Online Supplement Material and Methods

$SPHK1^{-/-}$ mice in C57/B6 background: $SPHK1^{-/-}$ mice (1) were mated into the background of *wt* C57/B6129 mice to generate animals with same genetic background. $SPHK1^{-/-}$ mice were born normally and were otherwise indistinguishable from their wildtype (WT) littermates. We observed that 100% (n=200) of $SPHK1^{-/-}$ mice survived up to 18 months similar to *wt* mice. To eliminate background effects from $SPHK1^{-/-}$ 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_2 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 $SPHK1^{-/-}$ BMPCs in lungs was carried out as described (2). Briefly,

the mice were injected with 3 x 105 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 by the equation of Simpson's rule for the volume of a truncated cone: volume= ([area basal section + area middle section] x height of the lung)/ 3+ (area apical section/ 2 x height of lung / 3) + λ/6x [height of the lung/ 3]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) (1600rpm for 30 min) and population was enriched for $CD45^+$ cells by incubation with rat anti-mouse CD45 antibody (BD Pharmingen). The resulting cell population was shaken gently at 4oC for 30 min, washed three times with HBSS and centrifuged at 1000rpm for 10 min at 4oC. Dynabeads M-450 (labeled with sheep anti-rat IgG; Dynals, Oslo, Norway) (100 µl) were added to cells and incubated at 4oC 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_3VO_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}I-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}I-albumin tracer was added to measure transendothelial ^{125}I-albumin permeability(5). Aliquots of 50 μl were sampled from lower chamber at 20 min, gamma radioactivity was measured, and transendothelial ^{125}I-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 ^{111}Indium 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. SIP was generated only in *wt*BMPCs. Values are mean ± SEM (n=3 per group). **, $p<$ 0.05 *wt* BMPCs versus *SPHK1*$^{-/-}$ BMPCs medium at the same time point.

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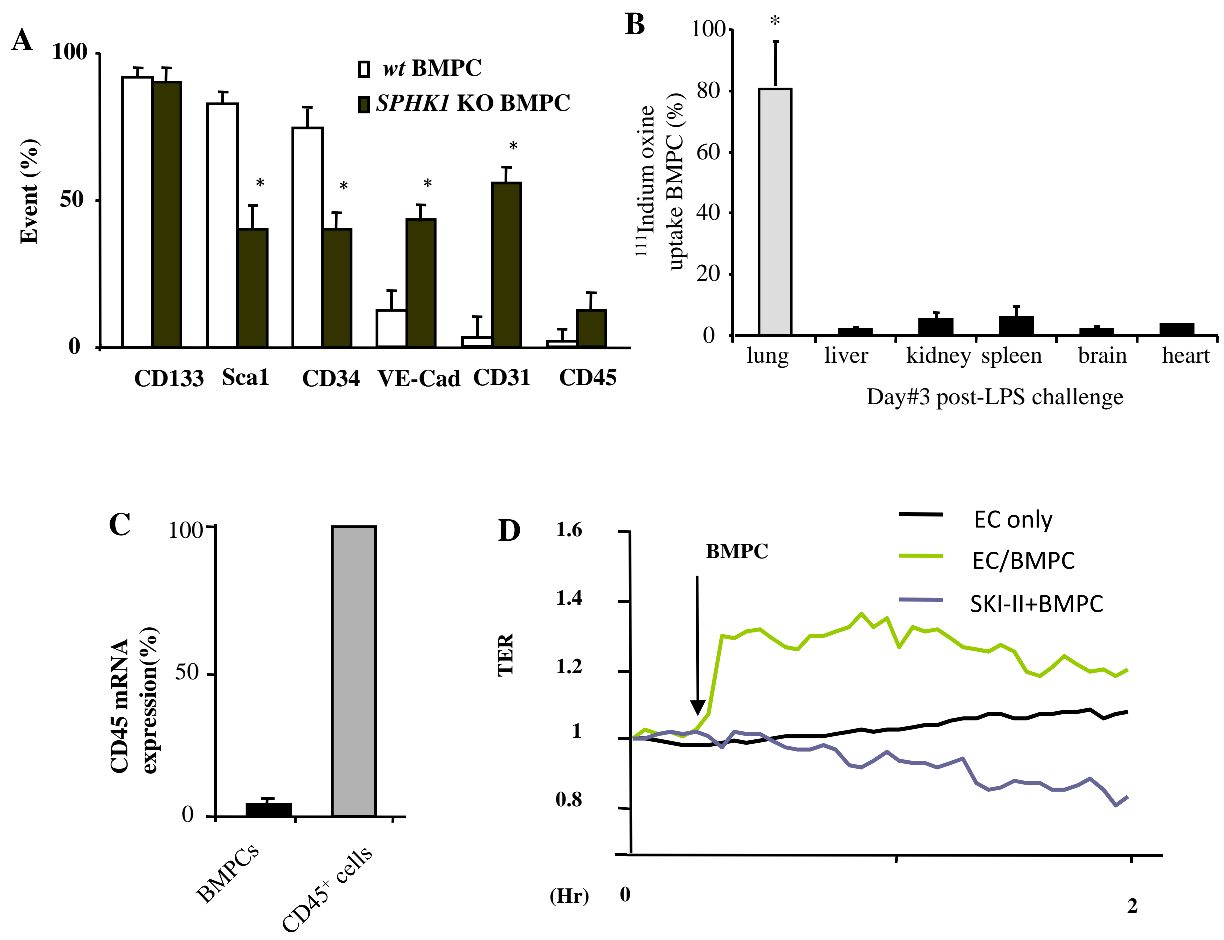
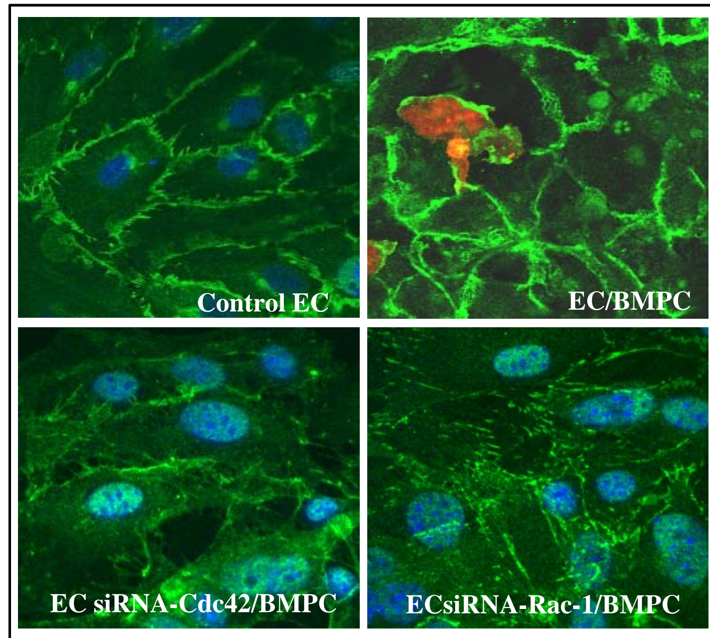


Online Figure 1

A



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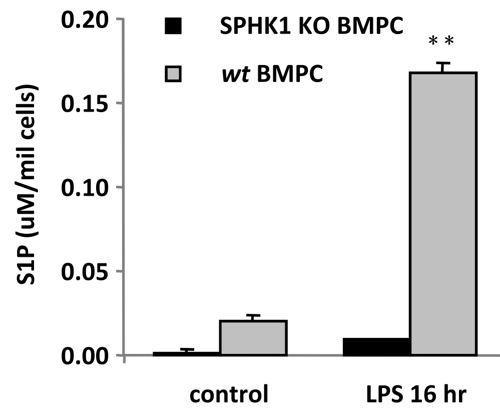

Online Figure 2