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 Rac1 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. Recent studies showed that transplantation of bone marrow–derived mesenchymal cells can also limit lung inflammation in experimental models. 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. Sphingosine kinases phosphorylate sphingosine leading to formation of S1P. Platelets lack the S1P degradation enzyme sphingosine-1-lyase and thus release S1P in the circulation. In addition, RBCs and endothelial cells produce abundant amounts of S1P. The endothelial barrier promoting effect of S1P is concentration-dependent and characterized by a rapid increase in transendothelial electric resistance. S1P signaling on the basis of its effects on immune and endothelial cells is also a modulator of immune and inflammatory responses. In the murine model of LPS-mediated acute lung injury, S1P was shown to attenuate pulmonary vascular leakage and inflammation. The S1P effects depend on ligation of Gi-coupled Edg-1 and Edg-3 receptors. 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. 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.
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 fluorescent chloromethyl tetramethyl rhodamine (CMTRM, Molecular Probes) were injected into mice (3×10⁶ 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 3×10⁵ BMPCs with ¹¹¹Indium oxine20 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 anti-murine CD31 (Becton-Dickinson); allophycocyanin-labeled antimurine c-Kit and CD34 antibodies (BD Pharmingen); mouse anti-human 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 3×10⁵ 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 (Kₑₑₚ) 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 GSTHotekin-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 lyso-
rally in vessels (Figure 1B). At 3 days after injection, there was a further decrease in accumulation of both wt and SPHK\textsuperscript{1\textminus/\textminus} BMPCs (34\% and 20\%, respectively, \(P<0.05\) between 2 groups), with most cells localized in the parenchyma (Figure 1B).

**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 \(\mu\)m). C, Quantification of total number of CMTMR-labeled wt BMPCs and SPHK\textsuperscript{1\textminus/\textminus} BMPCs at 20 minutes, 24 hours, and 72 hours after cell injection.

**BMPCs Prevent Increase in Lung Vascular Permeability**

We quantified alterations in pulmonary microvascular permeability by determining the \(K_{c,c}\) in mice receiving either BMPCs or mouse lung microvessel endothelial cells (ECs; in each case \(3\times10^5\) 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 \(K_{c,c}\) 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 \(3\times10^5\) BMPCs i.v. exhibited 83\% survival. In contrast, LPS-challenged mice receiving \(3\times10^5\) ECs (Figure 2C) or \(3\times10^5\) blood monocytes (not shown) showed no reductions in mortality.
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. Transeendothelial electric resistance (TER) was measured in confluent endothelial cell monolayers to provide assessment of junction alterations. BMPCs added to cultured EC monolayers (≈15 × 10^5 ECs in the confluent monolayer) in increasing numbers (from 0 to 5 × 10^5 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, 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, 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 125I-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 125I-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...
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, 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...
of CD45$^+$ cells (50×10$^5$) 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 $K_{tc}$ in mice injected with either wt BMPCs or SPHK1$^{-/-}$ BMPCs. Administration of LPS to SPHK1$^{-/-}$ mice resulted in similar increase in $K_{tc}$ 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 pan-leukocytic marker CD45 or a battery of mature endothelial cell markers. The study was designed specifically to test the...
function of BMPCs in the mechanism of endothelial barrier repair. We carried out the studies in cells, which we defined as 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 111indium 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 immunostaining 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

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 (~1x10^4 ECs) to which BMPCs (5x10^3 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 per group). *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, 12 hours post LPS challenge (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, 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
intrinsic 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 SK-I1 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.

References


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Online Supplement Material and Methods

SPHK1<sup>−/−</sup> mice in C57/B6 background: SPHK1<sup>−/−</sup> mice (1) were mated into the background of wt C57/B6129 mice to generate animals with same genetic background. SPHK1<sup>−/−</sup> mice were born normally and were otherwise indistinguishable from their wildtype (WT) littermates. We observed that 100% (n=200) of SPHK1<sup>−/−</sup> mice survived up to 18 months similar to wt mice. To eliminate background effects from SPHK1<sup>−/−</sup> line on the observed phenotype, F8 or greater generations were used.

Isolation of mouse BMPCs: Femoral and tibial bones were stripped of muscle and connective tissue and cut at both ends, and the bone marrow was flushed out with HBSS using a 25ga syringe. To harvest maximal amount of the bone marrow cells, the femora and tibias were cut into small pieces, including epiphyseal line and endosteum and incubated with 10 mL of collagenase A solution (1.0 mg/mL in HBSS) in 50 ml tube for 3-4 min at 37°C with gentle shaking. The digested mixture of small pieces of bone together with the initial bone marrow HBSS flush were then filtered using a 40μm nylon filter. Mononuclear cells were isolated by density gradient (Ficoll-Paque, Amersham) following centrifugation at 1600rpm for 30 min. The cells were re-suspended in EBM-2MV endothelial culture media using the supplement kit except for the FGF substrate (EBM-2, Clonetics, LONZA) made 10% FBS, 50 U/ml penicillin and streptomycin, 2 mMol/l L-glutamine (Invitrogen), and additional VEGF (5ng/ml). The cells were then plated in fibronectin-collagen-gelatin (ratio 1:1:1)-coated tissue culture flasks and incubated for 48 hr at 37°C made 5% CO<sub>2</sub> when the non-adherent cell population, 90-95% of the initial culture, was washed away. The cells were then cultured for 21 d.

Quantification of CMTMR-labeled BMPCs in lungs: The quantification of the total number of CMTMR-labeled wt BMPCs and SPHK1<sup>−/−</sup> BMPCs in lungs was carried out as described (2). Briefly,
the mice were injected with 3 x 10^5 CMTMR-labeled cells and sacrificed at 3 time points after injection. Lung tissue specimens were embedded in OCT compound (Sakura Finetek, Torrance, CA) and then flash-frozen in liquid nitrogen. Ten-micron sections were cut from the frozen blocks. The number of fluorescent cells was counted in sections taken from basal, medial, and apical segments of the left lung and average was calculated. The total number of labeled cells present within the lung was calculated based on the equation of Simpson's rule for the volume of a truncated cone: $\text{volume} = \frac{[\text{area basal section} + \text{area middle section}] \times \text{height of the lung}}{3} + \frac{[\text{area apical section}] \times \text{height of lung}}{2} + \frac{\lambda}{6} \times \left( \frac{\text{height of lung}}{3} \right)^3$ (2).

**CD45^+ cells:** Mouse bone marrow-derived CD45^+ cells were cultured as described (24, 25). Briefly, bone marrow obtained from femurs and tibias of C57/B6 mice was used to isolate mononuclear cells by density gradient centrifugation (Ficoll-Paque, Amersham) (1600 rpm for 30 min) and the population was enriched for CD45^+ cells by incubation with rat anti-mouse CD45 antibody (BD Pharmingen). The resulting cell population was shaken gently at 4°C for 30 min, washed three times with HBSS and centrifuged at 1000 rpm for 10 min at 4°C. Dynabeads M-450 (labeled with sheep anti-rat IgG; Dynals, Oslo, Norway) (100 µl) were added to cells and incubated at 4°C for 30 min. The cells attached to Dynabeads through the anti-rat IgG were passed over a magnetic column to remove unbound cells. The cells were next suspended in trypsin-EDTA to release the bound cells from beads. The trypsin-released cells were centrifuged, suspend with EBM-2MV, and cultured for 21-30 days. This population was assessed for presence of CD45^+ cells using FITC-labeled anti-CD45 IgG by FACS analysis.

**RT-PCR:** RNA was isolated using an RNeasy Mini kit including DNase I digestion (Qiagen). Two-step QRT-PCR analysis was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with TaqMan® Fast Universal PCR Master Mix, which contains a hot-start enzyme
system specific for fast quantitative PCR. CD45 Probes was used for quantitative detection of gene expression and normalized to cyclophilin as an internal control using the mouse cyclophilin primer/probe set (3).

**Pulmonary microvascular permeability:** Pulmonary microvessel filtration coefficient \(K_{f,c}\) was measured to determine microvascular permeability as described (4).

**Western blotting:** Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.25% sodium deoxycholate; 1.0% Nonidet P-40; 0.1% SDS; 1 mM Na\(_3\)VO\(_4\); 1 mM NaF; 44 µg/ml phenylmethylsulfonyl fluoride; and a protease inhibitor mixture). Equal amounts of lysate were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked, and incubated with an Ab specific to RhoA, Rac1, or Cdc42. After incubation of the membrane with the appropriate secondary antibody, protein bands were detected using an ECL reagent (Pierce). The relative intensity of bands was measured using Scion Image (Scion Image, NIH).

**Transendothelial \(^{125}\text{I}-\text{albumin permeability:}** Endothelial cells were grown on the upper side of a Transwell microporous filter (12 mm diameter, 1 cm\(^2\) growth area, 0.4 µm pore diameter; Becton Dickinson). Either BMPCs or endothelial cells were grown at the bottom of Transwell chamber to 60-70% confluence for 72 hr at 37°C. Endothelial cells on the upper side of the filter were challenged with 10µg of LPS for 2 hr in EBM-2MV, washed in PBS, and 1 ml of incubate containing \(^{125}\text{I}-\text{albumin tracer was added to measure transendothelial}^{125}\text{I}-\text{albumin permeability}(5). Aliquots of 50 µl were sampled from lower chamber at 20 min, gamma radioactivity was measured, and transendothelial \(^{125}\text{I}-\text{albumin permeability was calculated (6, 7).}

**S1P production:** S1P generation was measured as described (8). Mouse lung endothelial cells were co-cultured with either \(wt\) BMPCs or \(SPHK1^{-/-}\) BMPCs in the Transwell microporous filter co-culture system (BMPCs on top of the filter and endothelial cells in lower chamber). After 2 days, cells were
treated with LPS (2μg/ml) for 8 hr and media were collected for determination of S1P production by high performance liquid chromatography. We also determined time course of S1P generation (using ELISA kit, Echelon) in albumin-enriched (0.2% BSA) media of wt and SPHK1-/ BMPCs treated with LPS.

Transendothelial electrical resistance (TER): Endothelial junctional changes were determined using ECIS (9). TER was measured using mouse pulmonary microvessel endothelial cell monolayer to which were added BMPCs or endothelial cells. In a separated experiment, BMPC cells were incubated with vehicle alone (DMSO) or 3μM SK inhibitor, SKI-II (Sigma) for 1h before added into EC culture for TER measurement.

Online Figure Legends

Online Figure 1: Analysis of BMPCs from SPHK1/- mice. A) Comparison of FACS analysis of mouse wt and SPHK1/- BMPCs. FACS analysis was made using cultured wt and SPHK1/- BMPCs on Day#21 for the hematopoietic progenitor/stem cell markers (Sca-1, CD133, and CD34). Results are representative for 3 experiments. *, p < 0.05 versus either wt BMPCs or SPHK1/- BMPCs. B) Lung tissue of BMPCs after i.v. injection of 111Indium oxine-labeled BMPCs (3x10^5) was determined in lungs and other organs. We observed that 80% of the injected BMPCs were localized in lungs at 3 days after injection. Data are shown as mean ± SEM; n= 3. C) CD45 expression in BMPCs. Quantification of CD45 mRNA expression in BMPCs and CD45+ cells by real-time RT-PCR analysis. D BMPC-mediated endothelial barrier protection is blocked by SKI-II as SPHK inhibitor. BMPC cells were incubated with vehicle alone (DMSO) or 3μM SK inhibitor, SKI-II (Sigma) for 1h before added into EC culture for TER measurement. BMPC-mediated endothelial barrier protection
of EC was markedly attenuated by SKI-II. Data show that SKI-II significantly reduced BMPC-induced enhancement of endothelial junction barrier. (Online-Figure 1D)

**Online Figure 2:** A) **BMPCs induce AJ assembly dependent on Rac1 and Cdc42 activation.** ECs were grown to confluence on gelatin-coated Lab-Tek II Chamber Slide for 3 d and incubated with rhodamine-labeled BMPCs at a fixed ratio (1 BMPC: 3 EC) for 18 hr and stained for VE-cadherin (green). Interaction of BMPCs with ECs for 60 min increased VE-cadherin staining in control ECs but not in ECs depleted of either Rac1 (ECs siRNA-Rac1) or Cdc42 (ECs siRNA-Cdc42). Data are representative of 3 experiments. Red=rhodamine-labelled BMPCs. Bars=10μm.

B) **Time course of LPS-induced generation of S1P in wt BMPCs and SPHK1−/− BMPCs.** S1P generation in media was determined after LPS (2 μg/ml) stimulation of either wt BMPCs or SPHK1−/− BMPCs. S1P was generated only in wtBMPCs. Values are mean ± SEM (n=3 per group). **, p<0.05 wt BMPCs versus SPHK1−/− BMPCs medium at the same time point.

**References:**


R1

Online Figure 1
Online Figure 2