in mortality.

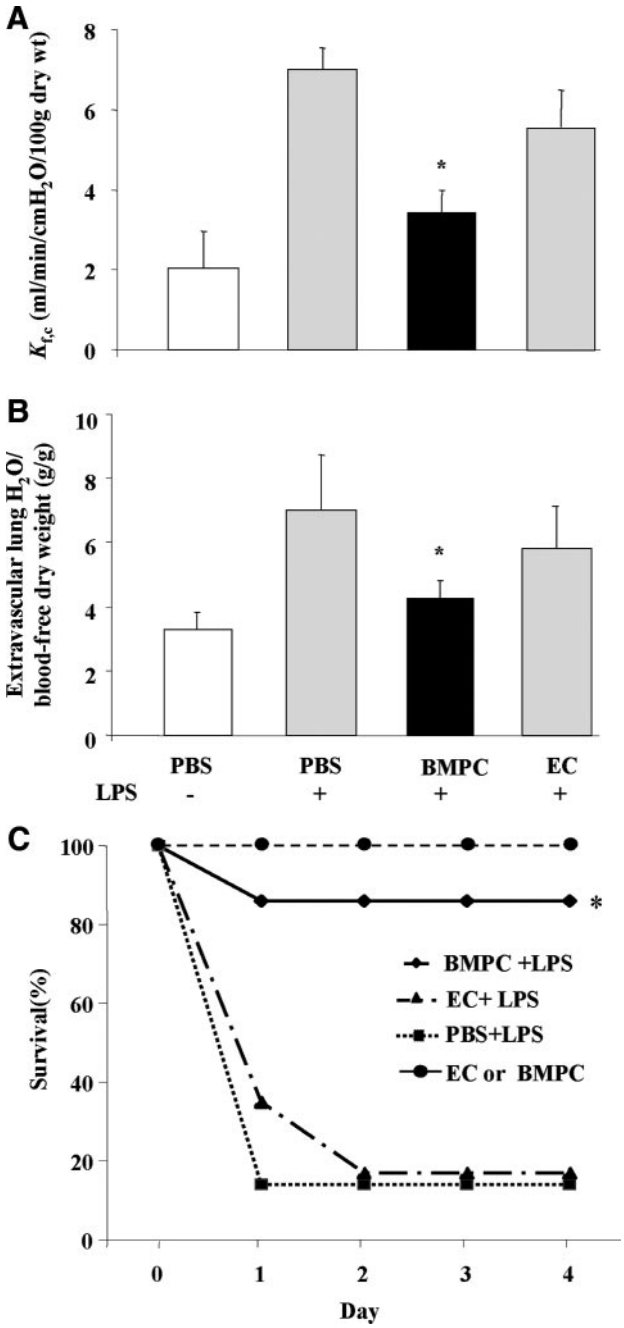

Figure 2. Transplantation of BMPCs in mice prevents increased lung vascular permeability and edema and improves survival after LPS challenge. A, $K_{f,c}$ was measured to determine changes in pulmonary microvascular permeability. In PBS-treated lungs, $K_{f,c}$ increased 3-fold compared to basal (PBS alone) at 12 hours after i.p. LPS injection (10 mg/kg BW). By contrast, transplantation of BMPCs (3×10^5 cells, i.v.) prevented the LPS-induced increase in $K_{f,c}$, whereas injection of same number of ECs provided no protection. Data are shown as mean±SEM (n=5 mice/group). *$P<0.05$ vs either LPS or EC+LPS. B, BMPC transplantation prevents LPS-induced pulmonary edema formation. Extravascular lung water content was determined 12 hours post-LPS challenge (10 mg/kg BW, i.p.). Data are shown as mean±SEM (n=8 mice per group). *$P<0.05$ vs LPS alone or EC+LPS. Extravascular lung water content was significantly reduced in BMPC-transplanted group compared to transplantation of ECs or injection of PBS. C, BMPCs transplantation promotes survival in mice challenged with LPS. At 3 days post-transplantation of either BMPCs or mouse lung microvascular

BMPCs Induce Rac1 and Cdc42 Activation in Endothelial Cells

To address mechanisms of BMPC-induced endothelial barrier protection, we first studied alterations in endothelial junctions that are crucial for maintenance of barrier integrity. Transendothelial electric resistance (TER) was measured in confluent endothelial cell monolayers to provide assessment of junction alterations.[19] BMPCs added to cultured EC monolayers ($\approx15\times10^3$ ECs in the confluent monolayer) in increasing numbers (from 0 to 5×10^3 cells) increased TER (Figure 3A). The response peaked at 30 minutes, then decreased but remained elevated above baseline during the experiment period (Figure 3A). In contrast, ECs added in same numbers had no effect on TER (Figure 3B). The maximum increase in TER induced by BMPCs was 4-fold greater than that by ECs (Figure 3C).

Because monomeric RhoGTPases Rac1 and Cdc42 regulate AJ assembly,[11,15] we next investigated their role in signaling junction barrier enhancement observed with BMPCs. BMPC addition to EC monolayers induced activation of Rac1 and Cdc42 in ECs within 30 minutes of BMPC addition, and activities of both GTPases remained elevated for the study duration (Figure 3D and 3E). Using siRNA to suppress the expression of Rac1 or Cdc42 in ECs (Figure 3F), we addressed the role Rac1 and Cdc42 in signaling the barrier enhancement. Reduction in the expression of either Rac1 or Cdc42 in ECs prevented the BMPC-induced increases in TER (Figure 3G through 3I), suggesting that BMPCs induce endothelial barrier enhancement by the activation of Rac1 and Cdc42. Specificity of siRNA-mediated silencing was verified by Western blot using Rac1 siRNA–treated cells. Rac1 siRNA reduced Rac1 activity without affecting Cdc42 (not shown).

As VE-cadherin localization at junctions is required for AJ barrier integrity,[15] we next determined the effects of BMPCs on VE-cadherin assembly. VE-cadherin immunostaining showed increased membrane localization of VE-cadherin in ECs in the presence of BMPCs (Online Figure IIA). However, knockdown of Rac1 or Cdc42 in ECs inhibited BMPC-induced AJ annealing (Online Figure IIA).

We quantified transendothelial ^{125}I-albumin permeability using Transwell microporous filters coated with a confluent endothelial monolayer to determine the functional effects of BMPCs on barrier function (Figure 4A). Permeability of ^{125}I-albumin increased after LPS exposure (Figure 4B), and addition of 5×10^4 BMPCs in the lower chamber not only prevented the increase in endothelial permeability but also BMPCs reduced basal endothelial permeability to a value below baseline. This experiment also demonstrated that endothelial barrier protection in-

Figure 2 (Continued). ECs (3×10^5 cells per each, i.v.), or PBS control, mice were challenged with LPS (22 mg/kg BW, i.p.). In PBS-treated mice, only 11% of mice survived 3 days post-LPS challenge. In comparison, BMPC transplantation significantly reduced LPS-induced mortality with 83% survival seen at 3 days post-LPS. Differences in survival between BMPC-treated group vs either EC-treat or PBS-treated group were significant (n=15 to 18 mice per group; *$P<0.001$ BMPC treated vs either EC or PBS).

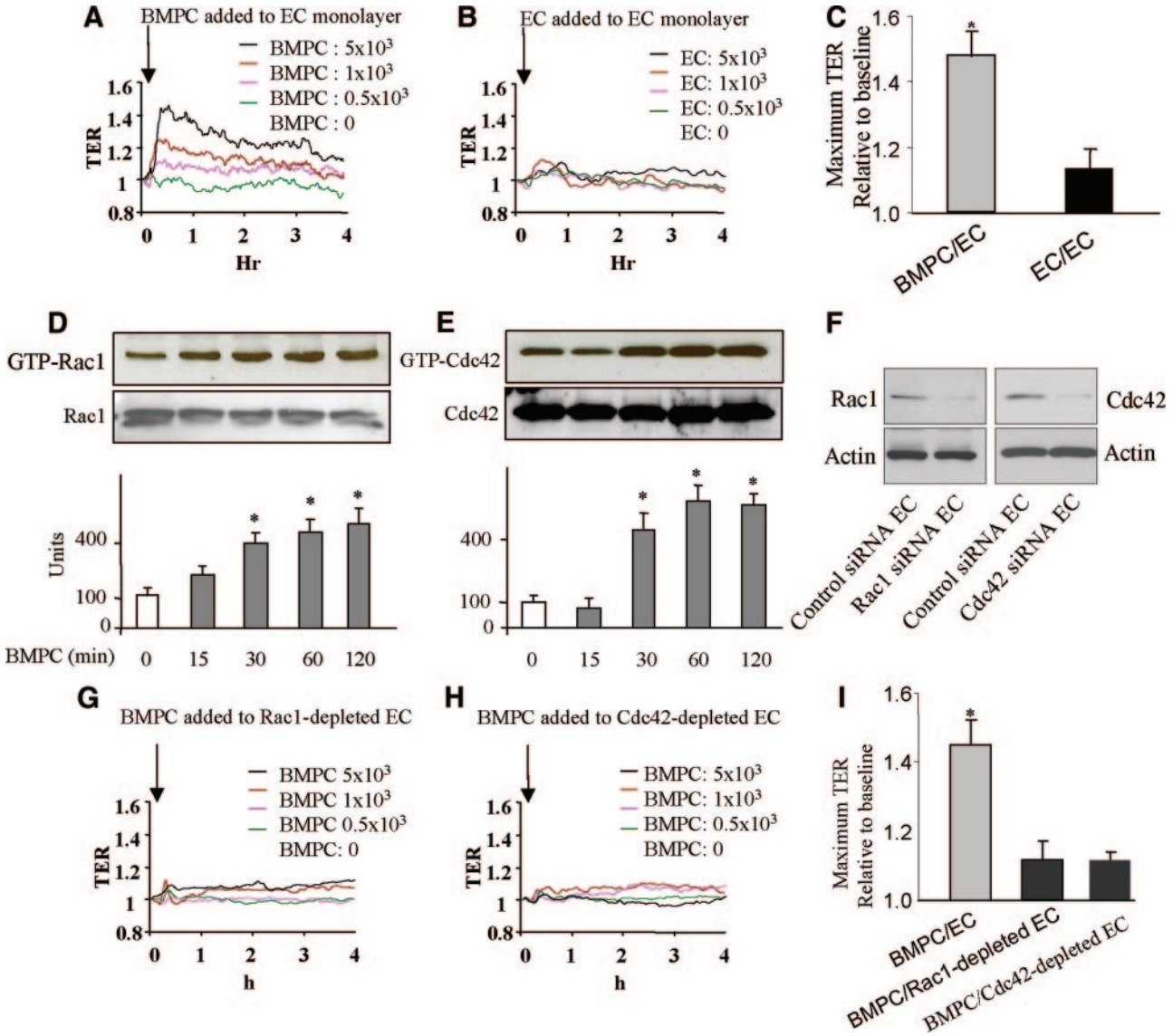

Figure 3. BMPC-mediated endothelial barrier protection requires activation of Rac1 and Cdc42 in endothelial cells. A through C, Real-time TER was measured after addition of increasing numbers of BMPCs (A) or ECs (B). BMPCs were added to EC monolayers ($\approx 15\times10^3$ ECs in the confluent monolayer) in increasing numbers (from 0 to 5×10^3 BMPCs). Data are representative of 3 experiments and show that BMPCs enhance endothelial junction barrier in a cell number-dependent manner (A). Addition of ECs had no significant effect on TER (B). Maximum TER values seen with addition of either BMPCs or ECs are shown as mean±SEM in (C) (n=3 per group). D and E, BMPCs differentially activate Rac1 and Cdc42 in mouse lung microvessel ECs. ECs were grown to confluence on gelatin-coated 100-mm dishes for 3 days and then coincubated with BMPCs for up to 120 minutes (ratio of ECs to BMPCs 3:1). Active GTP-bound Rac1 or Cdc42 in ECs was determined using anti-Rac1 or anti-Cdc42 antibodies. BMPCs addition activated Rac1 and Cdc42 in ECs within 30 minutes, which remained elevated after BMPC addition. Total amount of Rho GTPases did not change in any group. Data are representative of 3 experiments. Densitometric analysis is shown in bar graphs as mean±SEM (n=3). *$P<0.05$ vs basal (0 minutes). F through I, Depletion of Cdc42 and Rac1 in ECs by siRNA prevent BMPC-mediated stabilization of endothelial junction barrier. siRNA-mediated suppression of Rac1 and Cdc42 in ECs 96 hour posttransfection (F). ECs treated with scrambled siRNA did not reduce expression of Rac1 and Cdc42 (F). TER was measured in confluent Rac1-depleted (G) or Cdc42-depleted (H) ECs. BMPCs did not alter TER in either Rac1- or Cdc42-depleted ECs. Data are representative of 3 experiments. Maximum TER values after addition of BMPCs to either control ECs or ECs depleted of either Rac1 or Cdc42 (in each case 5×10^3 cells BMPCs were added) are shown as mean±SEM (n=3; I). *$P<0.001$ vs either Rac1- or Cdc42-depleted ECs.

duced by BMPCs did not require direct contact with the EC monolayer, indicating the involvement of a paracrine mechanism. Depletion of either Rac1 or Cdc42 in EC monolayers prevented the BMPC-induced reduction in transendothelial ^{125}I-albumin permeability that was seen in control cells (Figure 4B). Neither Rac1 nor Cdc42 silencing in ECs significantly altered the basal endothelial permeability values relative to that observed in control EC monolayers (Figure 4B).

As paracrine mediators of progenitor-like cells from a monocyte/myeloid lineage could also mediate endothelial repair,[24] we addressed the possibility that barrier protection induced by BMPCs was the result of a CD45$^+$ myeloid cell subpopulation. However, addition of an enriched population

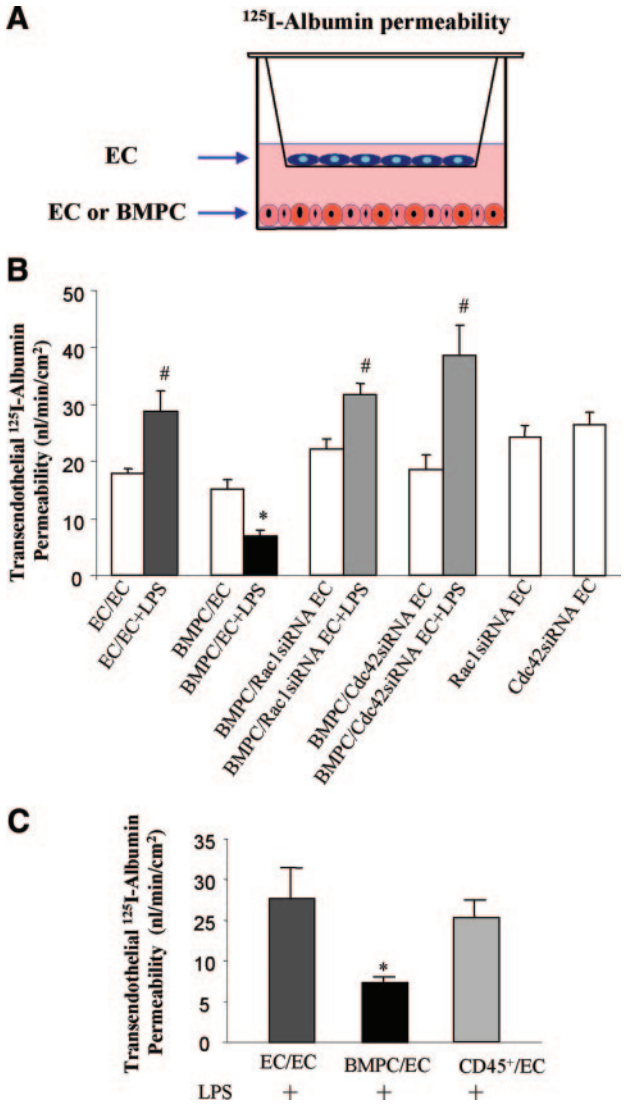

Figure 4. BMPCs prevent increase in transendothelial ^{125}I-albumin permeability by Rac1 and Cdc42 activation in endothelial cells. A, Schematic of Transwell chamber used to separate BMPCs from ECs and measure transendothelial ^{125}I-albumin permeability. ECs were grown to a confluent monolayer on top of the microporous filter for 48 hours and either ECs or BMPCs were grown at bottom of the lower chamber to 60% to 70% confluence for 48 hours. We used 50×10^3 of each cell type on the filter and the bottom chamber. In some experiments, endothelial monolayers were depleted of either Rac1 or Cdc42 using siRNAs. Endothelial monolayers were challenged with LPS (2 μg/mL) for 2 hours, and transendothelial ^{125}I-albumin permeability was measured. B, LPS addition shows an increase in transendothelial ^{125}I-albumin permeability whereas the increase is prevented by addition of BMPCs. Knockdown of Cdc42 or Rac1 in endothelial monolayer prevented the BMPC-mediated protective effect on transendothelial ^{125}I-albumin permeability. Values are mean±SEM (n=4 to 6 per group). *P<0.01 vs other 3 LPS-treated groups. #P<0.05 LPS challenged vs Basal without LPS challenge. C, BMPCs but not bone marrow–derived CD45$^+$ cells blocked LPS-induced increase in transendothelial ^{125}I-albumin permeability. Values are mean±SEM (n=4 to 6 per group). *P<0.01 vs either ECs or CD45$^+$ cells treated group.

of CD45$^+$ cells (50×10^3) did not increase endothelial barrier function (Figure 4C).

BMPC Expression of SPHK1 Is Required for Endothelial Barrier Annealing

We next addressed the possibility that endothelial barrier protection was the result of production of S1P by BMPCs because of the importance of S1P in regulating endothelial AJ barrier integrity.[11] We thus isolated BMPCs from sphingosine kinase-1–null mice (*SPHK1*$^{-/-}$) in which S1P production is severely impaired, and measured TER in the presence of *SPHK1*$^{-/-}$ BMPCs added in the lower chamber. Addition of *SPHK1*$^{-/-}$ BMPCs failed to increase TER in contrast to *wt* BMPCs (Figure 5A through 5C). The SPHK inhibitor, SKI-II, significantly decreased the BMPC-induced enhancement of endothelial junction barrier (Online Figure ID). We also determined $K_{f,c}$ in mice injected with either *wt* BMPCs or *SPHK1*$^{-/-}$ BMPCs. Administration of LPS to *SPHK1*$^{-/-}$ mice resulted in similar increase in $K_{f,c}$ as in *wt* mice challenged with LPS (Figure 5D). Transplantation of BMPCs from *SPHK1*$^{-/-}$ mice into either *wt* mice or *SPHK1*$^{-/-}$ mice failed to prevent the LPS-induced increase in lung vascular permeability. However, transplantation of *wt* BMPCs into *SPHK1*$^{-/-}$ mice prevented the increase in lung vascular permeability induced by LPS (Figure 5D). Pulmonary edema formation followed the same pattern as alterations in lung vascular permeability; pulmonary edema did not develop after transplantation of *wt* BMPCs into lungs of *SPHK1*$^{-/-}$ mice (Figure 5E).

Using the Transwell microporous filter described above, we next determined the activities of Rac1 and Cdc42 in ECs in the presence of *wt* or *SPHK1*$^{-/-}$ BMPCs cultured on the filter while *wt* ECs were cultured in the lower chamber. Activities of Rac1 and Cdc42 were markedly reduced in LPS-challenged ECs with the addition of either *SPHK1*$^{-/-}$ BMPCs or with conditioned medium from *SPHK1*$^{-/-}$ BMPCs compared to ECs with the addition of either *wt* BMPCs or medium from *wt* BMPCs (Figure 5F). We measured generation of S1P in the presence of LPS by either *wt* BMPCs or *SPHK1*$^{-/-}$ BMPCs cultured with ECs in the Transwell chamber (Figure 5G). S1P production increased with addition of *wt* BMPCs but not with *SPHK1*$^{-/-}$ BMPCs (Online Figure WS-IIB), indicating the paracrine role of BMPC-derived S1P in mediating endothelial barrier protection.

Discussion

Here we have addressed the role of BMPCs in regulating endothelial barrier function and defined the crucial underlying signaling mechanisms mediating this response. We demonstrate that transplantation of BMPCs prevents the increase in lung vascular permeability and edema formation and significantly reduces mortality in mice in response to LPS challenge, and does so primarily through a S1P-mediated paracrine mechanism. The studies were made using BMPCs expressing the progenitor/stem cell markers Sca-1 and CD133 as well as hematopoietic stem/progenitor cell marker CD34. These cells importantly did not express the panleukocytic marker CD45 or a battery of mature endothelial cell markers. The study was designed specifically to test the

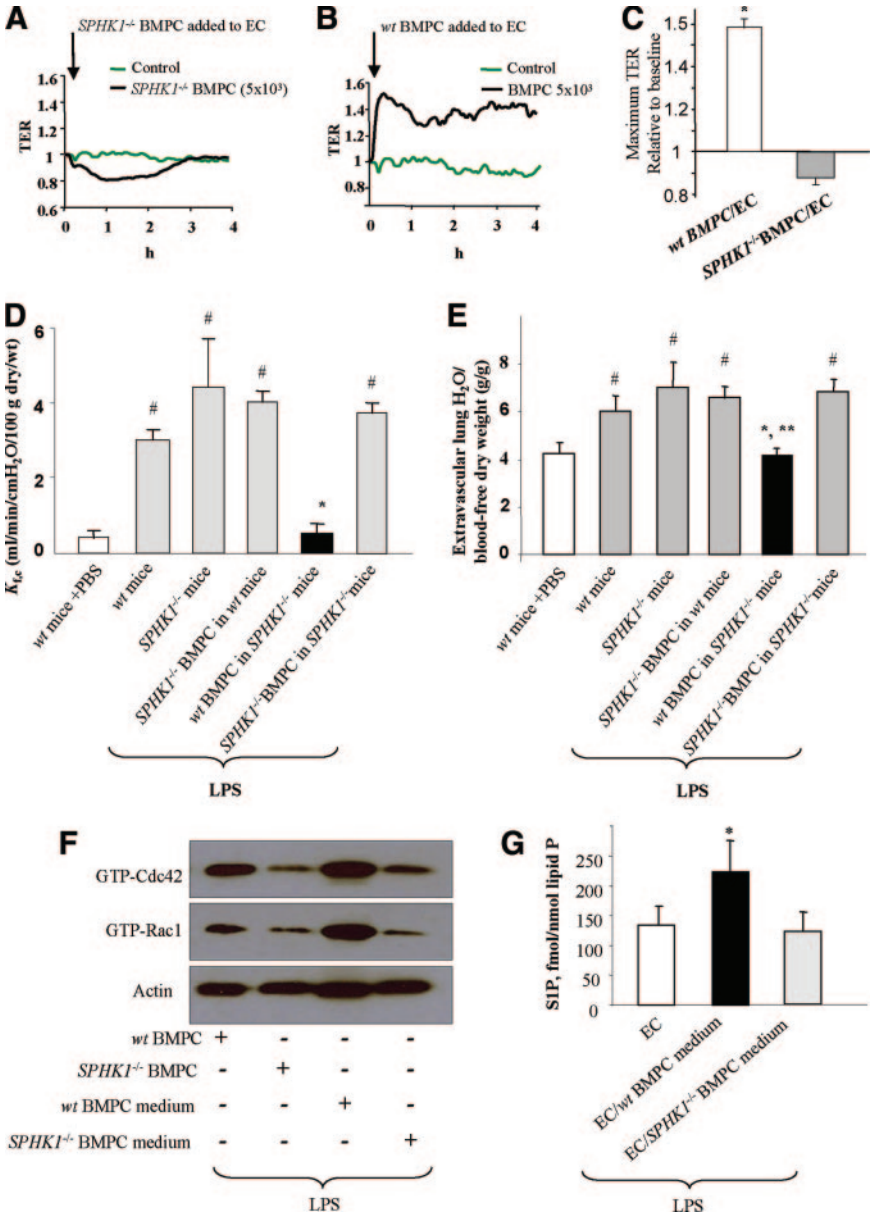

Figure 5. BMPC-generated S1P prevents increased lung vascular permeability and edema in mice after LPS challenge. A, SPHK1 in BMPCs is required for endothelial barrier protection. TER was measured in EC monolayers ($\approx 15\times10^3$ ECs) to which BMPCs (5×10^3 cells) from either *SPHK1*$^{-/-}$ (A) or *wt* (B) mice were added. C, Results are representative of 3 experiments. Bar graph indicates the maximum TER values (mean±SEM; n=3). *$P<0.001$ vs *SPHK1*$^{-/-}$ BMPC treated ECs. D, Pulmonary microvascular permeability was measured in *wt* and *SPHK1*$^{-/-}$ mice after BMPC transplantation by determining $K_{f,c}$ 12 hours post LPS challenege (10 mg/kg BW, i.p.). Values are mean±SEM (n=5 to 7 mice per group). *$P<0.001$ vs other LPS-treated groups. #$P<0.05$ vs *wt* mice+PBS. Transplantation of BMPCs from *wt* mice prevented the LPS-induced increase in $K_{f,c}$ in *SPHK1*$^{-/-}$ mice. In contrast, transplantation of *SPHK1*$^{-/-}$ BMPCs was not protective. E, Extravascular lung water content was assessed 12 hours post-LPS challenge (10 mg/kg BW, i.p.). Values are mean±SEM (n=5 to 12 mice per group). *$P>0.05$ vs *wt*+PBS; **$P<0.01$ vs other LPS treated groups. #$P<0.05$ vs *wt* mice+PBS. Wt BMPCs prevented lung edema formation whereas *SPHK1*$^{-/-}$ BMPCs had no effect. F, Interaction of *SPHK1*$^{-/-}$ BMPCs to ECs (BMPC:EC ratio of 1:3) or *SPHK1*$^{-/-}$ BMPC-conditioned medium reduced Cdc42 and Rac1 activation in ECs induced by LPS (2 μg/mL). Studies were made using the Transwell chamber to determine the effects of *SPHK1*$^{-/-}$ BMPCs or *SPHK1*$^{-/-}$ BMPC conditioned medium in modulating Rac1 and Cdc42 activities LPS-induced activation in ECs. Both *SPHK1*$^{-/-}$ BMPCs or *SPHK1*$^{-/-}$ BMPC-conditioned medium reduced Rac1 and Cdc42 activities in ECs compared to *wt* BMPCs or *wt* BMPC conditioned medium. Data are representative of 3 experiments. G, LPS induces generation of S1P by *wt* BMPCs but not *SPHK1*$^{-/-}$ BMPCs. S1P generation in the media was determined after LPS (2 μg/mL) stimulation of ECs alone and ECs treated with either *wt* BMPCs or *SPHK1*$^{-/-}$ BMPCs. Values are mean±SEM (n=3 per group). *$P<0.05$ vs either EC or EC/*SPHK1*$^{-/-}$ BMPC medium.

function of BMPCs in the mechanism of endothelial barrier repair. We carried out the studies in cells, which we defined a BMPCs, to avoid the implication that they were a uniform population. In a sense this was fortuitous because a mixed cell population of progenitor cell can act synergistically to induce a high degree of revascularization and tissue repair.[24]

Analysis of uptake of i.v.-injected ^{111}indium oxine–labeled BMPCs showed the sequestration of cells persisting up to 3 days after injection with relatively few cells localizing in other organs. This was also evident by immunostainng of BMPCs in lungs on day 3 after transplantation when BMPCs were localized in the lung parenchyma. The basis of preferential lung uptake is not clear. The uptake is likely the result of BMPCs being trapped in the first vascular bed encountered after their i.v. injection.[17]

Based on both cell culture and lung vascular permeability results, it is evident that BMPC transplantation marked endothelial barrier protection. However, mature mouse endothelial cells, CD45$^+$ leukocytes, or blood monocytes afforded no protection. We also observed that transplantation of BMPCs induced greater than 7-fold increase in survival compared to *wt* mice after a lethal dose of LPS challenge. Such a marked reduction in mortality may be the result of multiple factors besides the reannealing of endothelial junctions. It is possible that BMPCs also have a direct antiinflammatory effect that could improve survival.[2,25]

Addition of BMPCs induced the activation of Cdc42 and Rac1 in endothelial cells, whereas siRNA-mediated suppression of Cdc42 or Rac1 expression in endothelial cells abolished BMPC-mediated endothelial barrier protection. These

intriguing findings suggest a cross-talk between BMPCs and endothelial cells that resulted in the Rac1 and Cdc42 activation in endothelial cells. Both Cdc42 and Rac1 have been shown to induce the formation of membrane protrusions, resulting in the formation of the junction adhesion complexes that thereby enhances barrier integrity.[26] We determined alterations in VE-cadherin, the AJ protein regulating endothelial permeability,[27] to address whether BMPCs mediated their barrier protective effect by promoting junctional integrity. We observed that BMPCs induced Rac1- and Cdc42-dependent assembly of AJs in endothelial cells, indicating that BMPC-induced activation of Rac1 and Cdc42 was required for AJ assembly and thus the repair of endothelial barrier.

To identify mechanisms of BMPC-mediated endothelial barrier protection, we focused on the endothelial barrier annealing mediator S1P, which promotes endothelial junction integrity via Rac1 and Cdc42 signaling.[11] Our results here demonstrated that S1P generation by BMPCs and the activation of Rac1 and Cdc42 in endothelial cells plays a crucial role in restoring the integrity of the endothelial barrier. We observed that BMPCs not only released S1P to protect the endothelial barrier but also S1P generation exerted its maximal effects in the presence of LPS. This latter finding is consistent with studies showing that LPS induces the activation of SPHK1 in macrophages and hepatic cells, which can serve to dampen the hyper-immune response induced by Gram-negative bacteria.[28] Our studies made using BMPCs isolated from *SPHK1*$^{-/-}$ mice,[29] the key enzyme responsible for S1P production in endothelial cells,[9] showed that *SPHK1*$^{-/-}$ BMPCs failed to prevent the increase in lung vascular permeability induced by LPS whereas *wt* BMPCs were protective. In addition, an SK inhibitor SKI-II prevented BMPC-induced endothelial barrier protective effect, indicating a causal relationship between BMPC SPHK functional activity and endothelial barrier protection. Thus, these findings demonstrate the fundamental importance of the SPHK1-generated S1P in the mechanism of endothelial barrier protection. Although we have identified a novel mechanism, the present findings do not preclude the possibility that other mediators such as IL-10 released by BMPCs may also be involved in the endothelial barrier protection and dampening the inflammatory response in vivo.

Other factors should also be considered to explain the role of SPHK in the mechanism of endothelial barrier protection. We observed that some antigen markers in *SPHK1*$^{-/-}$ BMPCs were different from *wt* BMPCs and also that fewer *SPHK1*$^{-/-}$ BMPCs were sequestered in lungs after LPS challenge than in *wt* BMPCs. Although we cannot the rule out contribution of these factors in the reduced endothelial barrier protection in lungs seen with *SPHK1*$^{-/-}$ BMPCs, we observed in the cell culture experiments that when the same number of *wt* and *SPHK1*$^{-/-}$ BMPCs were added to endothelial monolayer only the *SPHK1*$^{-/-}$ BMPCs failed to prevent increased endothelial permeability. Thus, although SPHK1-generated S1P is an important paracrine mediator responsible for BMPC-induced endothelial barrier protection, other factors in the whole lung such as the reduced uptake of *SPHK1*$^{-/-}$ BMPCs could also explain our results.

In conclusion, BMPC sensing of LPS and the consequent activation of SPHK1 and generation of S1P is a critical factor preventing increased vascular permeability. The S1P release function of BMPCs stabilizes endothelial junction barrier by a paracrine mechanism that activates Cdc42 and Rac1 signaling in endothelial cells. The present results provide a rationale for the therapeutic use of BMPCs in inflammatory diseases such as acute lung injury.

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Disclosures

None.

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