SUPPLEMENTAL DATA

Cytokines trigger disruption of endothelium barrier function and p38 MAP kinase activation in *BMPR2*-silenced human lung microvascular endothelial cells

MATERIALS AND METHODS

Materials

Cell culture medium RPMI, fetal bovine serum (FBS), penicillin, streptomycin, fungizone, dispase, phosphate buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI), TRIzol, SuperScript™ III First-Strand Synthesis System, primers and RT2 SYBR Green/ROX qPCR Master Mix were purchased from Life Technologies. TNFα was purchased from R&D Systems. IL18 was from Medical and Biological Laboratories. Gelatin, bovine serum albumin (BSA) and fluorescein isothiocyanate (FITC)-labelled albumin were purchased from Sigma-Aldrich. [3H]-thymidine (specific activity: 74 GBq.mmol-1) was from Perkin Elmer. RNeasy Mini Kit and RNase-Free DNase set were purchased from Qiagen. Anti-human CD31 antibody coupled to allophycocyanin fluorochrome (CD31-APC) was from Miltenyi Biotec. Lab-Tek chamber slides from Nunc. THP-1 cells were a gift from the Lung Toxicology Research Group (KU Leuven). Monoclonal antibodies raised in mouse against human CD31 (clone JC70A, #M0823) and human von Willebrand factor (vWF; clone F8/86, #M0616) were purchased from Dako. Monoclonal antibodies raised in mouse against human ICAM-1 (#BBA3) and VCAM-1 (#BBA5) were from R&D Systems. Rabbit anti-phospho-p38 MAPK (clone D3F9, #4511), anti-p38 MAPK (#9212), anti-phospho SMAD1/5/8 (#13820), anti-SMAD1/5/8 (#6944) and VE-cadherin (clone D87F2, #2500) monoclonal antibodies were from Cell Signaling. Polyclonal antibodies raised in rabbit against peptide comprising amino-acids 950 to C-terminus of human BMPRII (ab115239) and mouse monoclonal antibody against human β-actin (clone AC-15, #ab6276) were from Abcam. Secondary antibodies conjugated with horseradish peroxidase were from Jackson Immuno Research and secondary antibodies Alexa Fluor™ 488 goat anti-mouse and anti-rabbit were from ThermoFisher. Acetylated low-density lipoprotein (LDL) coupled to a fluorescent carbocyanine dye, 1,1\'-dioctadecyl – 3,3,3\',3\'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was purchased from Tebu-Bio.

Human lung microvascular endothelial cells (HLMVECs) were purchased from Cell Applications. HLMVECs were cultured in microvascular endothelial cell growth medium (Cell Applications) containing 6% growth supplement, 100 U/mL penicillin, 100 µg/mL streptomycin and 1.25 µg/mL fungizone and starved in microvascular endothelial cell basal medium (Cell Applications) supplemented with 0.2% growth supplement.

BMPR2, IL18 and IL18R mRNA expression

Primers used were as follows: BMPR2, forward 5'-TGCAGGTTCTCGTGTCTAGG-3' and reverse 5'-GGTCCCAACAGTCTTCGATT-3'; IL18, forward 5'-GGGAAGAGGAAAGGAACCTC-3' and reverse 5'-CCATCTTTATTCCTGCGACA-3'; IL18R, forward 5'-TGGTCAACAGCACATCATTG-3' and reverse 5'-ACCCCTGATCTTCAAACTCG-3'; β-actin, forward 5'-GGACATCCGCAAAGACCTGT-3' and reverse 5'-CTCAGGAGGAGCAATGATCTTGAT-3'.

Western blotting

HLMVECs were grown to 90% confluence in 6 well-plates and starved in starving medium for 24h and further stimulated with 10 µg/mL TNFα or IL18 for 1 h at 37°C. HLMVECs were washed twice in ice-cold phosphate-buffered saline, pH 7.4 and lysed for 30 min at 4°C in ice-cold RIPA buffer (50 mM Tris [pH 7.4], 150 mM sodium chloride, 1% [vol/vol] NP 40, 0.5% [wt/vol] sodium deoxycholate, 1 mM ethyleneglycoltetraacetic acid [EGTA], 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin [10 µg/mL] and antipain [10 µg/mL]. Samples were centrifuged at 12,000 × g for 15 min and protein concentrations were determined by the Bradford method. Proteins were further separated on a 10% acrylamide gel by SDS-PAGE and transferred to polyvinylidenefluoride filters by electroblotting for 3 h in a transfer buffer containing 25mM Tris, pH 8.1-8.5, 192 mM glycine and 20% methanol. Filters were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) and 3% BSA (β-actin antibody) or 5% nonfat dry milk (phospho-SMAD1/5/8, phospho-p38 MAPK, total SMAD1/5/8, total p38 MAPK and BMPRII antibodies) for 1h at RT. Filters were incubated with primary antibodies overnight at 4°C in TBS-Tween and 3% BSA (anti-β-actin) or 5% nonfat dry milk (anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho SMAD1/5/8, anti-SMAD1/5/8, BMPRII). The following horseradish peroxidase-conjugated secondary antibodies: donkey anti-rabbit immunoglobulin (Ig)G (anti-BMPRII, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho SMAD1/5/8, anti-SMAD1/5/8) and donkey-anti-mouse (Ig)G (anti-β-actin) antibodies were incubated in TBS-Tween for 1h at room temperature. Peroxidase staining was revealed by chemiluminescence and imaged with the Proxima 2850T imaging system (Isogen life technologies, NL) and analyzed with Totallab 1D (Isogen life technologies, NL).

FIGURES

Figure S1

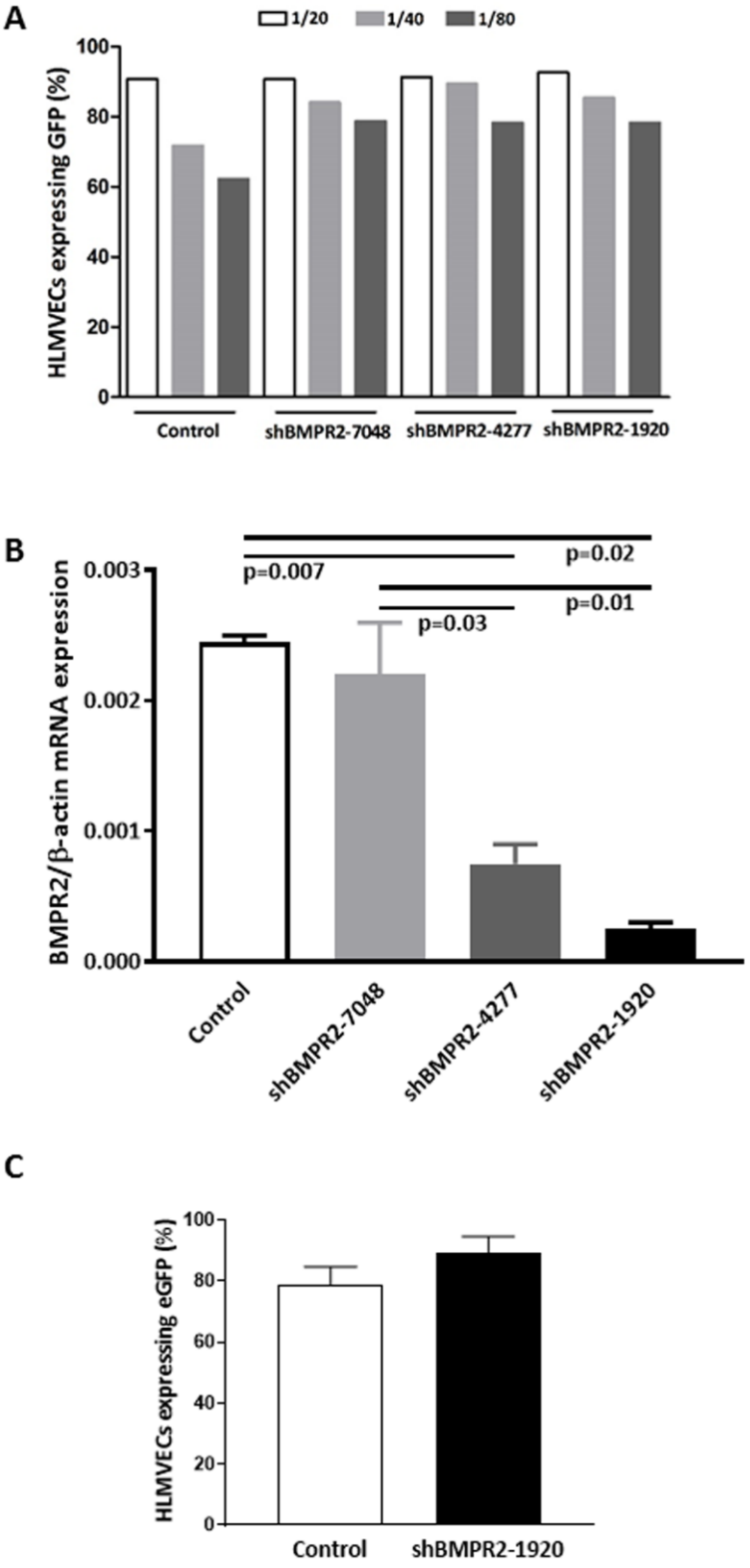

Figure S1. Lentiviral vector transduction efficiency in HLMVECs. Percentage of HLMVECs expressing eGFP **(A)** and *BMPR2* mRNA expression in HLMVECs **(B)** transduced with control and shBMPR2-7048, 4277 and

1920 lentiviral vectors. ANOVA, p<0.005. **(C)** Efficiency of transduction of HLMVECs with control and LV_miR_BMPR2_1920 lentiviral vectors was assessed by flow cytometry. Experiments were performed in triplicate in HLMVECs at passage 4. Panel **(A)** shows the results of one independent experiment.

Figure S2

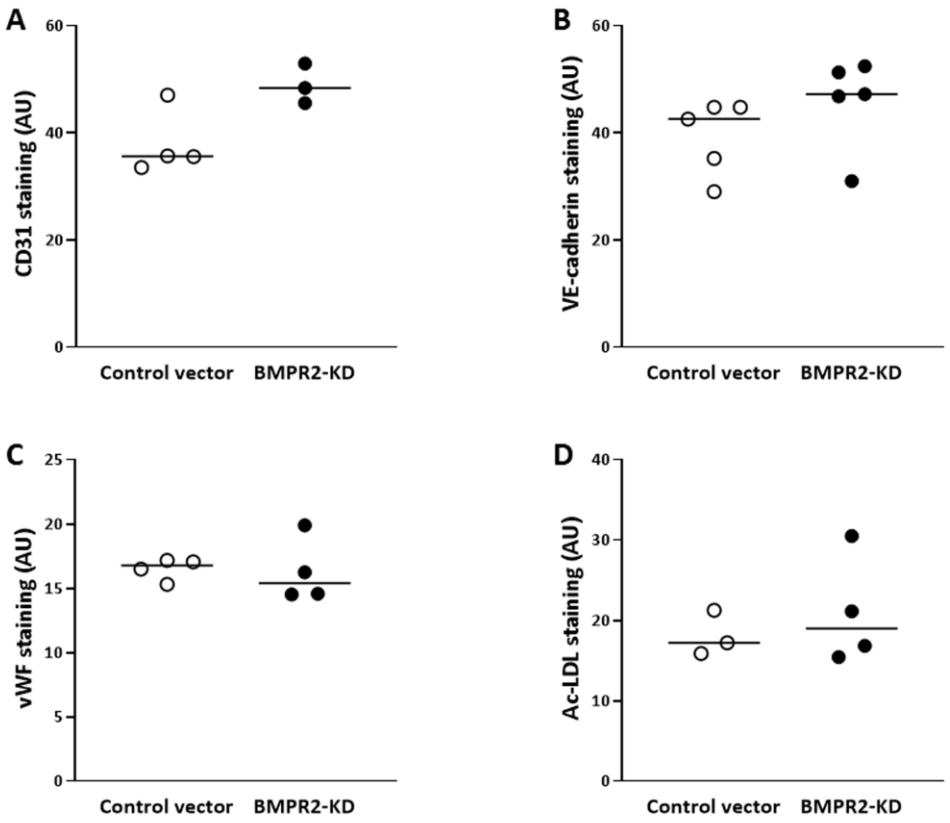

Figure S2. Quantification of the expression of specific endothelial markers in HLMVECs. Expression of CD31 **(A)**, VE-cadherin **(B)**, vWF **(C)** and Ac-LDL uptake **(D)** were quantified both in control and BMPR2-KD HLMVECs.