VEGF-C/VEGFR-3 signaling in macrophages ameliorates acute lung injury

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Materials and Methods

Mice

Wild-type C57BL/6J mice and C57BL/6J-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J mice, i.e., macrophage Fas-induced apoptotic mice (Mafia mice), were purchased from NIHON SLC (Tokyo, Japan) and the Jackson Laboratory (Sacramento, CA, USA), respectively. Because Mafia mice exclusively express enhanced green fluorescent protein (EGFP) only in cells expressing the macrophage colony-stimulating factor (M-CSF) receptor, we used these mice as reporters to detect monocyte-lineage cells in the present experiments.

Experiments using mice with a LysM-specific VEGFR-3 deletion, generated by mating LysM-Cre (1) mice with VEGFR-3-floxed mice (2), were conducted in accordance with the guidelines set by the Regional State Administrative Agency for Southern Finland. Both mouse models were crossed with the C57BL/6J background. The experimental protocols for using C57BL/6J, Mafia, and VEGFR-$3^{flox/flox}$;LysMCre and littermate mice were approved by the Committee for Animal Experiments of Iwate Medical University.

Generation of adenoviruses (Ad-VEGF-C, Ad-soluble VEGFR-3-Ig, and Ad-LacZ)

Adenoviral (Ad) vectors encoding VEGFR-3-Ig, VEGF-C, and LacZ as a negative control were used (3, 4). Briefly, cDNAs were subcloned into the pAdCMV plasmid, constructed via subcloning of the human CMV immediate early promoter, multiple cloning site, and the bovine growth hormone polyA signal from the pcDNA3 plasmid (Invitrogen, Groningen, Netherlands) into a pAdBgl*II* vector. Replication-deficient E1- to E3-deleted clinical adenovirus vectors (good manufacturing practices [GM] grade) were produced in 293T cells.

Kinetics of adenovirus-mediated transgene expression

To investigate the kinetics of adenovirus-mediated transgene expression, 1.0×10^9 PFU/mouse Ad-human soluble human VEGFR-3, Ad-human VEGF-C, and Ad-LacZ as a control were intratracheally injected into C57BL/6J mice. The expression levels of soluble

VEGFR-3 and VEGF-C protein in bronchoalveolar lavage (BAL) fluid over time after vector injection were estimated via immunoblotting and enzyme-linked immunosorbent assay (ELISA), respectively.

Lipopolysaccharide (LPS)-ALI model

LPS (Sigma-Aldrich, St. Louis, MO, USA; from *Escherichia coli* O111) in sterile saline (100 μL) was administered intranasally into 8- to 12-week-old female mice anesthetized with sevoflurane. We used several doses of LPS to estimate the course during acute lung injury, including 1.9 and 3.8 mg/kg body weight as lung injury models. An equal volume of sterile saline was used as a vehicle control in the experiments using an adenovirus vector. In experiments using adenoviral vectors, 1.9 mg/kg LPS was intranasally administered to mice on day 3 after intranasal injection of the adenovirus vector (1.0×10^9 PFU/mouse), and the mice were euthanized on the next day or after 4 days (Figure S2E). In experiments using the VEGFR-3 blocking antibody (mF4-31C1; Eli Lilly and Company, Indianapolis, IN, USA), 20 μg of primary antibody or isotype IgG was intranasally administered into mice (5). In experiments using mice with VEGFR-$3^{flox/flox}$;LysMCre (a LysM-specific VEGFR-3 deletion) and VEGFR-$3^{flox/flox}$ as a control, mice were euthanized on day 0 and days 1, 3, and 5 after injection of LPS 3.8 mg/kg. In survival analysis, we adapted 20% or more reduction rates of body weight as humane endpoints. For lung administration of mouse extrinsic ultraviolet (UV)-radiated PKH-labelled apoptotic neutrophils (3×10^6 in 100 μL of phosphate-buffered saline (PBS) per mouse) at 5 days after instillation with 1.9 mg/kg LPS, the extrinsic apoptotic neutrophils were intranasally administered to LPS-treated mice. For inhibitory experiments for the digestion of apoptotic neutrophils, 20 μg of cytochalasin D (Sigma-Aldrich) or vehicle control was intranasally injected on a day before ultraviolet (UV)-radiated PKH-labelled apoptotic neutrophils (1.0×10^6 in 100 μL of phosphate-buffered saline (PBS) per mouse) were injected into mice treated with 0.38 mg/kg of LPS.

Bronchoalveolar lavage (BAL) and tissue collection

Mice were euthanized at the appropriate time points, using pentobarbital, and the BAL cells, fluid, and lung tissues were harvested. After euthanasia, the lungs were perfused through the right ventricle with 5 mL of PBS. Two 1.0-mL aliquots of PBS were used for lavage. Recovery rates were 89.0 ± 0.3% (range 80-90%). Red blood cells (RBCs) were lysed using VersaLyse (Beckman Coulter, Brea, CA, USA). The resulting cells were enumerated using a cell counter. The dead cells were identified by trypan blue staining and were excluded from the analysis. For differential cell counts, BAL cells were attached to glass slides using the cytospin method, followed by May-Giemsa staining. BAL polynuclear and mononuclear cells were divided via the specific gravity centrifugal method using 63% Percoll (GE Healthcare, Chicago, IL, USA) to estimate the mRNA levels of lymphangiogenic factors. The purity of the polynuclear and mononuclear cells obtained by the specific gravity centrifugal method was more than 95%. Cell-free BAL fluid was stored in a -80°C freezer for cytokine measurements.

Histopathology and cytochemistry

Mouse tissue samples were fixed with 10% formalin and embedded in paraffin. Thereafter, 4-mm-thick sections were cut and stained with haematoxylin and eosin. In histologic evaluation by H&E staining, investigators were blinded to the treatment groups. The levels of lung injury were determined according to the semiquantitative scoring outlined below. All lung fields at ×20 magnification were examined for each sample. Histological lung injury was assessed by grading as follows: 1, very focal (<10% of lung section); 2, focal (<25% of lung section); 3, moderate (25%< and <50%); 4, diffuse (>50% of lung section) interstitial congestion and inflammatory cell infiltration; 5, focal (<50% of lung section) consolidation and inflammatory cell infiltration; 6, diffuse (>50% of lung section) consolidation and inflammatory cell infiltration. The mean score was used for comparison between groups. During cytochemistry, BAL cells were attached to slides, using cytospin, and fixed with 4% paraformaldehyde to observe phagocytosis of the EGFP-positive monocyte-lineage cells and

pHrodo-labelled neutrophils. Cell nuclei were labelled with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) (6). Dual-immunofluorescence analysis was performed via triple-channel confocal laser scanning microscopy using a NIKON C1SI microscope (Nikon Corp., Tokyo, Japan).

ELISA

We estimated BAL fluid levels of pro-inflammatory cytokines responsive to inflammation, including myeloperoxidase, C-X-C Motif Chemokine Ligand (CXCL) 1, CXCL2, interleukin (IL)-1beta, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, IL-6, and IL-17A, as well as anti-inflammatory cytokines including IL-10. Duoset or Quantikine ELISA kits obtained from R&D Systems (Minneapolis, MN, USA) were used for mouse CXCL1, CXCL2, IL-1beta, TNF-α, MCP-1, IL-6, and IL-17A, and human soluble VEGFR-3, VEGF-C and IL-10 in BAL fluid, in accordance with the manufacturer's instructions. ELISA kits obtained from eBioscience (Santa Clara, CA, USA) were used to evaluate mouse IL-10.

Immunoblotting

The intensities of BAL sVEGFR-3-Ig were determined via immunoblotting using primary anti-human VEGFR-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology). Relative protein band intensity was estimated using Fiji image analysis software (Version 2.0.0).

Fluorescence-activated cell sorting (FACS)

Monocyte lineage cells, neutrophils, and lymphatic endothelial cells were identified via FACS. Mice were euthanized and their lungs were perfused through the right ventricle with 5 mL of PBS. Peripheral lung tissue was cut into small pieces, transferred into conical tubes, and processed in digestion medium (RPMI1640; Thermo Fisher Scientific, Waltham, MA, USA) containing 1 mg/mL type I collagenase (Worthington, Lakewood, NJ, USA) and 0.1 mg/mL DNase I (Wako Corp., Osaka, Japan), followed by gentle pipetting several times. Homogenized lungs were filtered through a 40-μm cell strainer to obtain a single-cell

suspension. Cells obtained from tissue or BAL samples were incubated with anti-mouse CD16/32 antibodies (BD Biosciences) for Fc blocking, followed by staining with mixtures of fluorochrome-conjugated antibodies (Supplementary Table S2). Data acquisition and analysis were performed using a BD Canto II Flow Cytometer and BD FACS Diva software (Version 8). Cell sorting was performed using a FACSAria I instrument (BD Biosciences, Franklin Lakes, NJ, USA). The dead cells were distinguished by 7-amino-actinomycin D (7-AAD) staining. The purity of individual cells was generally greater than 95%.

Quantitative RT-PCR

RNA was extracted from sorted cells and cell pellets obtained by BAL and specific gravity centrifugal methods, and from pellets of bone marrow-derived macrophages cultured with medium, using a total RNA extraction kit (Qiagen, Venlo, the Netherlands). For quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis, 0.5 μg of total RNA was used for reverse transcription with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative fluorogenic real-time PCR was performed to detect *Vegfa*, *Vegfc*, *Vegfd*, *Flt4*, *Axl1*, *Cd81*, *Itgav*, *Cd36*, *Merkt*, *Gpnmb*, and *FLT4* using TaqMan (Thermo Fisher Scientific) kits and an ABI 7500 Fast Real-Time PCR system (Thermo Fisher Scientific) (Supplementary Table S7). All mRNA expression levels were normalized to *Gapdh* and *GAPDH* mRNA, and differences between treatment groups were quantified in accordance with the manufacturer's instructions. Relative gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, and the results are presented as fold increase relative to the expression of these genes in naive WT controls or alveolar macrophages before LPS administration.

Generation of apoptotic neutrophils

Purified neutrophils were harvested from the spleens of the same animals in which extrinsic neutrophils were injected. Single-cell suspensions were obtained from spleen tissues using collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA), and neutrophils

were separated using a magnetic microbead-based antibody technique, which positively selects for the granulocyte-specific antigen Ly6G (autoMACS; Miltenyi Biotec, Bergisch, Germany). Neutrophils obtained from spleens showed greater than 95% purity, as evident from May-Giemsa staining. Neutrophils were labelled using a PKH26 Red Fluorescent Dye Linker Kit (Sigma-Aldrich) or pHrodo Red (Thermo Fisher Scientific) for *in vivo* and *in vitro* experiments, respectively, in accordance with the manufacturer's instructions. Dye-labeled neutrophils were exposed to ultraviolet radiation for 15 min and cultured in RPMI 1640 for 3 h at 37°C. This method helped to determine apoptosis rates of greater than 85% for unlabelled neutrophils, as assessed via flow cytometry, using an Annexin V binding assay in combination with 7-AAD staining.

In vitro phagocytosis assay

Murine macrophages located in alveolar spaces were harvested from mice injected at 0 and 4 days after LPS administration with two 1.0-mL aliquots of RPMI1640 containing 10% fetal calf serum. The harvested macrophages in 400 µL of medium used for BAL were cultured at 1.0×10^5 cells/well in four-chamber slides (chambers mounted on glass slides with covers; Eppendorf, Hamburg, Germany) for 1 h. Subsequently, 5.0×10^5 UV-irradiated pHrodo-labelled neutrophils suspended in 100 µL of RPMI1640 containing 10% fetal calf serum were seeded on the cultured macrophages. Simultaneously, 10 nM VEGFR-3 blocking antibody (mF4-31C1) or CD51/61 blocking antibody (23C6, 10 g/mL), or respective isotype controls were added to the chamber slides and incubated for 2 h at 37.0°C in 5% CO_2. In the chamber slide, non-ingested neutrophils were eliminated by washing twice in PBS. The phagocytosis index was calculated as EGFP-M-CSFR$^+$ cells with phagocytosis in pHrodo$^+$ cells divided by the total number of EGFP-M-CSFR$^+$ cells.

Human samples

BAL samples were collected from healthy volunteers, ARDS patients, and COP patients. ARDS was diagnosed based on the Berlin criteria (7). COP was diagnosed based on clinical,

radiological, and histopathological criteria (8). BAL was performed by instilling 150 mL of normal saline in 50-mL aliquots, each retrieved by low suction. The samples were filtered through a sterile gauze and centrifuged for 10 min at 500 × *g* at 4°C. The resulting cell-free solution was aliquoted and frozen immediately at -80°C. VEGF-C levels were estimated in a 5-fold concentration of BALF with Minicon® B15 concentrators (Merck Millipore, Billerica, MA, USA). Human monocyte-derived macrophages (hMDMs) were used for estimating its capacity of IL-10 expression and the phagocytic assay. Fresh human blood was treated with an anticoagulant and diluted with 2 times volume of PBS containing 0.5% BSA (PBS/BSA). Samples were then centrifuged using Ficoll-Paque™ Plus (GE Healthcare), and the mononuclear cell layer was collected by pipette and resuspended in Poly D-Lysine-coated flasks. The adherent cells were incubated with RPMI containing 10% FCS, 2 mM glutamine, 1% NEAA, 1% sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 ng/mL rHu M-CSF for 7 days, and the peripheral monocytes were differentiated into hMDMs. The intensity of VEGFR-3 expression on hMDMs was estimated via FACS, after 2-hour incubation in BAL fluid obtained from patients with COP or ARDS, and healthy volunteers, containing 1% BSA. In a similar fashion, the concentration of soluble VEGFR-3 in BAL fluids incubated with/without hMDMs for 2 h was determined via ELISA. Furthermore, the concentration of IL-10 in BAL fluids incubated with hMDM, and anti-VEGFR-3 antibody or isotype control was determined via ELISA. The hMDMCs were cultured at 1.0×10^5 cells/well in four-chamber slides for 1 h. Thereafter, BAL fluids, obtained from human healthy volunteers and ARDS and COP patients, containing 1% BSA, were exchanged after washing with PBS, and 5.0×10^5 UV-irradiated pHrodo-labelled neutrophils obtained from murine spleen were seeded on the hMDMs, and incubated for 2 h. The use of all human samples in the present study was approved by the Ethics Committees of Iwate Medical University (IRB H29-18 and MH2018-053). Informed consent was obtained from all patients and volunteers.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) values. The normality of distribution was estimated using the Kolmogorov–Smirnov test. All other data other than VEGF-C levels estimated via ELISA in BAL fluid obtained from healthy volunteers and patients with ARDS and COPD were normally distributed. Differences in measured variables between the experimental and control groups were assessed using Student's *t*-tests. One-way analysis of variance (ANOVA) followed by Tukey's or Steel-Dwass's post-hoc test were used for multiple comparisons in parametric or nonparametric analyses, respectively. The Kaplan-Meier method was used to estimate mouse survival, and differences were analyzed using the log-rank test. The comparison between two related samples was estimated using a paired sample t-test. Statistical analyses were performed using IBM SPSS Statistics (IBM Japan, Tokyo, Japan). Differences with a *P* value less than 0.05 were considered statistically significant.

Supplementary References

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Figure S1. Comparison of the individual rates of macrophages and neutrophils relative to total blood cells between bronchoalveolar lavage (BAL) fluid and tissue samples. (a,b) Mice (C57BL/6J, n = 3 mice per treatment group) were intranasally injected with 1.9 μg/kg body weight lipopolysaccharide (LPS). (c) LPS of 1.9 mg/kg body weight (n = 10 mice per group) was intranasally injected into mice (C57BL/6J) on day 3 after the intranasal injection of Ad-VEGF-C or Ad-LacZ (Lac) vectors (1.0×10^9 PFU/mouse), and the mice were euthanized the next day or 4 days later. (d) LPS of 1.9 mg/kg body weight (n = 10 mice per group) was intranasally injected into mice (C57BL/6J) on day 3 after the intranasal injection of Ad-VEGFR-3-Ig (R3) or Ad-LacZ (Lac), and the mice were euthanized the next day or 4 days later. (e, f) Anti-VEGFR-3 blocking antibody (R3 Ab) or isotype control (n = 8 mice per group) was intranasally injected into mice (C57BL/6J) on day 3 after intranasal injection of LPS of 1.9 mg/kg body weight, and the mice were euthanized 2 days later. (g) LPS of 3.8 mg/kg body weight (n = 10 mice per group) was intranasally injected into LysM-specific knockout mice of VEGFR-3 (LysMCre;VEGFR-$3^{flox/flox}$ [KO]) and the littermates. Mice were euthanized on days 0, 1, 3, and 5. The rates of macrophages are not shown in the experiments where lymphocytes did not appear in samples. Data represent means ± standard error of the mean values. $^{*}p < 0.05$, Student's *t*-test.

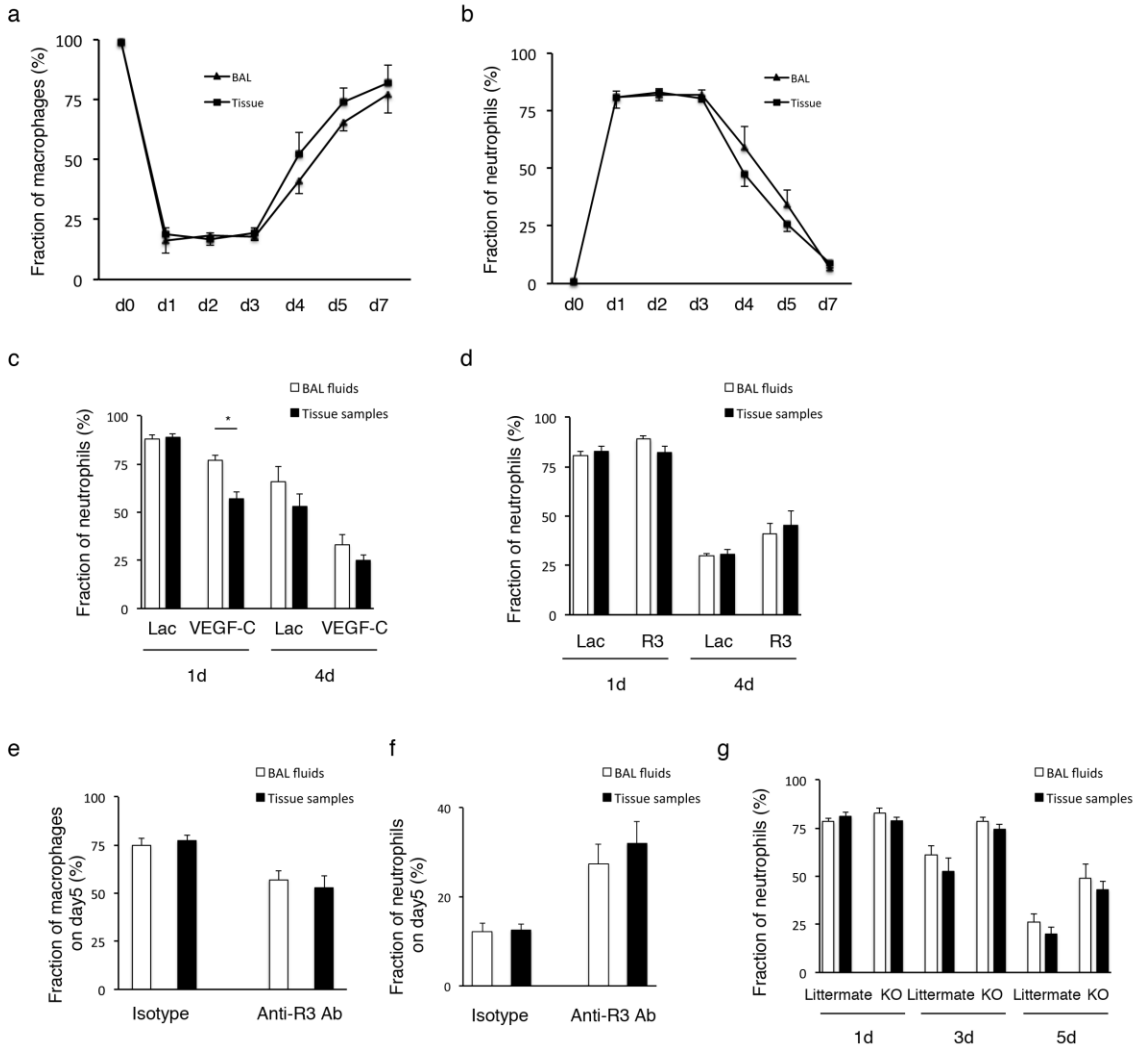
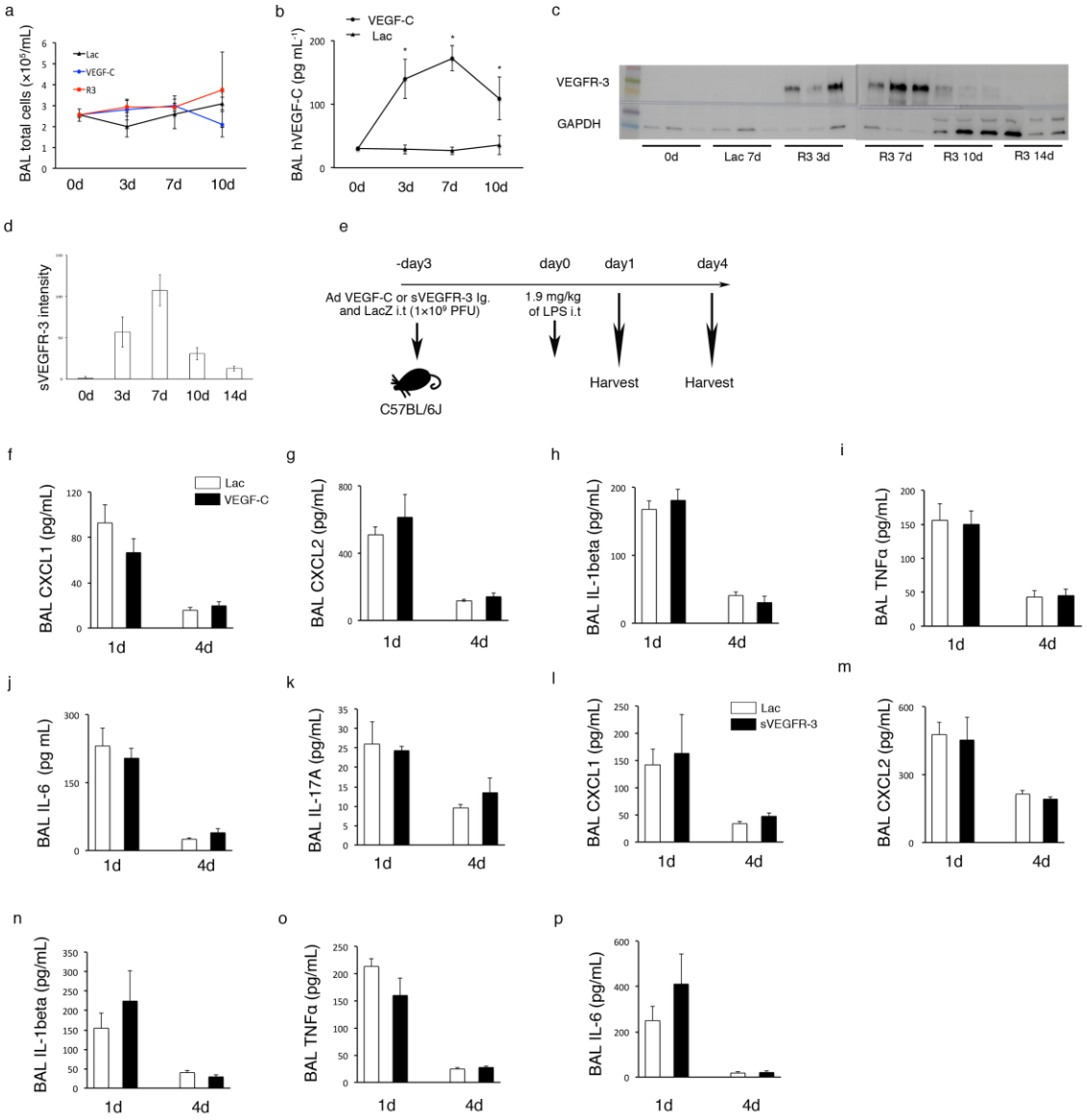

a
Fraction of macrophages (%)
BAL
Tissue
d0 d1 d2 d3 d4 d5 d7
b
Fraction of neutrophils (%)
c
BAL fluids
Tissue samples
Lac VEGF-C Lac VEGF-C
1d 4d
d
Lac R3 Lac R3
e
Fraction of macrophages on day5 (%)
Isotype Anti-R3 Ab
f
Fraction of neutrophils on day5 (%)
g
Littermate KO Littermate KO Littermate KO
1d 3d 5d

Figure S2. (a) Mice (C57BL/6J, n = 3 mice per treatment group) were intranasally injected with Ad-LacZ (Lac), Ad-VEGF-C vectors and Ad-human sVEGFR-3-Ig (R3) (1.0×10^9 PFU). Kinetics of the total cell counts in bronchoalveolar lavage (BAL) samples. (b) BAL human VEGF-C concentration determined via an enzyme-linked immunosorbent assay (ELISA) (c) Immunoblotting of human sVEGFR-3 and GAPDH in the BAL fluid harvested at 0, 3, 7, and 10 days after intranasal injection of Ad vectors. BAL fluid was concentrated 10-fold with a Minicon B15 Concentrator (Merck Millipore, Billerica, MA, USA). (d) Expression levels of soluble VEGFR-3. (e) Schematic of the experimental protocol using adenoviral vectors. (f-k) Lipopolysaccharide (LPS) of 1.9 μg/kg body weight (n = 10 mice per group) was intranasally administered to mice (C57BL/6J) on day 3 after the intranasal delivery of Ad-VEGF-C (VEGF-C) or Ad-LacZ (Lac) vectors (1.0×10^9 PFU/mouse), and the mice were euthanized on the next day or after 4 days. The concentrations of C-X-C Motif Chemokine Ligand (CXCL) 1, CXCL2, tumor necrosis factor α (TNFα), interleukin (IL)-1beta, IL-6 and IL-17A protein in the bronchoalveolar lavage fluid were quantified via ELISA. (l-p) Lipopolysaccharide (LPS) of 1.9 μg/kg body weight (n = 10 mice per group) was intranasally administered to mice (C57BL/6J) on day 3 after the intranasal delivery of Ad-sVEGFR-3 Ig. (R3) or Ad-LacZ (Lac) vectors (1.0×10^9 PFU/mouse), and the mice were euthanized on the next day or after 4 days. The concentrations of CXCL1, CXCL2, TNFα, IL-1beta and IL-6 protein in the bronchoalveolar lavage fluid were quantified via ELISA. Data are presented as the means ± standard error of the mean. $^*p < 0.05$, Student's *t*-test.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BAL total cells (×10⁵/mL)
Lac
VEGF-C
R3
0d
3d
7d
10d
b
BAL hVEGF-C (pg mL⁻¹)
VEGF-C
Lac
c
VEGFR-3
GAPDH
0d
Lac 7d
R3 3d
R3 7d
R3 10d
R3 14d
d
sVEGFR-3 intensity
e
-day3
day0
day1
day4
Ad VEGF-C or sVEGFR-3 Ig. and LacZ i.t (1×10⁹ PFU)
1.9 mg/kg of LPS i.t
Harvest
C57BL/6J
f
BAL CXCL1 (pg/mL)
g
BAL CXCL2 (pg/mL)
h
BAL IL-1beta (pg/mL)
i
BAL TNFα (pg/mL)
j
BAL IL-6 (pg mL)
k
BAL IL-17A (pg/mL)
l
BAL CXCL1 (pg/mL)
sVEGFR-3
m
BAL CXCL2 (pg/mL)
n
BAL IL-1beta (pg/mL)
o
BAL TNFα (pg/mL)
p
BAL IL-6 (pg/mL)
1d
4d

Figure S3. Mafia mice and vascular endothelial growth factor receptor 3-expressing (VEGFR-3^{+}) monocyte lineages. (a) Comparison of bronchoalveolar lavage (BAL) cell counts between C57BL/6J WT and Mafia mice receiving treatment with 1.9 mg/kg body weight of lipopolysaccharide (LPS; n = 3 mice per group). Time courses during LPS-induced injury were similar between Mafia mice and C57BL/6J mice in terms of the peak of inflammation and subsequent resolution. (b) The presence of VEGFR-3^{+} monocyte lineage cells in the BAL at 4 days after LPS injection. Flow cytometry plots demonstrated VEGFR-3 expression in macrophage colony-stimulating factor receptor-expressing (M-CSFR^{+}) monocyte lineage cells in Mafia mice. VEGFR-3^{-} (Gate 1) and VEGFR-3^{+} (Gate 2) cells were sorted. (c) M-CSFR^{-}CD11b^{+}Ly6G^{+} neutrophils on day 2 were also sorted (Gate 3). (d) Relative *Flt4* mRNA expression in neutrophils and VEGFR-3^{-} and VEGFR-3^{+} monocyte lineage cells were estimated via real time reverse transcription polymerase chain reaction (RT-PCR) (n = 3 mice per group). Sorted VEGFR-3^{+} cells expressed markedly higher *Flt4* mRNA levels relative to that of VEGFR-3^{-} cells, validating the activity of the primary antibody against VEGFR-3. (e) Protocol schematic of a model experiment using VEGFR-3$^{flox/flox}$;LysMCre (KO) and VEGFR-3$^{flox/flox}$ (Littermates) mice. Abbreviations: Neu, neutrophil; Macs, macrophages. Data are presented as the means ± standard error of the mean. Differences between multiple groups were assessed using one-way analysis of variance, and individual comparisons were analyzed using Tukey's *post hoc* tests. $^{**}p < 0.01$.

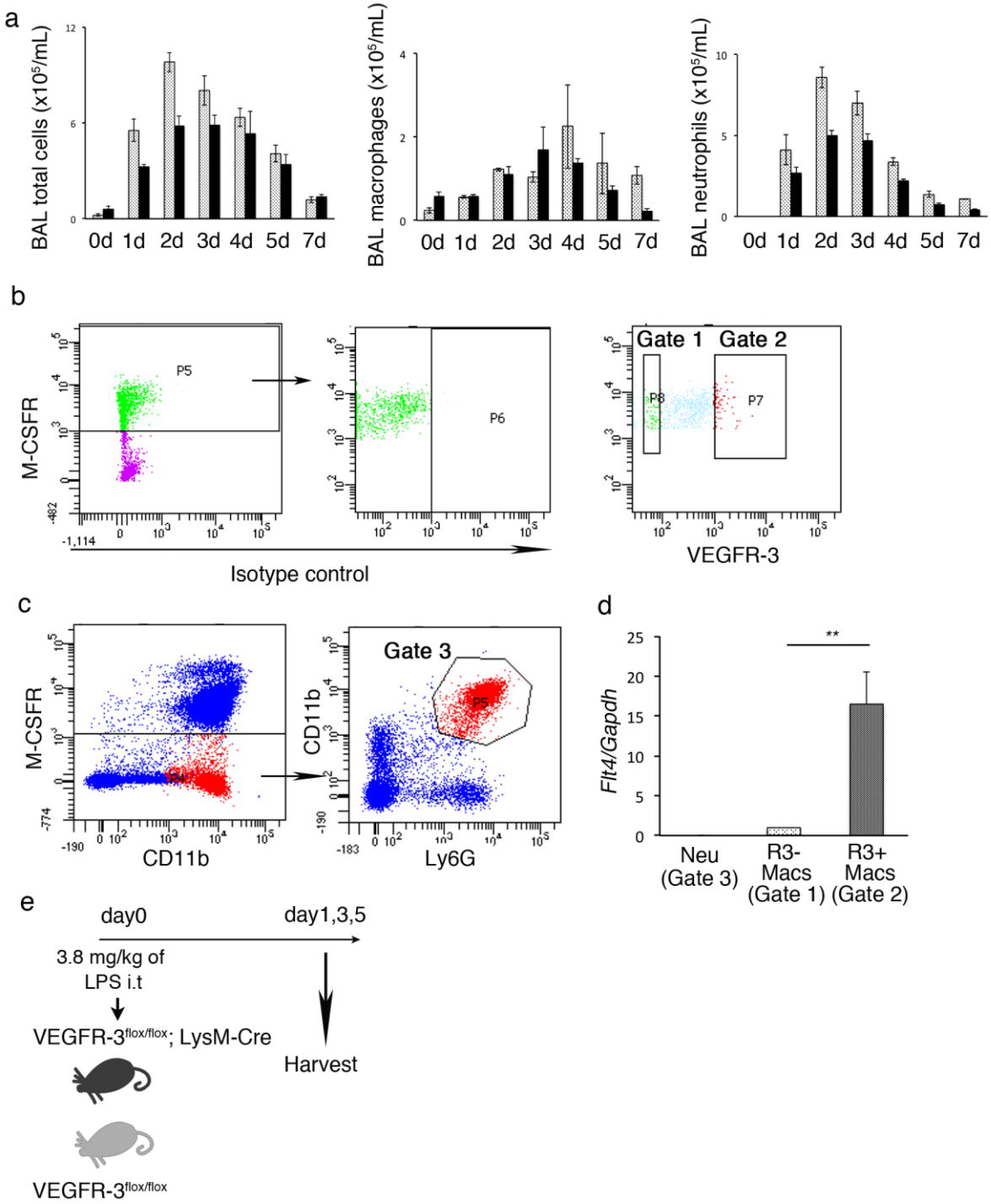
a
BAL total cells (x10⁵/mL)
BAL macrophages (x10⁵/mL)
BAL neutrophils (x10⁵/mL)
0d 1d 2d 3d 4d 5d 7d
b
M-CSFR
P5
P6
Isotype control
Gate 1
Gate 2
P8
P7
VEGFR-3
c
M-CSFR
CD11b
Gate 3
P5
CD11b
Ly6G
d
Flt4/Gapdh
**
Neu (Gate 3)
R3- Macs (Gate 1)
R3+ Macs (Gate 2)
e
day0
day1,3,5
3.8 mg/kg of LPS i.t
VEGFR-3flox/flox; LysM-Cre
Harvest
VEGFR-3flox/flox

Figure S4. Lipopolysaccharide (LPS) of 1.9 μg/kg was intranasally administered to mice with deletion of LysM-specific VEGFR-3 gene and the littermates. The mice were harvested before LPS injection (n = 5 mice per group) and on day 1, 3, 5 after (n = 10 mice per group). (a-h) The concentrations of C-X-C Motif Chemokine Ligand (CXCL) 1, CXCL2, monocyte chemoattractant protein 1, tumor necrosis factor α, interleukin (IL)-1beta, IL-6, IL-17A, and IL-10 protein in the bronchoalveolar lavage fluid were quantified via an enzyme-linked immunosorbent assay. Data are presented as the means ± standard error of the mean. Comparison between the two groups were analyzed using Student *t*-test.

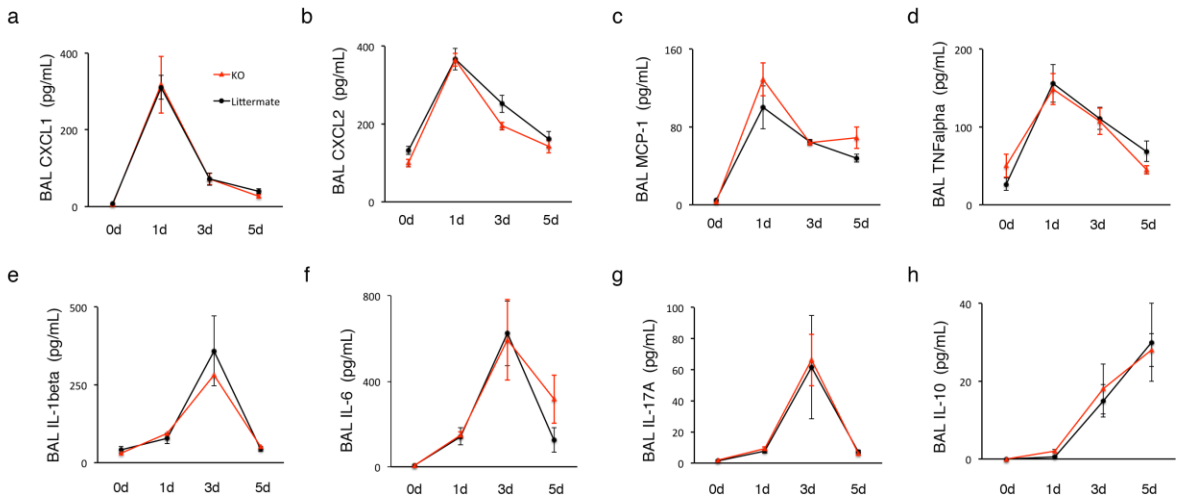

Figure S5. The effects of vascular endothelial growth factor (VEGF)-C/VEGF receptor (VEGFR)-3 signaling on the efferocytosis of apoptotic neutrophils in alveolar macrophages (AMs). (a) Schematic showing the phagocytosis of extrinsic apoptotic neutrophils by alveolar macrophages of mice with the deletion of LysM-specific-VEGFR-3 gene and the littermates. (b) Histogram of VEGFR-3 expression on AMs on days 0, 1, 2 and 5 during the course of lung injury induced by 1.9 mg/kg of lipopolysaccharide. (c) Intratracheally injection of cytochalasin D markedly decreased the percentage of $PKH26^{+}$M-$CSFR^{+}$ $CD11c^{+}CD11b^{-}F4/80^{+}I\text{-}A^{-}$ AMs compared with that of vehicle controls (n = 3 per each group) ($p < 0.05$). Data represent as percentage of PKH^{+}M-$CSFR^{+}CD11c^{+}CD11b^{-}F4/80^{+}I\text{-}A^{-}$ AMs for M-$CSFR^{+}CD11c^{+}CD11b^{-}F4/80^{+}I\text{-}A^{-}$ AMs. (d-h) Efferocytosis-related gene profiles on the alveolar macrophages and their effect on vascular endothelial growth factor (VEGF)-C/VEGF receptor (VEGFR)-3 signaling. Alveolar macrophages were harvested by bronchoalveolar lavage on day 0. After incubation in 12-well plates for 2 h, the alveolar macrophages were treated with VEGF-C recombinant protein with/without VEGFR-3 blocking antibody (anti-R3 Ab). After 1 h of incubation, macrophages were harvested. Relative mRNA levels of *Axl1, Cd81*, *Cd36*, *Mertk*, and *Gpnmb* in the three groups were determined via quantitative real-time reverse transcription polymerase chain reaction. Data are presented as the means ± standard error of the mean. Comparison between the two groups was analyzed using Student *t*-test. Differences between multiple groups were assessed using one-way analysis of variance.

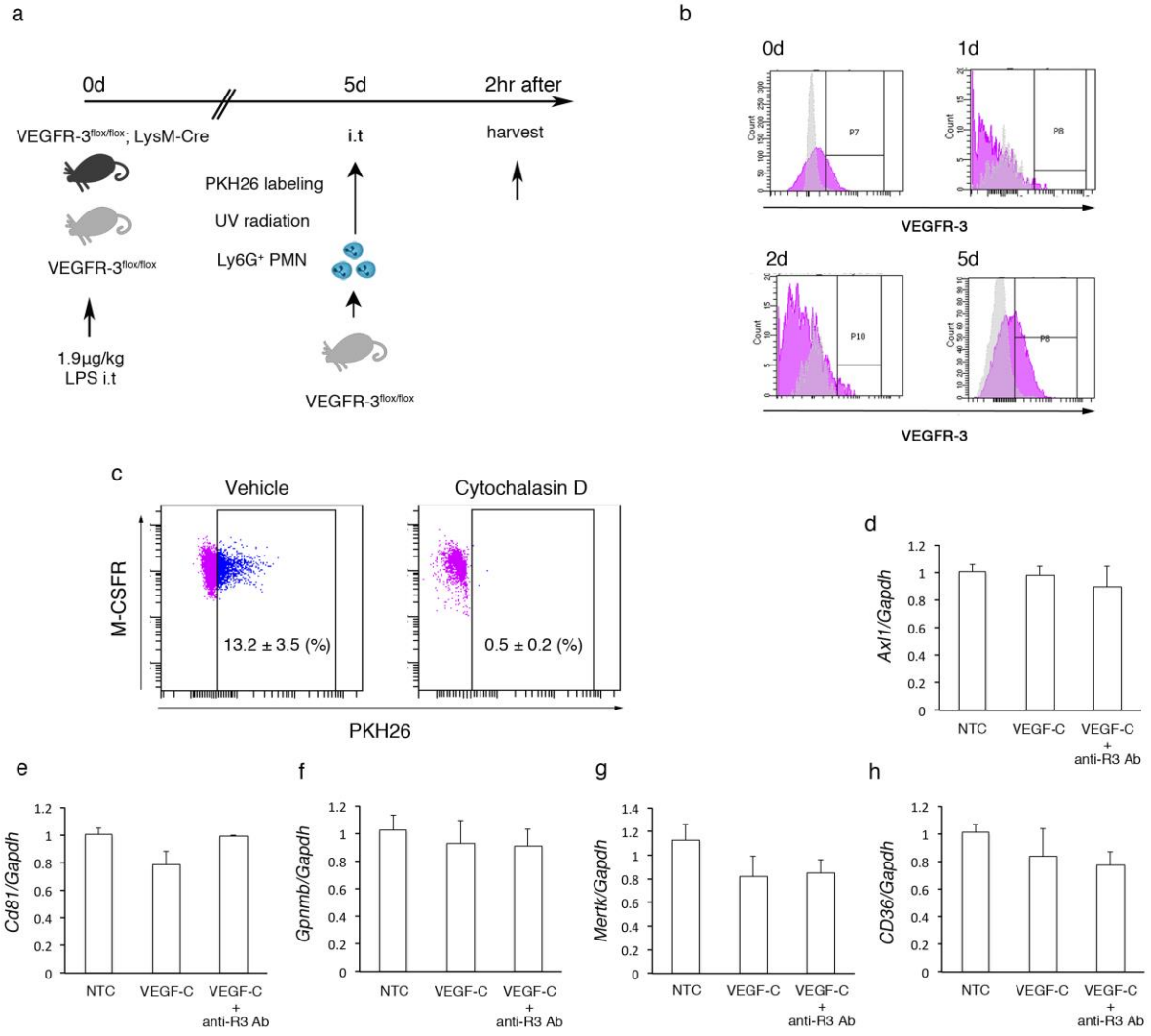
a
0d
5d
2hr after
VEGFR-3flox/flox; LysM-Cre
i.t
harvest
PKH26 labeling
UV radiation
Ly6G+ PMN
VEGFR-3flox/flox
1.9μg/kg
LPS i.t
VEGFR-3flox/flox
b
0d
1d
2d
5d
P7
P8
P10
P8
Count
VEGFR-3
VEGFR-3
c
Vehicle
Cytochalasin D
M-CSFR
13.2 ± 3.5 (%)
0.5 ± 0.2 (%)
PKH26
d
Axl1/Gapdh
e
Cd81/Gapdh
f
Gpnmb/Gapdh
g
Mertk/Gapdh
h
CD36/Gapdh
NTC
VEGF-C
VEGF-C + anti-R3 Ab

Figure S6. (a) Histogram of flow cytometry results for VEGFR-3 expression in human peripheral blood-derived colony-stimulating factor (M-CSF)-stimulated macrophages. VEGFR-3^{-} (Gate 1) and VEGFR-3^{+} (Gate 2) macrophages were sorted. (b) Relative FLT4 mRNA expression in VEGFR-3^{-} and VEGFR-3^{+} human monocyte-derived macrophages (hMDMs) was estimated via real-time reverse transcription polymerase chain reaction (n = 3 mice per group). Sorted VEGFR-3^{+} hMDMs expressed markedly higher FLT4 mRNA levels than those of VEGFR-3^{-} cells, validating the activity of the primary antibody against VEGFR-3. (c) The concentrations of VEGF-C in BAL fluid obtained from patients with ARDS (n = 12) and COP (n = 14) as well as healthy volunteers (n = 18) were determined by ELISA. (d) The concentrations of IL-10 in BAL fluid obtained from patients with ARDS (n = 12) and COP (n = 14) were determined by ELISA. (e) In a chamber slide assay, phagocytosis rates in AMs obtained from LysMCre;VEGFR-3$^{flox/flox}$ (KO) mice and the littermates incubated with COP (n = 14) or ARDS BALF (n = 12). $^{**}p < 0.01$, $^{***}p < 0.001$ by Steel-Dwass's *post hoc* tests when one-way ANOVA tests were $p < 0.05$.