Endothelial Cell Protein C Receptor Deficiency Attenuates *Streptococcus Pneumoniae*-induced Pleural Fibrosis

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Supplementary data

Methods

Generation of stable transfectants and cell line maintenance. Immortalized and non-cancerous human pleural mesothelial cell line MeT-5A was obtained from American Type Culture Collection (ATCC; Manassas, VA). EPCR was selectively knocked-down in MeT-5A by specific shRNA constructs (cloned into pSilencer 2.1 U6-Puro expression vector) using Fugene HD transfection reagent, according to the manufacturer's protocol (Roche Diagnostics Corp. Indianapolis, IN). MeT-5A cells were also stably transfected with empty pSilencer 2.1 U6-Puro vector to serve as a negative vector control (VC). Wild-type MeT-5A, MeT-5A(VC) and MeT-5A(-EPCR) cells were grown in RPMI 1640 basal medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco/BRL, Grand Island, NY).

Pulmonary function testing and CT imaging. Seven days post-infection, before euthanasia and collecting the lungs, mice were subjected to pulmonary function testing followed by computed tomography (CT) imaging, as described previously (1). Briefly, mice were anesthetized with a ketamine/xylazine mixture and were intubated by inserting a sterile, 20-gauge intravenous cannula into the trachea. Lung compliance, elastance, and resistance values were obtained by the "snapshot perturbation method" using the flexiVent system (SCIREQ, Tempe AZ) as per the manufacturer's specifications. Chest CT imaging was performed on mice to obtain lung volumes, as described earlier (1). To minimize spontaneous breaths and to ensure that mice remained anesthetized throughout the procedure, ketamine/xylazine anesthetized mice were maintained under the flow of isoflurane/oxygen mixture. Images were obtained using the Explore Locus Micro-CT Scanner. CT scans were performed during full inspiration and at a resolution of 93 µm. Microview software was used to analyze lung volumes and render three-dimensional images. Lung volumes were calculated from renditions collected at full inspiration.

Pleural lavage. Pleural lavage was obtained by gently pipetting one mL of sterile PBS into the thoracic cavity after making a small opening in the chest. Cell analysis was done using the HEMAVET-HV950FS Hematology System (Drew Scientific, Miami Lakes, FL, USA). Pleural lavages were spun at 250 x g for 5

min, and cells were resuspended in PBS and processed for flow cytometric analysis. Supernatants were used to analyze cytokines.

Cytokine measurements. Individual sandwich ELISA was used to measure the levels of IL-6, IL-1β, TNFα, IFNγ, and TGF β using commercially available kits (e-Bioscience, San Diego, CA) as per the manufacturer's instructions.

TAT assay. Thrombin levels in the pleural lavages were assessed as the generation of thrombin: antithrombin (TAT) complexes, which were measured using the AssayMax mouse ELISA kit (AssayPro, St. Charles, MO).

Hydroxyproline assay. Collagen was indirectly measured by estimating the hydroxyproline content in the lung tissues using hydroxyproline assay kit according to the manufacturer's instructions (Chondrex, Inc, Woodinville, WA) with some minor modifications. Briefly, peripheral lung tissues from saline and *S. pneumoniae*-infected (7 days post-infection) were homogenized in RIPA buffer containing protease inhibitors (approximately 50 mg of tissue in 500 µl buffer). While collecting lung tissues, the gelatinous outer coating on the lungs of *S. pneumoniae* infected mice was also included across the genotypes. Tissue homogenates (100 µl per mouse) were hydrolyzed by incubating with an equal quantity of 10 N HCl at 120^{o}C for 24h. Following the hydrolysis, the samples were centrifuged at 10 000 x g for 10 min to remove the debris. The supernatants were used to determine the hydroxyproline content according to the manufacturer's instructions. Appropriate blanks were used to adjust the O.D. for the background color. Final O.D. values were obtained by subtracting O.D. values of the blanks from the sample O.D. values. Hydroxyproline levels were normalized to the protein content for each sample.

Determination of activated protein C levels. Activated protein C (APC) levels in pleural lavages were determined by first capturing protein C/APC from the samples with murine protein C antibody coated on a 96-well microplate and then measuring the chromogenic activity of the bound sample. Briefly, 96-well microplates were coated overnight with rat anti-mouse protein C monoclonal antibody (AMGDPC 1587). After blocking the wells with 2% bovine serum albumin, pleural lavages were added to the wells and incubated for 2 h at room temperature. The plates were then washed four times, and APC chromogenic substrate (S-2366; Diapharma, KY) was added to the wells. The extent of hydrolysis of the chromogenic substrate was determined after overnight incubation by measuring the absorbance at 405 nm using a microplate reader. Known concentrations of murine activated protein C were used to generate a standard curve, from which the absorbance values were deduced to determine APC levels in samples.

Immunohistochemistry and Immunofluorescence. Mice were perfused with saline to remove blood in tissues, as described earlier (2). Lungs were inflated and fixed in Excel fixative solution (StatLab, McKinney, TX) for 48 h. Fixed tissues were then processed in graded alcohols and xylenes, embedded in paraffin. Five µm-thin sections were cut, and the sections were de-paraffinized and rehydrated using standard procedures. Rehydrated sections were then stained with Masson's trichrome stain, tissue gram stain, or immunostained with isotype control IgG, goat anti-mouse EPCR antibody, rabbit anti-mouse tissue factor antibody (a gift from Novo Nordisk, Denmark), rabbit anti-pneumococcal antibody against serotype 2 capsular protein (Pool A, Ref-16725, SSI Diagnostica, Hillerϕd, Denmark), rabbit anti-F4/80 (Biolegend), or rabbit anti-mouse Ly6G (Novus, St. Charles, MO). Human lung tissue sections were immunostained with isotype control IgG or goat anti-human EPCR antibody. Immunostained sections were developed using AEC (aminoethyl carbazole, Sigma Aldrich) chromogen and counterstained with hematoxylin. Trichrome stained sections were visualized under BioTek citation 5 microscope (BioTek, Winooski, VT), and whole lung images were reconstructed at 4x magnification using Gen5-3.05 software. Gram-stained tissue sections were imaged under BioTek Lionheart-Fx automated microscope. Immunostained images were viewed and imaged using an Olympus Bx43 microscope and photographed using DP27 digital camera and Olympus Cellsens software. Collagen was also detected by picrosirius red staining protocol as per the manufacturer's instructions (ScyTek, Utah).

To quantify infiltration of macrophages and neutrophils by immunohistochemistry, F4/80 and Ly6G positive cells from multiple fields (14 to 20 fields) from tissue sections originated from five mice/group were counted at 20x magnification. To obtain unbiased data, counting was done by two investigators who were blinded to the information about treatments and genotypes. The count was restricted to fibrotic pleural regions.

For immunofluorescence studies, lung tissues were inflated and fixed with 4% paraformaldehyde (PFA) and dehydrated with a 15% sucrose solution followed by 30% sucrose solution until the lung tissues settled at the bottom. Tissues were then embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and 5 mm-thin sections were cut. The sections were fixed in ice-cold acetone for 10 min and were blocked using a proprietary blocking solution from a mouse on mouse immunodetection kit (M.O.M, Vector Labs, Burlingame CA) as per the manufacturer's protocol. Blocked sections were stained overnight at 4°C with rabbit anti-mouse calretinin (Sigma-Aldrich, St. Louis, MO) and goat anti-Collagen-1a (Southern Biotech, Birmingham, AL) followed by incubation with mouse mAb anti-alpha SMA (R&D Systems, Minneapolis, MN) for 10 min. Sections were stained with rat anti-mouse

CD31 antibody (Biolegend), an endothelial-specific marker, to detect neoangiogenesis and blood vessel staining. After primary antibody binding, sections were incubated with appropriate AF488-, AF568-, or AF647-conjugated secondary antibodies and DAPI (5 μg/ml; to stain nuclei). Confocal images of the sections were obtained using an LSM 510 confocal system (Carl Zeiss).

Flow cytometry. Pleural lavage cells were immunophenotyped using flow cytometry. The cells were stained to assess cell viability with amine-reactive dye Aqua (Invitrogen; 1:500 dilution) for 30 min in the dark at room temperature (RT), followed by a single wash in 1 x PBS. For all flow cytometric analyses, cells were incubated in 0.5 lg Fc Block (BD Biosciences) for 10 min at RT. Surface staining was performed in the dark for 30 min at RT in staining buffer. Cells were then washed twice with staining buffer followed by fixation in 1% paraformaldehyde (VWR, West Chester, PA). A comprehensive list of surface markers for these experiments includes: CD45R (B220) clone RA3-6B2 PE-Texas Red (1:250, BD Biosciences), CD4 clone RM4-5 PerCP-Cy5.5 (1:160, BD Biosciences), CD8 clone 53-6.7 PerCP-Cy5.5 (1:160, BD Biosciences), CD8 clone 53- 6.7 eFluor 450 (1:333, eBioscience), CD11b clone M1/70 eFluor 450 (1:160, eBioscience, San Diego, CA), CD11b clone M1/70 PE-Texas Red [1:500, Invitrogen (Caltag)], CD11c clone HL3 PE-Cy7 (1:125, BD Biosciences), F4/80 clone BM8 APC (various dilutions, optimized at 1:100, eBioscience), F4/80 clone BM8 FITC (various dilutions, eBioscience), Ly6G clone 1A8 PE (1:416, BD Biosciences), MHC Class II clone M5/114.15.2 FITC (1:167, eBioscience). For flow cytometry and immunophenotyping experiments, cells were analyzed using an LSR Fortessa x-20 cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with 405 nm, 488 nm, 561 nm, and 640 nm excitation lasers. Fluorescence minus one (FMO) controls were used for gating analyses to distinguish positively from negatively staining cell populations. Compensation was performed using single color controls prepared from BD Comp Beads (BD Biosciences) for cell surface staining or Arc Beads (Invitrogen) for Aqua live/dead discrimination. Compensation matrices were calculated and applied using FlowJo software (Tree Star). Macrophages ($F4/80^+$), neutrophils ($Ly6G^+$), and γδ-T cells are expressed as percent frequency over total leukocyte population ($CD45^+$ cells). M1 and M2 phenotypes are expressed as percent frequency over $F4/80^+$ cells.

Assessment of *in vivo* bacterial burdens. To evaluate *in vivo* survival and dissemination of *S. pneumonia* in WT, EPCR-deficient, and Tie2-EPCR mice, mice were infected with *S. pneumoniae* as described in Methods sections of the main article, except that the mice were euthanized at 48 h post-infection. Blood was collected into citrate anticoagulant (10% v/v) via the submandibular vein. Pleural lavages were collected, as mentioned earlier. After collection of blood and pleural lavage, mice were perfused saline, and various organs were collected. Tissue homogenates of kidney, lung, spleen, liver were prepared by

tissue homogenization using ice-cold sterile PBS and a tissue homogenizer. Lung tissue homogenates, pleural lavage, and blood were diluted serially, and 100 µL of diluted samples were plated on blood agar plates. Similar volumes of other tissue homogenates were plated without dilution. The plates were incubated at 37^{o}C in a humidified 5% CO_2 incubator overnight, and colonies were counted.

Antibiotic protection assay. EPCR-dependent internalization of bacteria was assessed by antibiotic protection assay, as described earlier (3, 4), with a few minor modifications. Briefly, MeT-5A, MeT-5A (VC), and MeT-5A(-EPCR) cells were grown to confluence in 12-well plates coated with fibronectin and bovine type I collagen. Log-phase *S. pneumoniae* was resuspended in RPMI 1640 containing 1% FBS without any antibiotics at a concentration of 1 x 10^7 CFU/mL. One mL of bacterial suspension was added to each well and incubated for 2 h at 37^{o}C in a humidified 5% CO_2 incubator. The same size culture dish wells with only bacteria, but no cells were used to assess the replication of the bacteria during incubation. After incubation, cell monolayers were washed three times with sterile PBS. After that, 1 mL of RPMI 1640 containing 1% FBS supplemented with ampicillin (10 µg/ml) and gentamicin (200 µg/ml) was added to each well and incubated for one hour to kill all extracellular bacteria. The same concentration of antibiotics was added to wells containing only bacteria (no cells) to check the efficacy of antibiotics in killing the bacteria. After one hour, the monolayers were again washed three times with ice-cold PBS, and the cells were lysed in 1 mL 0.025% ice-cold Triton X-100 with gentle pipetting. To determine the total number of bacteria internalized, 100 µL of the cell lysate was plated in triplicates on blood agar plates. After overnight incubation, bacterial colonies were counted. To evaluate the involvement of caveolae-dependent endocytosis, confluent MeT-5A cells were incubated with 5 mM methyl-β-cyclodextrin (MβCD) or 50 µM Dynasore 1 h prior to infection with *S. pneumoniae.* After washing the cells once with serum-free medium, *S. pneumoniae* was added to the cells, and the internalization of *S. pneumoniae* was evaluated as described above.

Evaluation of *S. pneumoniae* adherence and internalization in mesothelial cells by fluorescence microscopy. *S. pneumoniae* was fluorescently labeled with 5, 6-carboxyfluorescein succinimidyl ester (FAM-NSE; Molecular Probes, Eugene, Oreg.). Briefly, log-phase bacteria were resuspended in sterile PBS (1x 10^9 CFU) and incubated with FAM-NSE (10 mg/ml in DMSO solution) at 37^{o}C for 1 h with mild shaking. After 1 h, the suspension was washed six times with PBS containing 0.2% bovine serum albumin. Labeled bacteria were resuspended at 1 x 10^9 CFU/mL in RPMI 1640 containing 1% FBS. To investigate EPCR dependency of bacterial adherence and internalization, labeled bacteria (1x10^7 CFU/well) were added to monolayers of MeT-5A, MeT-5A (VC), or MeT-5A(-EPCR) cells grown on glass coverslips coated with

fibronectin and bovine type I collagen. After 2 h of incubation at 37°C in a humidified 5% CO_2 environment, non-adherent bacteria were removed, and cells were washed three times with ice-cold PBS. Trypan blue dye (0.5%) was added to some wells and incubated for 10 min to quench the fluorescence of extracellular adherent but non-internalized bacteria. Trypan blue was removed, and cells were washed with PBS to remove excess Trypan blue. Quenching was confirmed by adding Trypan blue dye to the well containing only labeled bacteria (no cells), which showed a complete loss of fluorescence. The cells on coverslips were fixed with 2% paraformaldehyde and stained with antibodies against human EPCR (5 μg/mL) followed by AF594 labeled secondary antibody. The nuclei were stained with DAPI (5 μg/mL). Confocal images were obtained using an LSM 510 confocal system (Carl Zeiss) using a Plan-APOCHROMAT 63.3/1.4 NA oil objective.

APC-induced MesoMT markers and Immunoblot analysis. Human pleural mesothelial cells were grown to 80% confluency. Overnight serum-starved cells cells were treated with APC (100 nM), factor Xa (100 nM), or TGF-β (5 ng/mL) for 48 h. Equal amounts of cell lysates were subjected to SDS-PAGE and immunoblot analysis to evaluate the expression of α-SMA and collagen-1a. EPCR expression in various human cell types was probed by immunoblot analysis using human EPCR-specific mouse monoclonal antibody (JRK 1489).

References

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3. Tang P, Foubister V, Pucciarelli MG, Finlay BB. Methods to study bacterial invasion. *Journal of Microbiological Methods* 1993; 18 227-240.
4. Ring A, Weiser JN, Tuomanen EI. Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. *J Clin Invest* 1998; 102: 347-360.

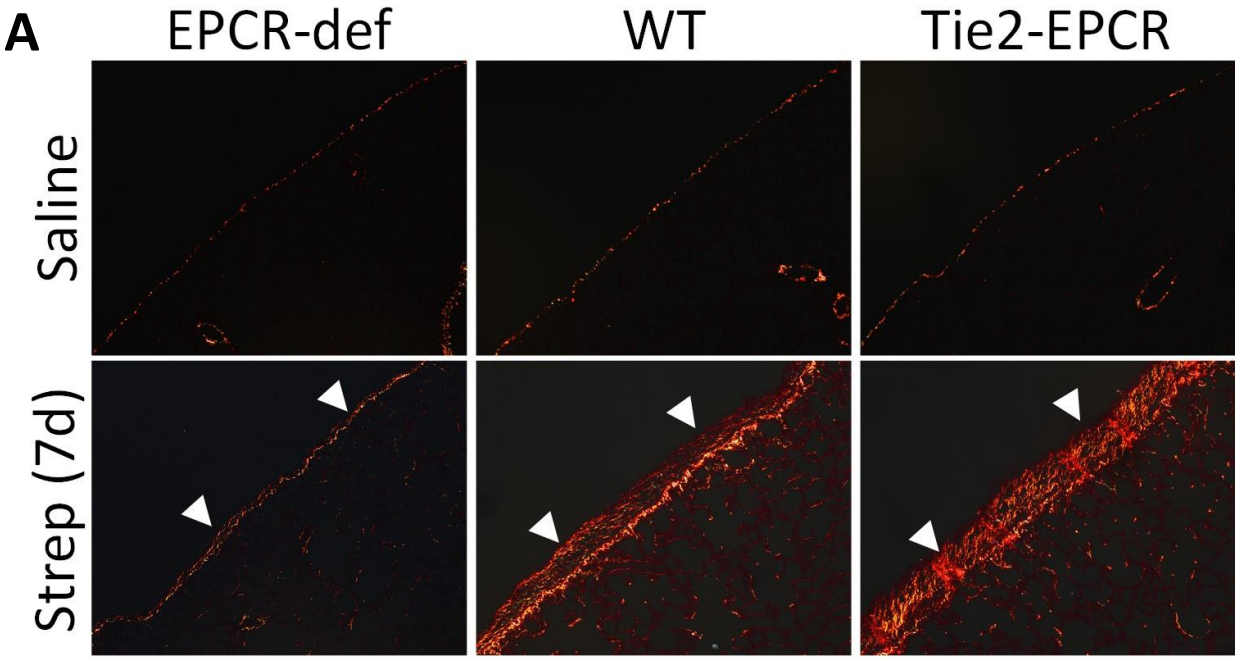
Supplemental Table 1. Mouse Sickness Score (MSS). Wild-type, Tie2-EPCR, or EPCR-deficient mice administered with either saline or *S. pneumoniae* intrapleural were evaluated for the sickness to calculate mouse sickness score (MSS). The following criteria were used to evaluate the mice. Scoring was done by two investigators who were blinded to the genotype. Although scoring was done once daily, mice were monitored at 12-hourly if the score was 1, 6-hourly if the score was 2, 4-hourly if the score was 3, and hourly if the score was 4.

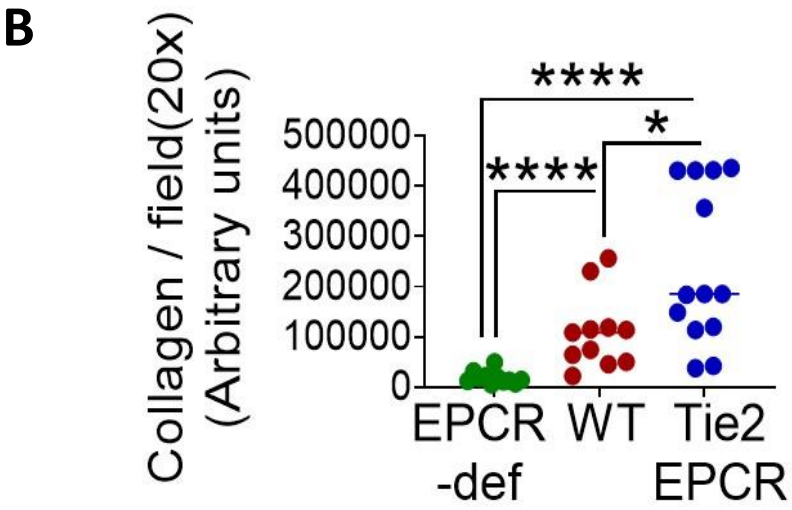
Score	1	2	3	4
Fur aspect and Appearance	Coat is smooth, actively grooming	Rough hair coat with dehydration checked with pinch test.	Piloerection and mouse appear puffy	Piloerection, Mouse appears emaciated
Eyes	Eyes fully open	Eyes partially open but without secretions	Eyes partially open with white secretions	Eyes closed completely with milky white secretions
Activity	Normal activity mouse is either: eating, drinking, climbing, running fighting	Reduced activity disturbed, avoids standing upright, mouse is moving around bottom of the cage	Suppressed activity, mouse moves only when provoked with possible tremors	No activity when disturbed or stimulated. Mouse experiencing tremors, particularly in hind legs
Posture	Normal	Slightly hunched, moving freely	Hunched with stiff movement/posture	Hunched with no movement stimulated
Response to stimulus	Mouse responds immediately to auditory stimulus or touch (moves to escape)	Slow response to auditory stimulus or but strong response to touch (moves to escape)	No response to auditory stimulus but slow response to touch	No response to auditory stimulus. Little or no response to touch (moves to escape)
Respiration	Normal. Rapid mouse respiration	Slight decrease in respiration. Brief periods of labored breathing.	Moderately dyspneic. Severely reduced respiration with labored breathing and occasional gasping Frequently chirping	Severely dyspneic with extremely reduced respiration and gasping for air with chirping sounds
Body weight loss	0%-5%	5%-10%	10%-15%	15%-20%

Supplemental Table 2. **APC levels in the pleural lavage of *S. pneumoniae*-infected mice.** EPCR-deficient, wild-type, and Tie2-EPCR were administered with *S. pneumoniae* intrapleural. After 48 hours, pleural lavages were obtained from the thoracic cavity, and APC levels were measured. Because of large variability, the substantial differences in APC levels among the three genotypes were not statistically significant.

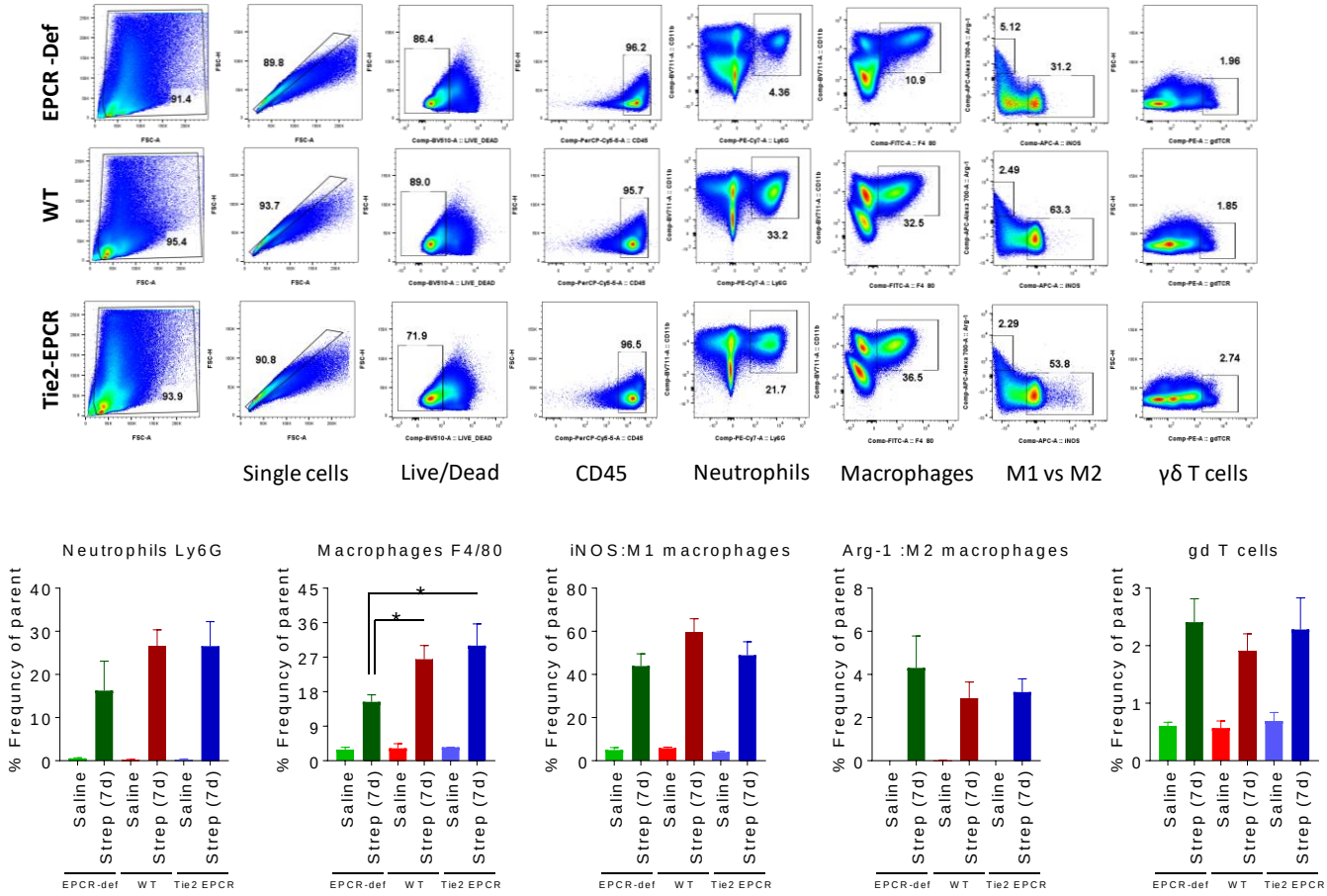
Genotype	APC (ng/mL)	
	Saline	*S. pneumoniae*-infected
EPCR-def	0.004 ± 0.002	0.019 ± 0.005
Wild-type	0.018 ± 0.017	0.26 ± 0.084
Tie2-EPCR	0.002 ± 0.0004	2.674 ± 2.189

<u>Supplemental Figure 1</u>. Increased collagen deposition in EPCR expressing mice infected with *S. pneumoniae*. (A) Lung tissue sections of *S. pneumoniae*-infected (7 days post-infection) wild-type (WT), Tie2-EPCR, or EPCR-deficient mice were stained with picrosirius red stain, and the natural birefringence of collagen was captured using polarized light microscopy (20x magnification). Top row, lung tissue sections of saline-administered mice; bottom row, lung tissue sections of *S. pneumoniae*-infected mice. Collagen deposition was indicated by white arrowheads (bottom panel) (20 x magnification). **(B)** The intensity of the picrosirius red stain was quantified by Image J (Fiji) software, and collagen deposition was expressed in arbitrary units per field. *, p<0.05; ****, p<0.0001.

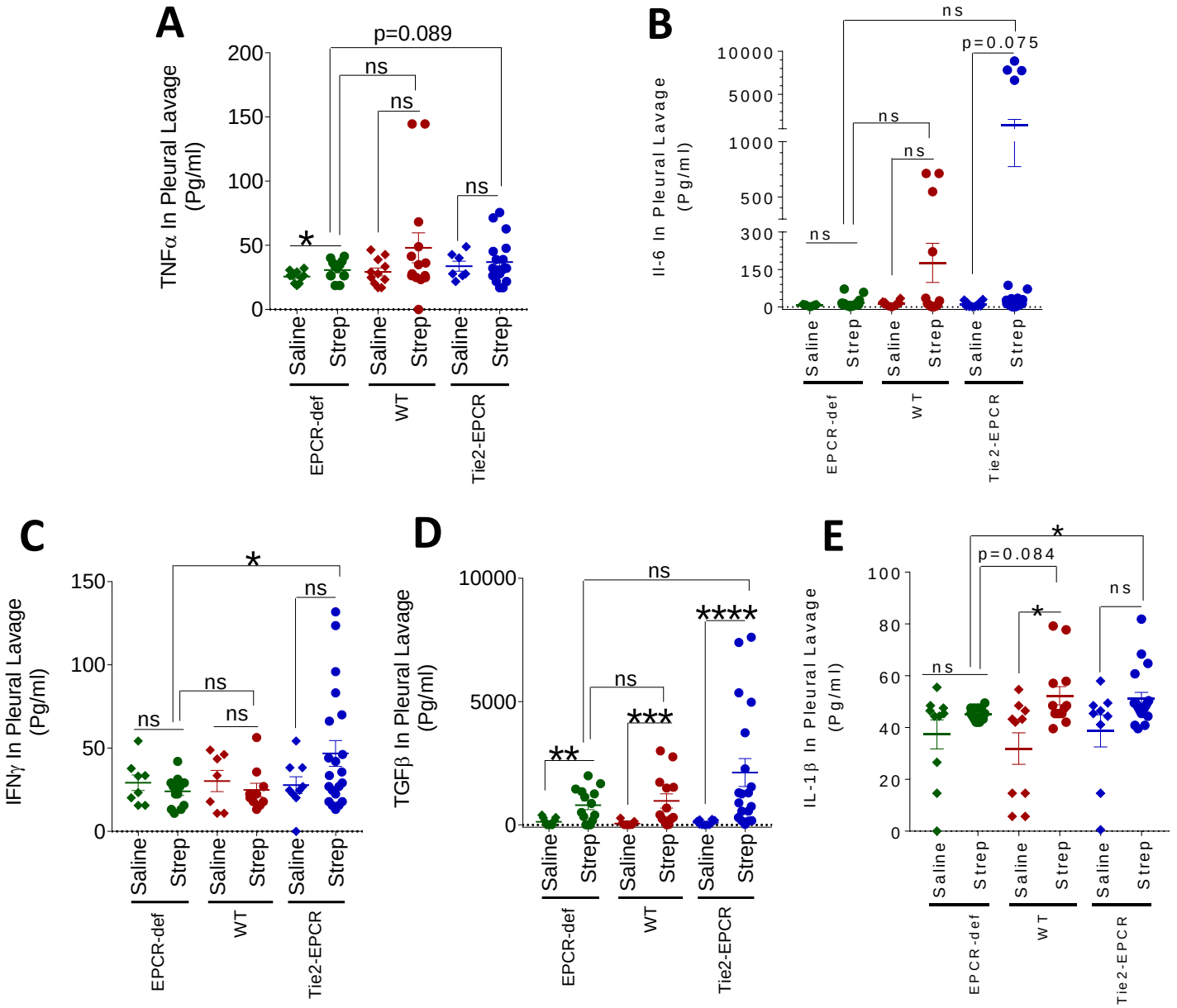




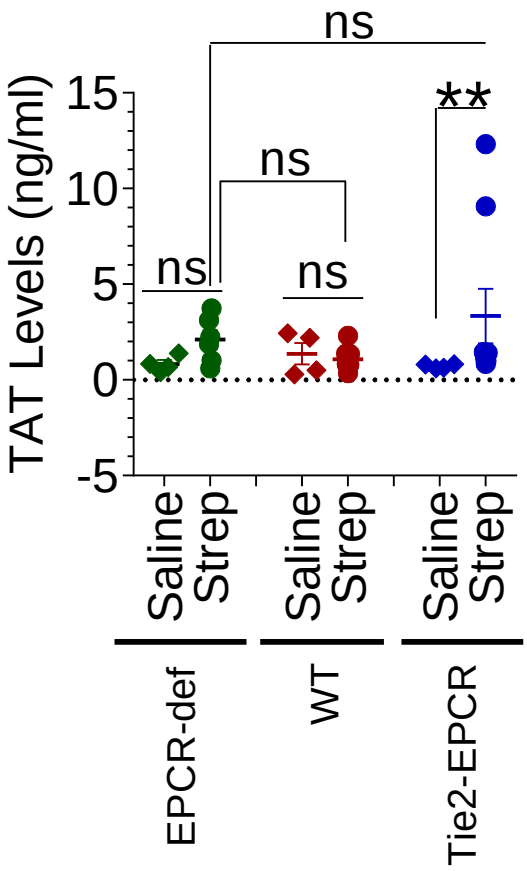
<u>Supplemental Figure 2</u>. Immune cell phenotyping in pleural lavages of *S. pneumoniae*-infected mice. Wild-type, Tie2-EPCR, or EPCR-deficient mice were administered with saline or *S. pneumoniae* intrapleural. After 7 days, pleural lavages were obtained from the thoracic cavity of these mice, and cells were isolated. These cells were immunostained and processed for flow cytometric analysis using a set of specific antibodies to identify neutrophils, macrophages, M1 macrophages, M2 macrophages, and γδ T cells. The bottom panels depict quantified data from flow cytometry analysis. The results are expressed as percent frequency over parent ($CD45^+$ cells). *, $p<0.01$.



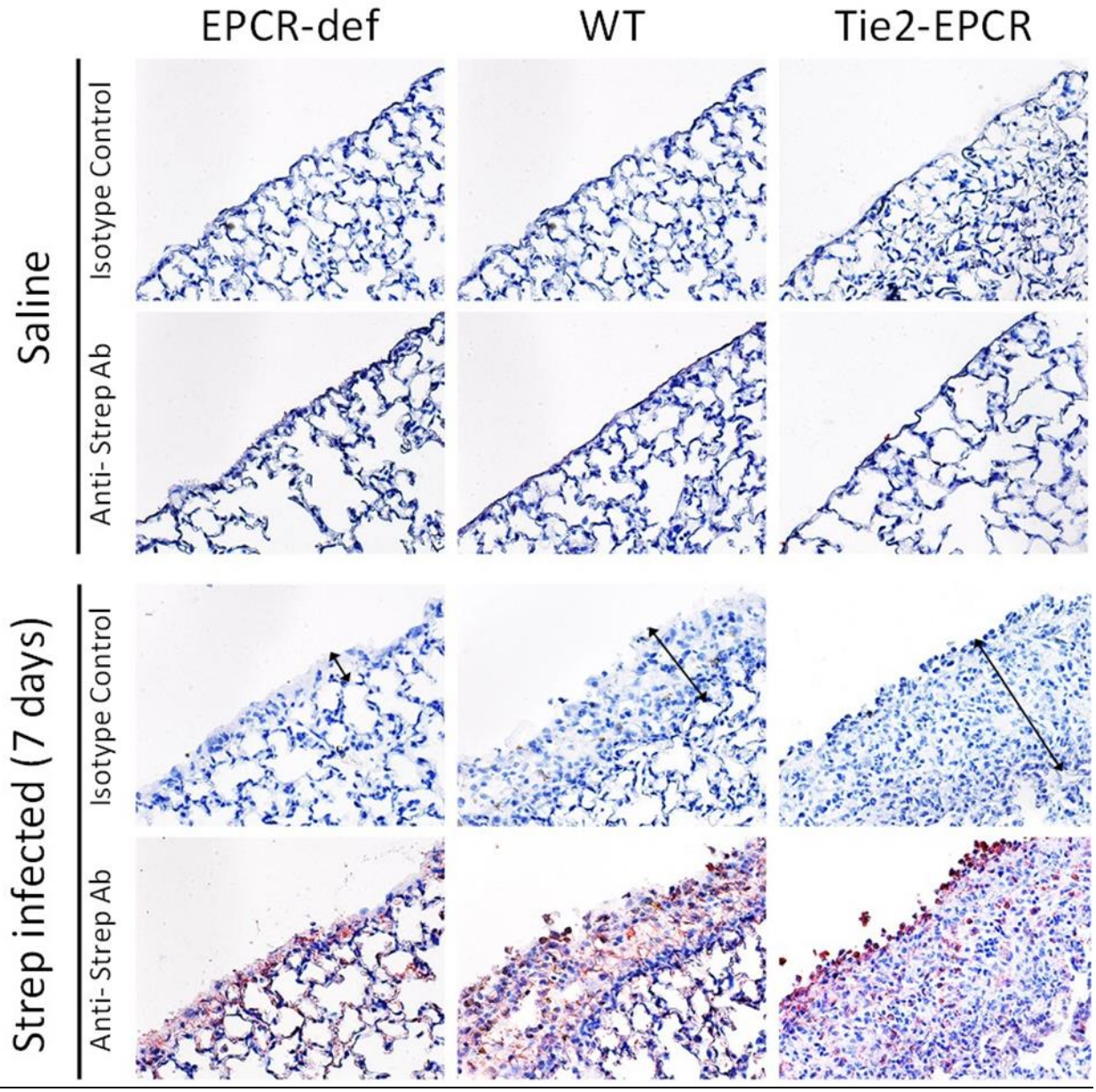
<u>Supplemental Figure 3</u>. Cytokine analysis of the pleural lavages of *S. pneumoniae*-infected mice. Pleural lavages *S. pneumoniae*-injected wild-type (WT), Tie2-EPCR, or EPCR-deficient mice were collected 7 days post-infection. Cells were removed and the supernatants were analyzed for proinflammatory cytokines **(A)** IL-6, **(B)** TNFα, **(C)** IL-1β, **(D)** IFN-γ, and **(E)** TGF-β. ns, not statistically significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.



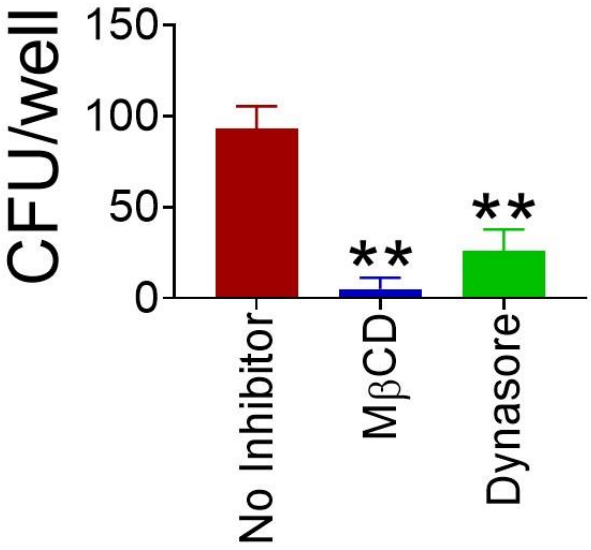
<u>Supplemental Figure 4</u>. Thrombin: Antithrombin (TAT) complex levels in the pleural lavages of *S. pneumoniae*-infected mice. TAT levels were estimated in the pleural lavages of saline-injected or *S. pneumoniae*-infected wild-type (WT), Tie2-EPCR, or EPCR-deficient mice, collected 7 days post-infection. ns, not statistically significant; **, p<0.01



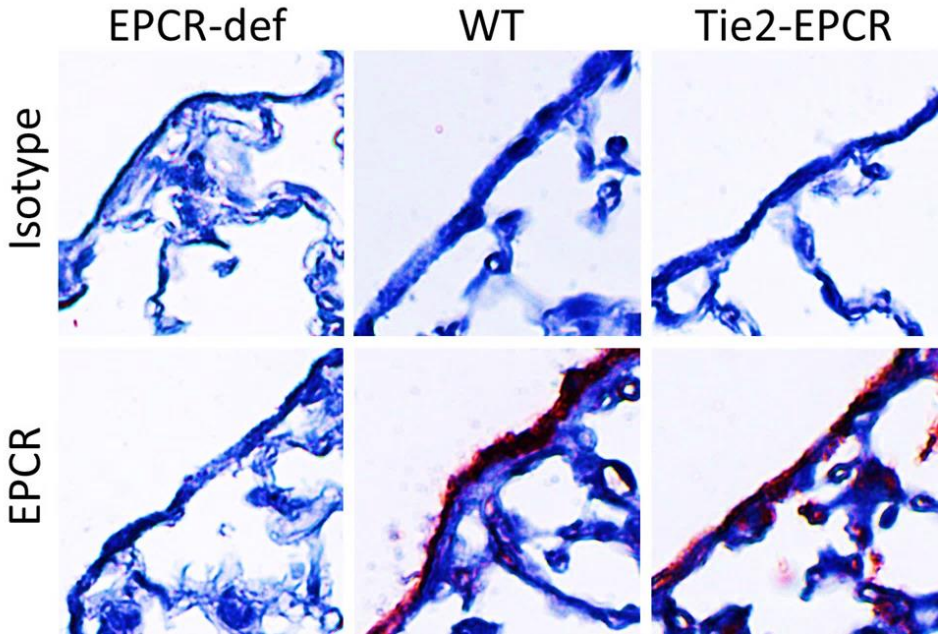
<u>Supplemental Figure 5</u>. EPCR expression increases bacterial burden and dissemination in *S. pneumonia* infection Lung tissue sections of *S. pneumoniae*-infected (7 days post-infection) wild-type, EPCR-overexpressing Tie2-EPCR, or EPCR-deficient mice and their saline counterparts were immunostained with an anti-pneumococcal antibody against serotype 2 capsular protein. The sections were developed using AEC substrate and counterstained with hematoxylin stain. Images were photographed using an Olympus BX41 light microscope at 40x magnification. Red staining represents *S. pneumoniae* bacteria. The bar with two-sided arrows marks the fibrotic region.

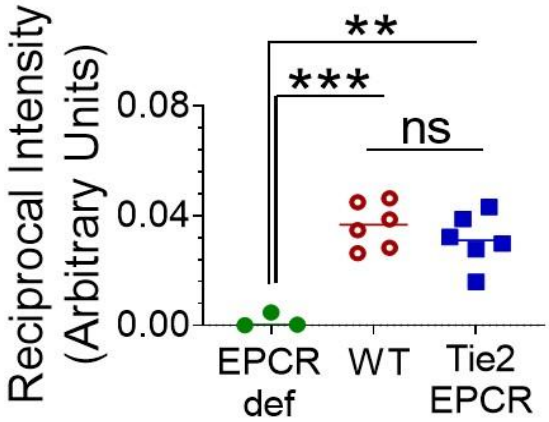


<u>Supplemental Figure 6</u>. Inhibition of caveolae- and dynamin-dependent endocytosis inhibits *S. pneumoniae* internalization by mesothelial cells. Confluent MeT-5A cells were pretreated with 5 mM methyl-β-Cyclodextrin (MβCD) or 50 µM Dynasore 1 h prior to the infection with *S. pneumoniae. S. pneumoniae* (50 moi/cell) were added to the cells and internalization was allowed for 2 h. The number of bacteria internalized was determined as described in Methods. **, p <0.01.

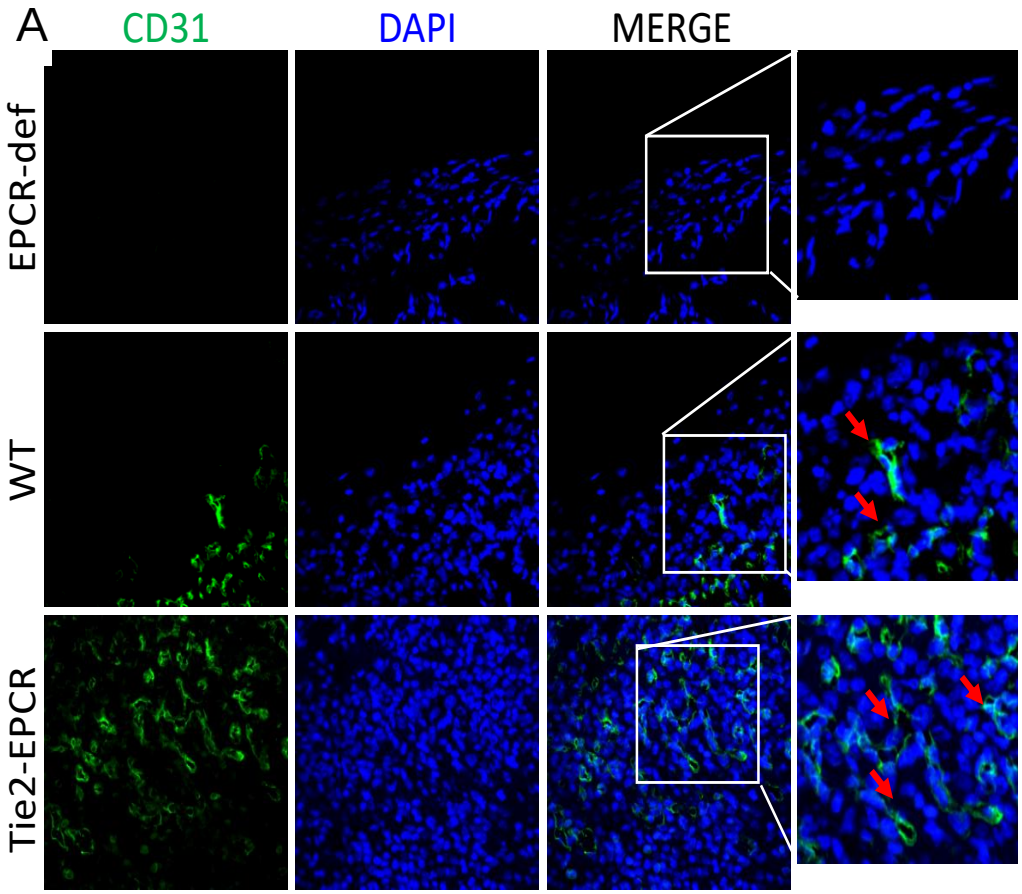


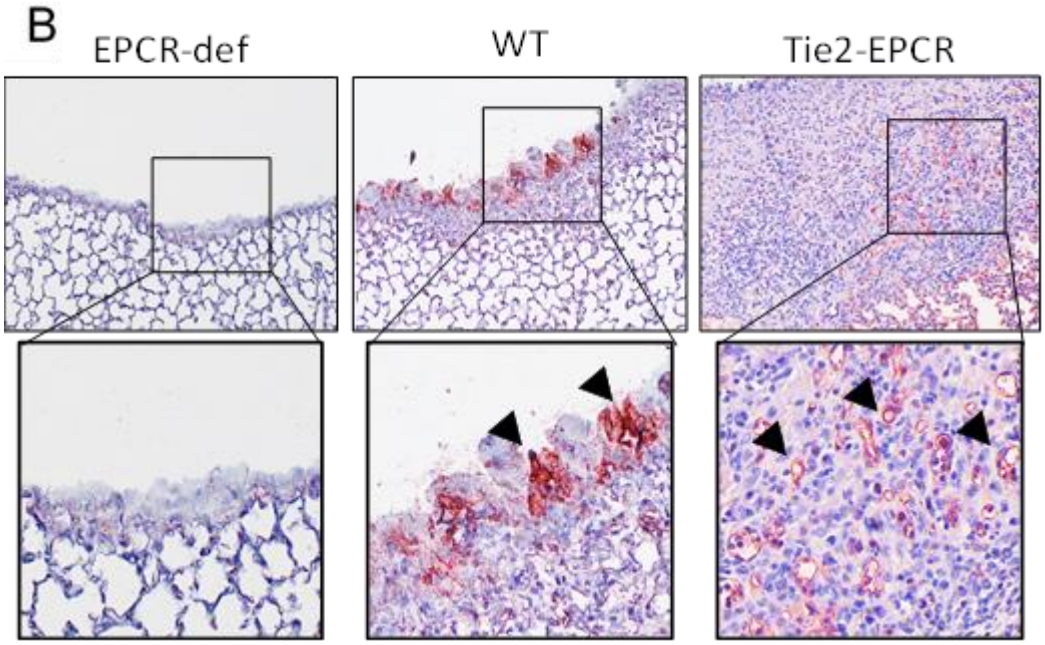
<u>Supplemental Figure 7</u>. EPCR expression on the mesothelium of EPCR-deficient, wild-type, and Tie2-EPCR mice. Lung tissue sections of uninfected EPCR-deficient, wild-type (WT), and Tie2-EPCR mice were immunostained with goat anti-mouse EPCR antibody or isotype control IgG. Immunostained sections were photographed using an Olympus BX41 light microscope at 40x magnification. RGB images of Immunohistochemistry were deconvoluted and the intensity of EPCR expression was quantified in the red panel using Image J software and expressed in arbitrary units. ns, not statistically significant; **, p<0.01; p<0.001.





<u>Supplemental Figure 8</u>. EPCR expressing wild-type and Tie2-EPCR mice exhibited increased neo-angiogenesis following *S. pneumoniae* infection. Lung tissue sections of *S.pneumoniae-infected* wild-type (WT), Tie2-EPCR, or EPCR-deficient mice (7 days post-infection) were stained with rabbit anti-murine CD31 **(A)** or goat anti-murine EPCR antibodies **(B).** CD-31 staining could be seen in the thickened fibrotic area of both wild-type and Tie2-EPCR mice (indicated by red arrowheads), indicating neoangiogenesis (40x magnification). Immunohistochemistry with anti-mouse EPCR antibodies shows EPCR expression in the newly formed blood vessels (indicated by black arrowheads) (20x magnification).




B
EPCR-def
WT
Tie2-EPCR

<u>Supplemental Figure 9</u>. Activated Protein C (APC) induces MesoMT in human pleural mesothelial cells *in vitro*. HPMCs were serum-starved overnight and treated with APC (100 nM) for 48 h. After 48 h, cell lysates were prepared and were subjected to immunoblot analysis to probe for MesoMT markers, α-SMA and collagen-1a. FXa (100 nM) treatment was used as a positive control for the induction of the MesoMT markers.