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Supplementary Information for

Cysteinyl leukotriene 2 receptor promotes endothelial permeability, tumor angiogenesis and metastasis

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SI Materials and Methods

Materials: Corning™ Matrigel growth factor-reduced (phenol free) and basic fibroblast growth factor were purchased from Corning (Bedford, MA), murine recombinant vascular endothelial growth factor (VEGF) was purchased from Peprotech (Rocky Hill, NJ) and human recombinant vascular endothelial growth factor (VEGF) was purchased from R&D Systems (Minneapolis, MN). LTD_4, MK571 and $BayCysLT_2$ were purchased from Cayman chemicals (Ann Arbor, MI). Mouse CD31 antibody was purchased from Life technologies (Grand Island, NY); CD45 antibody was purchased from (Cell Signaling Technology, Danvers, MA); NG2 antibody was purchased from Millipore (Temecula, CA). Collagenase I and IV was from Worthington Biochemical Corporation (Lakewood, NJ). Phospho-specific antibodies (phospho-ERK (1/2) and phospho-MLC-2) were purchased from Cell Signaling Technology (Danvers, MA). VE-Cadherin, $CysLT_1$ Receptor, and $CysLT_2$ Receptor antibodies were purchased from Santacruz Biotechnology (Dallas, TX). GAPDH antibody was purchased from Fitzgerald (Acton, MA). All HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Alexa Fluor 488- or 594-conjugated secondary antibodies, Calcein-AM, Fura-2 AM were obtained from Molecular Probes (Eugene, Ore).

Cell culture: Human Dermal Micro Vascular Endothelial Cells (HDMEC) (1) were maintained in EBM2 medium with EGM2 SingleQuots™ Kit (Lonza) and 10% fetal bovine serum (FBS). Mouse dermal endothelial cells (MDEC) (2, 3) and Mouse lung endothelial cells (MLEC) were cultured in DMEM (Low glucose) supplemented with 10% Nu-serum (Corning), 10% FBS (Atlanta Biologicals), 0.2% ITS (insulin, transferrin, selenium) 500X (BioWhittaker), 3 µg/L murine FGF (Corning), 80 µg/L murine VEGF (Peprotech), 1% glutamine 100X (Millipore), 100 mg/L heparin (Sigma Aldrich) and 1% streptomycin/penicillin 100X (GE-Healthcare). CHO-$CysLT_1R$ cells were cultured in DMEM supplemented with 10% FBS and Geneticin (40 µg/mL) while CHO-$CysLT_2R$ were cultured in in DMEM supplemented with 10% FBS, Geneticin (40 µg/mL) and Zeocin (250 µg/mL). All EC were grown as monolayer cultures at 5% CO_2, 37°C and 95% humidified cell culture incubator. MLEC were used up to passage 5. Cells were starved overnight with 0.5% FBS containing media and treated in serum free media with the respective agonists.

Matrigel tube formation assay: Matrigel tube formation assay was performed as described (3). Concentrated and Growth Factor-reduced Matrigel (Corning) was placed into a 48-well plate and allowed to solidify at 37°C for 30 minutes. MDEC and MLEC (8×10^4 cells) suspended in 0.5% serum containing media were added to wells and then treated with 500 nM LTC_4 or indicated concentrations of LTD_4 for 12-16 h. Cells were fixed with 4% paraformaldehyde for 15 minutes and images were taken on EVOS core XL phase contrast microscope (Evos core XL). In some experiments, cells were stained with Calcein AM fluorescent dye as per manufacturer procedure and fluorescent images were taken on EVOS *fl* fluorescence microscope (AMG, Mill Creek, WA). The number of tube-like structures with closed networks of vessel-like tubes were manually counted using image J software (NIH).

Ex-vivo mouse aortic ring assay: Aortas of 6-8 weeks old C57BL/6 mice were aseptically harvested, and the surrounding tissues were removed. The aortas were sliced into rings of approximately 1-mm thick, washed in PBS and were embedded in Matrigel in 24-well plates. 500 µL of 0.4% serum containing media with or without LTD_4 (500 nM) in the presence or absence of MK571 (1 µM) or $BayCysLT_2$ (1 µM) were added and incubated at 37°C. Media were replaced every third day and pictures of radial sprouting from the aortic rings were taken under a

phase contrast microscope (Evos core XL). Vessel sprout length was measured and quantified using image J (NIH).

Syngeneic LLC tumor model and metastasis: Mouse Lewis lung carcinoma (LLC) cells (2 x 10^6) in 100 µL of PBS were subcutaneously injected into both flanks of wild-type (WT), *Cysltr1*$^{-/-}$ and *Cysltr2*$^{-/-}$ mice. Tumor size was measured using a digital caliper at 7, 14, 18 and 21 days after tumor cells injections. Tumor volumes were determined using the formula $4/3*\pi*(l/2)*(w/2)^2$, where l is the longest diameter and w perpendicular length of the tumor. At day 21, mice were euthanized, primary tumors were harvested, embedded in OCT and stored at -80ºC while lungs were fixed in 4% paraformaldehyde for analyzing metastasis. For *in vivo* permeability assay, mice were injected with 0.25 mg Texas Red-conjugated dextran (70 kDa) in saline through the tail vein 20 minutes before mice were euthanized. In some experiments, when tumors were palpable on the seventh day, a cohort of mice received $BayCysLT_2$ (3 mg/kg body weight in 100 µL of saline) by intraperitoneal (i.p) injection. $BayCysLT_2$ was administered every three days till day 21, when mice were euthanized, and primary tumor and lungs were harvested.

Calcium flux: CHO-$CysLT_1R$ and CHO-$CysLT_2R$ overexpressing cells (0.5-1 x 10^6/sample) were washed and labeled with fura 2-AM for 30 minutes at 37ºC. Cells were stimulated with LTD_4 (500 nM) in the presence or absence of MK571 (1µM) or $BayCysLT_2$ (1µM) and the changes in intracellular calcium were measured using excitation at 340 and 380nm in a fluorescence spectrophotometer (Hitachi F-4500) as described earlier (4). The relative ratios of fluorescence emitted at 510 nm were recorded and displayed as a reflection of intracellular calcium concentration.

Isolation of MLEC: Sheep anti-rat-IgG Dynabeads® (Invitrogen) were coated with anti-PECAM-1 antibody (BD Bioscience, San Jose, CA) and ICAM-2 antibody (BD Bioscience, San Jose, CA) (1µg antibody for 1 x 10^6 beads) according to the manufacturer's instructions. Lungs from WT, *Cysltr1*$^{-/-}$ and *Cysltr2*$^{-/-}$ mice were harvested aseptically and were dissected out from bronchi and mediastinal connective tissue. Lungs were minced, digested with Collagenase-I solution (37°C; 45 minutes), mechanically dissociated, filtered through a 40-µm disposable cell strainer and centrifuged at 400 *g* for 8 minutes at 4°C. The cell pellet was re-suspended in 2 mL cold isolation buffer and EC were separated using PECAM-1 antibody coated Dynabeads® (15 µL/mL). Recovered cells were plated in a fibronectin coated tissue culture flask and sorted second time using ICAM-2 coated Dynabeads® (15 µL/mL cell suspension).

Gap formation: EC (2 x 10^4) were grown to a monolayer in a 48-well plate for 24 hours and stimulated with indicated agonists and antagonists. Following stimulation, cells were fixed (4% PFA for 20 minutes, RT), permeabilized (0.25% Triton-X100), blocked (10% FBS) and stained with fluorescein-conjugated phalloidin (Life technologies). Images were taken using EVOS *fl* fluorescence microscope using 20× objective and gaps were analyzed and quantified using Image J (NIH) software.

VE-cadherin staining: EC cultured, stimulated, fixed and blocked as detailed above were incubated with VE-cadherin primary antibody (1:200 dilution) for 1 h followed by fluorescent labeled secondary antibody for 30 minutes. Cells were washed, stained with DAPI and images taken on EVOS *fl* fluorescence microscope using 20× objective.

Immunofluorescence staining: Matrigel plugs and tumor tissues were cut into 5- to 7-µm sections and fixed in cold acetone for 15 minutes at 4ºC and blocked for 1 hour at room temperature with 10% FBS. Sections were incubated with control IgGs or anti-CD31 (1:100 dilution), anti-CD45 (1:200 dilution) and anti-NG2 (1:200 dilutions) followed by Alexa Fluor

488- or 594-conjugated secondary antibodies (1:500 dilutions) for 1 hour and imaged on EVOS *fl* fluorescence microscope. Images were processed and analyzed using Cellprofiler™ (Broad institute, MIT) as described previously (5). Briefly, the fluorescent intensity pipeline was used to determine fluorescent stained objects and the area covered per frame. Blood vessel (BV) density and pericyte coverage were determined by the total area covered by CD31 and NG2 stains to the total area per frame.

Real-time quantitative PCR: The expressions of bFGF and PDGF transcripts were determined by real-time PCR using Light cycler 480 (Roche) (6-8). Total RNA from WT, *Cysltr1*$^{-/-}$ and *Cysltr2*$^{-/-}$ EC was isolated, cDNA was synthesized, and real-time PCR was performed using the following primers.

mbFGF-Forward: 5'-GGACGGCTGCTGGCTTCTAA-3'
Reverse: 5'-CCAGTTCGTTTCAGTGCCACATAC-3'
mPDGF-Forward: 5'-TGAAATGCTGAGCGACCAC-3'
Reverse: 5'-AGCTTTCCAACTCGACTCC-3'
mCysLT$_1$R-Forward: 5'-TCTGTGTATGTACATTGCCTCTC-3'
Reverse: 5'-GTACAAGGCATAGGTGGTGAG-3'
mCysLT$_2$R-Forward: 5'-CTCTGTGGGATCATATGGGTTT-3'
Reverse: 5'-GACTCAGCTC CAAGCATGATA-3'

Levels of respective genes relative to the GAPDH were analyzed and the ΔΔCT values were calculated. Real time PCR for each sample was performed in at least triplicate and repeated three times.

Cell Lysates and Western Blotting: Following stimulations, cells were lysed with lysis buffer (BD Bioscience) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Pierce). In some experiments, cells were pre-treated with respective antagonists as specified. Immuno-blotting was performed as described previously (9). Briefly, lysates were subjected to 4–15% SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with respective primary antibodies diluted in 1× TBS, 5% dry milk, 0.1% Tween-20 (1:1000) overnight at 4°C on shaker, and then with secondary antibody (peroxidase-conjugated anti-rabbit or anti-mouse). Western blot was incubated with ECL and the bands were visualized using imager (Protein Simple) and quantified using Image J (NIH).

Hematoxylin and Eosin (H&E) staining: Lungs from mice were embedded in paraffin, cut into 7-μm sections, and stained for hematoxylin and eosin (H&E). Images of lung metastasis were taken and counted on bright field microscope and the metastasis area were measures using ImageJ (NIH).

ROCK assay: ROCK assay was determined using CycLex Rho-kinase assay kit (MBL international, Woburn, MA) according to the manufacturer's protocol. In brief, Cell lysates were made from EC after respective treatments and assay plates were pre-coated with a recombinant C-terminus of myosin-binding subunit of myosin phosphatase that can be phosphorylated by ROCK at a threonine residue. Phosphorylated myosin-binding subunit was detected with HRP-conjugated anti-phospho-MBS (Thr-697) antibody and change in color is monitored by Epoch microplate reader at 450 nm using TMB as substrate.

Statistical Analysis: Blots presented are representative of three experiments performed and data were expressed as mean ± SEM from at least three experiments except where otherwise indicated. Significance was determined using one-way analysis of variance (ANOVA) and comparisons between the groups were determined by Tukey's multiple comparisons test (GraphPad Prism 7.01). *P<0.05, **P<0.01, ***P<0.001.

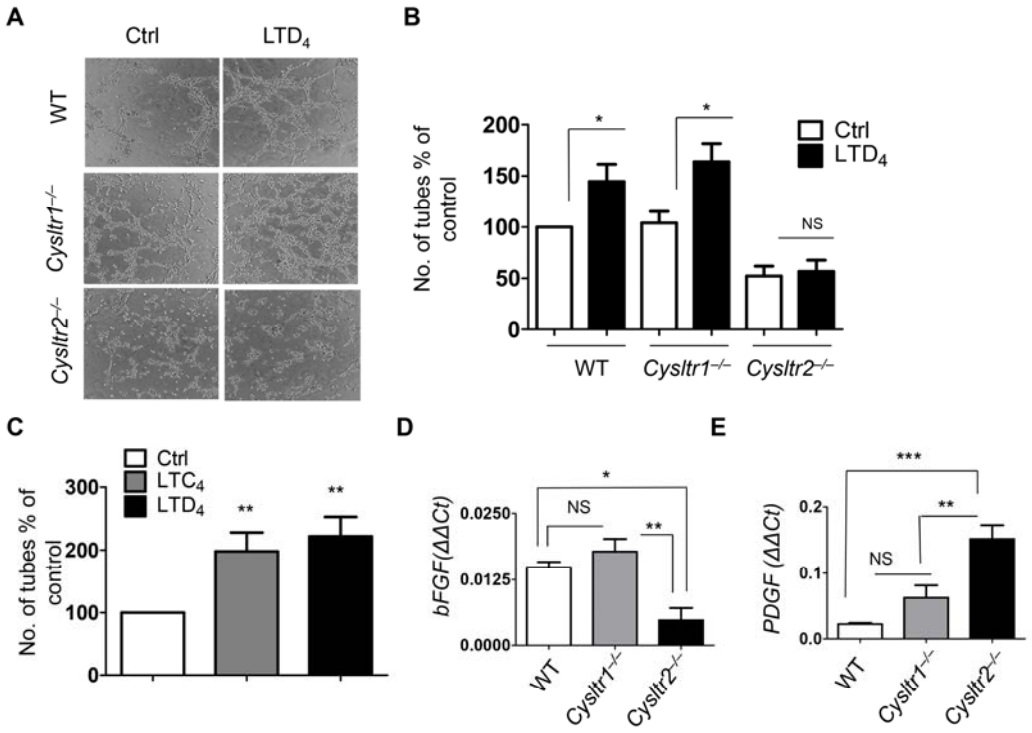

Fig. S1. LTD_4 induces tube formation via $CysLT_2R$ (A) Representative images showing the tube formation in WT, *Cysltr1*$^{-/-}$ and *Cysltr2*$^{-/-}$ MLEC with or without LTD_4 treatment (500 nM). (B) Quantification of tube formation from A. (C) Quantitative analysis of tube formation in MDEC with or without LTC_4 (500 nM) and LTD_4 (500 nM) treatment. Tubes from at least 10 representative images of each replicate were counted manually and presented as the percentage of average from three separate experiments. RT-PCR analysis of expression of bFGF (D) and PDGF (E) transcripts in WT, *Cysltr1*$^{-/-}$ and *Cysltr2*$^{-/-}$ MLEC. Data are shown as ± SEM, *P < 0.05; **P < 0.01; ***P<0.001, NS (non-significant) as determined by one-way ANOVA with Tukey post hoc test.

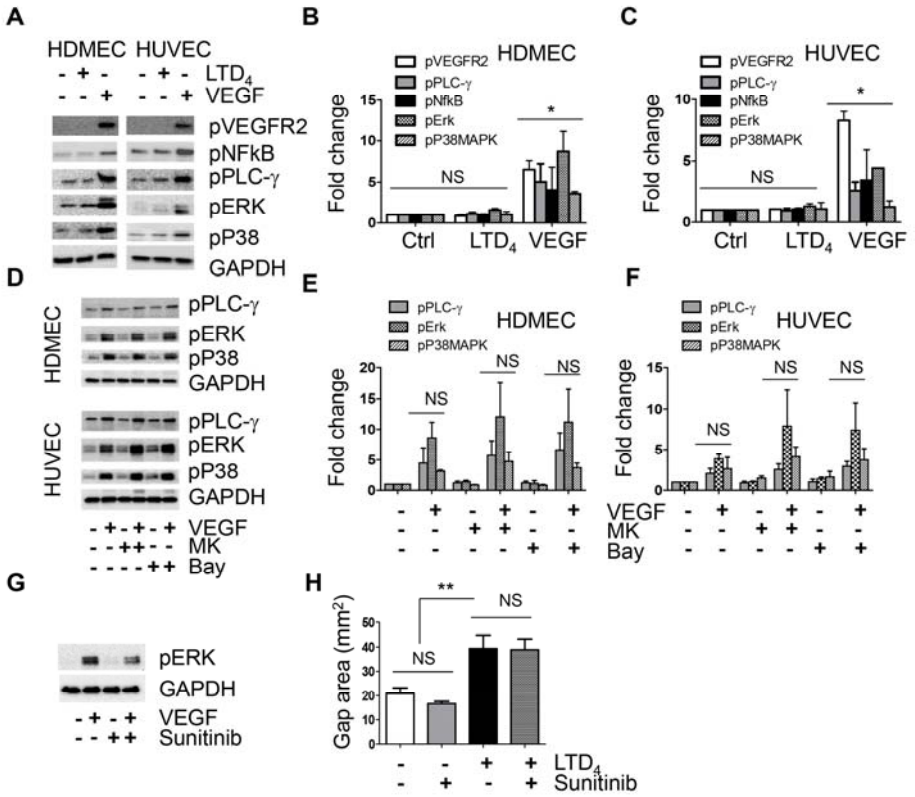

Fig. S2. LTD_4 does not trans-activate VEGFR and VEGF signals independent of CysLTR. (A) Western blot analysis and (B, C) quantification of phosphorylation of VEGFR2, NFκB, PLCγ, Erk and P38 MAPK in HMDEC and HUVEC stimulated with LTD_4 (500 nM) or VEGF (10 ng/mL). (D) Western blot analysis and (E, F) quantification of VEGF-induced phosphorylation of downstream signaling molecules in HDMEC and HUVEC in the presence or absence of MK571 (1 μM) and BayCysLT2 (1 μM). Blots were stripped and re-probed with GAPDH antibody and quantified using Image J (NIH). (G) Representative Western blot of VEGF-induced phosphorylation of Erk in the absence and presence of VEGFR inhibitor Sunitinib (100 μM; 2 h pre-incubation). (H) Quantification of LTD_4-induced gap formation with LTD_4 (500 ng/ml; 1 h) treatment in the presence or absence of Sunitinib (100 μM; 2 h pre-incubation) in WT MLEC. Results are mean ± SEM from 3 independent experiments. NS, not significant. *P< 0.05; **P < 0.01 as determined by one-way ANOVA with Tukey post hoc test.

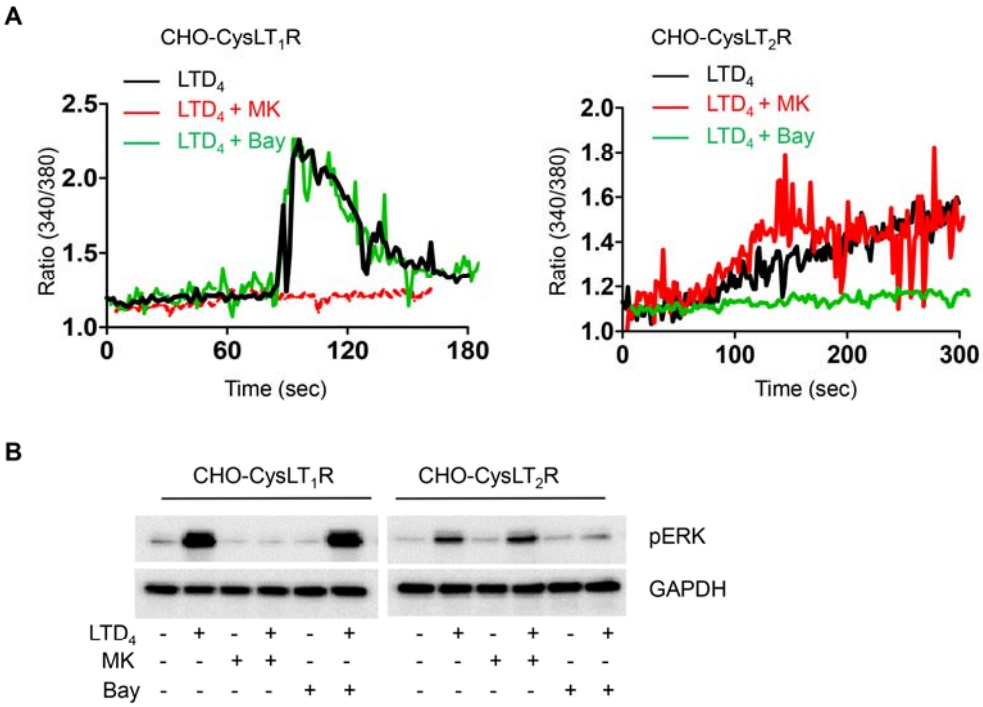

Fig. S3. Specificity of cys-LT antagonists to its receptor signaling. (A-B) CHO cells stably expressing $CysLT_1R$ and $CysLT_2R$ were stimulated with LTD_4 (500 nM) in the presence or absence of antagonists MK571 (1 μM) and BayCysLT2 (1 μM) and calcium influx (A) and Erk phosphorylation (B) were analyzed.

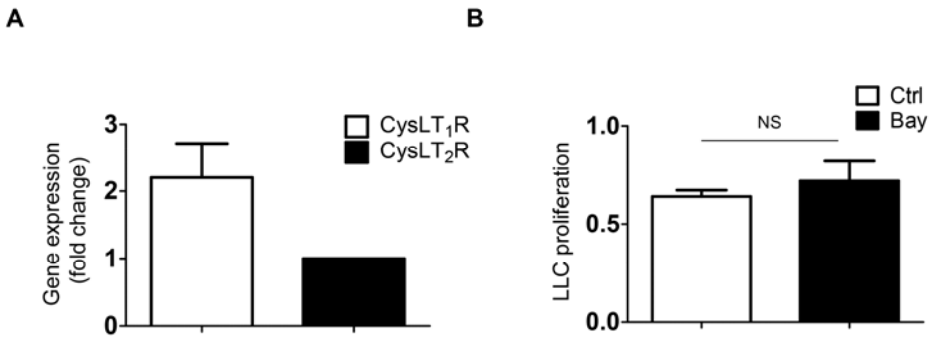

Fig. S4. BayCysLT2 does not affect the survival of LLC. (A) RT-PCR analysis showing the expression of $CysLT_1R$ and $CysLT_2R$ in LLC. (B) Proliferation of LLC in the absence or presence of BayCysLT2 (1 μM). LLC were treated with or without BayCysLT2 (1 μM) for 48 h and the proliferation was assayed by XTT assay. Data are shown as ± SEM, NS (non-significant) determined by Mann Whitney test.

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