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Supplemental Information

Controlling the Phenotype of Tumor-Infiltrating Macrophages via the PHD-HIF Axis Inhibits Tumor Growth in a Mouse Model

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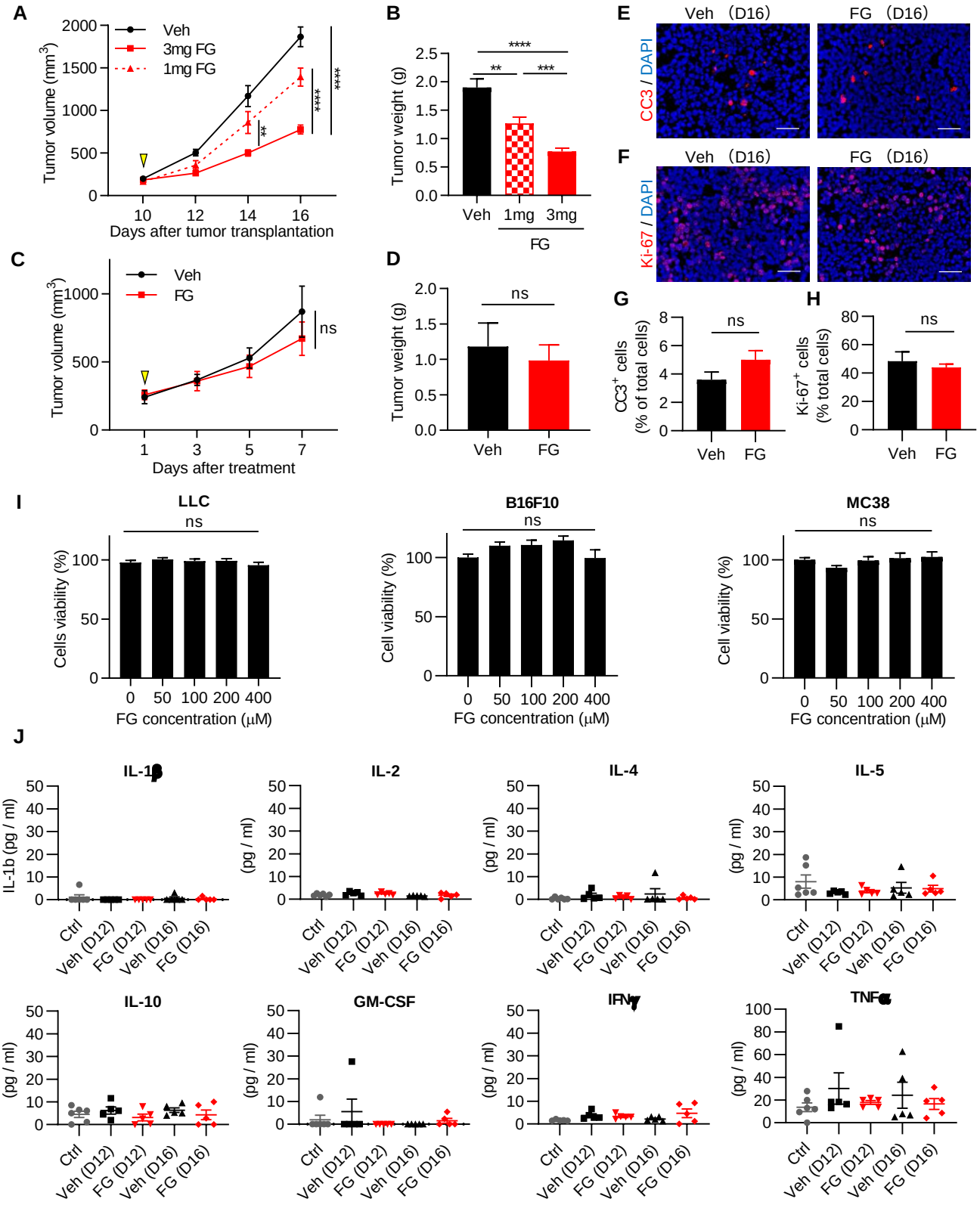
A
Tumor volume (mm3)
Veh
3mg FG
1mg FG
Days after tumor transplantation
B
Tumor weight (g)
Veh
1mg
3mg
FG
C
Tumor volume (mm3)
Veh
FG
ns
Days after treatment
D
Tumor weight (g)
ns
Veh
FG
E
Veh (D16)
FG (D16)
CC3 / DAPI
F
Veh (D16)
FG (D16)
Ki-67 / DAPI
G
CC3+ cells (% of total cells)
ns
Veh
FG
H
Ki-67+ cells (% total cells)
ns
Veh
FG
I
LLC
B16F10
MC38
ns
Cells viability (%)
Cell viability (%)
FG concentration (μM)
J
IL-1β
IL-2
IL-4
IL-5
IL-10
GM-CSF
IFNγ
TNFα
IL-1b (pg / ml)
(pg / ml)
Ctrl
Veh (D12)
FG (D12)
Veh (D16)
FG (D16)

Figure S1.

Figure S1. Effect of FG on tumor growth in LLC, B16F10, and MC38 tumor models. Related to Figure 1.

(A) Transplanted LLC (1×10^6 cells/mouse) tumor size in C57BL/6 mice was measured after treatment with vehicle (n = 15), 1 mg FG (n = 10), or 3 mg FG (n = 15) on day 10. **p < 0.01; ****p < 0.0001; two-way ANOVA. (B) Tumor weight was measured at the end of the experiment on day 16 (n = 10–15). **p < 0.01, ***p < 0.001, ****p < 0.0001; Mann-Whitney test. (C) Transplanted MC38 (1×10^6 cells/mouse) tumor size in C57BL/6 mice was measured after treatment with vehicle (n = 4) or 3 mg FG (n = 5) on day 10. ns, not significant; two-way ANOVA. (D) Tumor weight was measured at the end of the experiment on day 16 (n = 3–4). ns, not significant; Mann-Whitney test. (E) Immunofluorescence (IF) of cleaved caspase-3 (CC3; red)-stained sections of LLC tumors on day 16. Scale bar = 50 μm. (F) IF images of cleaved Ki-67 (red)-stained sections of LLC tumors on day 16. Scale bar = 50 μm. (G) Quantification of the $CC3^+$ cell ratio of total cells on day 16 (n = 5). ns, not significant; Mann-Whitney test. (H) Quantification of the $Ki\text{-}67^+$ cell ratio of total cells on day 16 (n = 3). ns, not significant; Mann-Whitney test. (I) Cell proliferation of LLC, B16F10, and MC38 cells were measured 72 h after FG treatment. ns, not significant vs. 0 μM, as determined by one-way ANOVA. Three independent experiments were performed in triplicate. (J) Blood plasma concentrations were determined via multiplex assays.

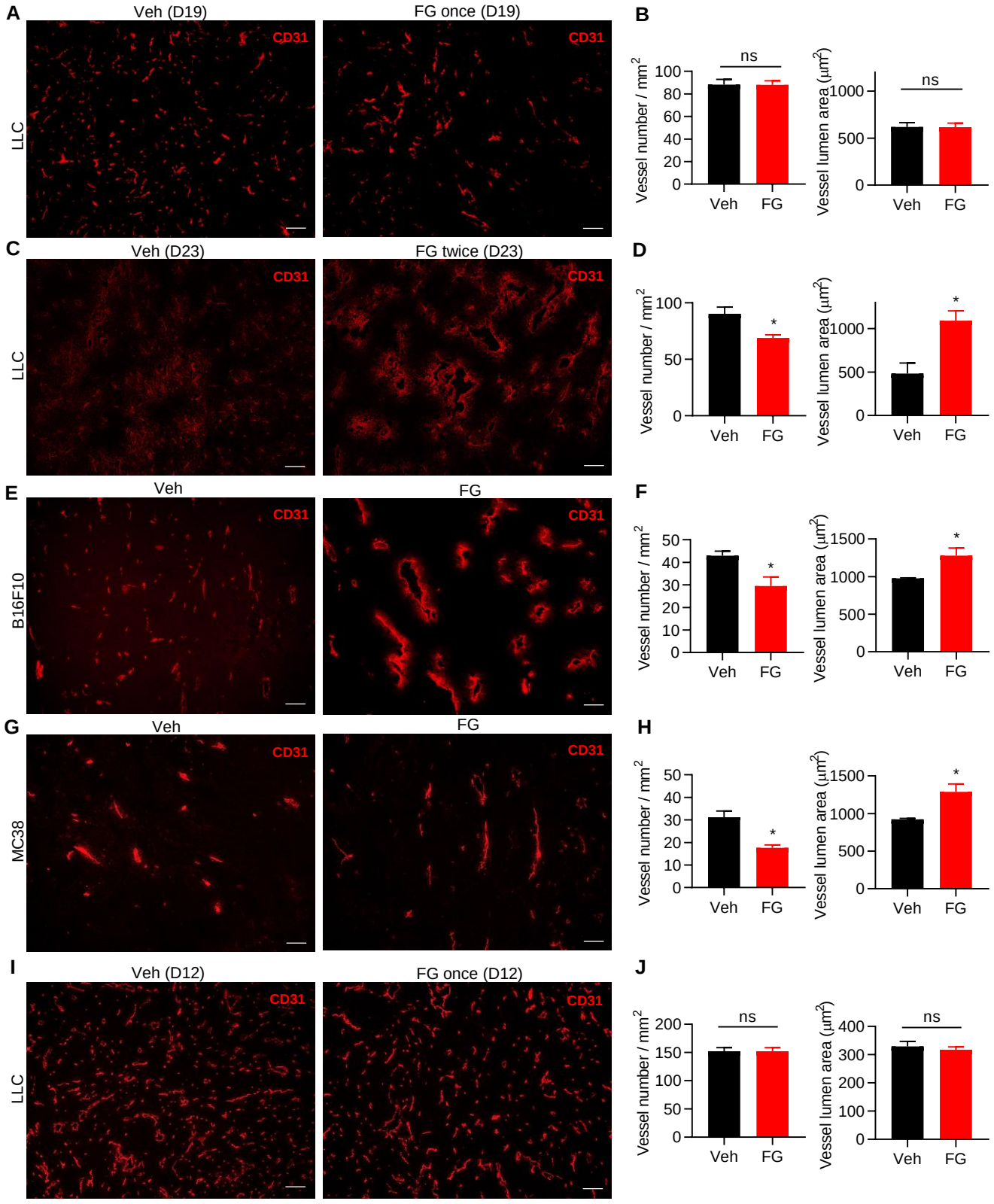
A
Veh (D19)
FG once (D19)
CD31
LLC
B
Vessel number / mm2
Vessel lumen area (μm2)
ns
Veh
FG
C
Veh (D23)
FG twice (D23)
D
*
E
Veh
FG
B16F10
F
G
MC38
H
I
Veh (D12)
FG once (D12)
J

Figure S2.

Figure S2. Effect of FG on tumor vessels in LLC, B16F10, and MC38 tumor models. Related to Figure 2.

(A) Immunofluorescence (IF) images of CD31-stained sections of LLC tumors on day 19 from vehicle- and one-time FG-treated mice. (B) Quantification of vessel density and vessel lumen area in s.c. LLC tumors from vehicle- and FG-treated mice (n = 3). ns, not significant; unpaired Student's t-test. (C) IF images of CD31-stained sections of LLC tumors on day 23 from vehicle- and FG-treated (multiple times) mice. (D) Quantification of vessel density and vessel lumen area in s.c. LLC tumors of vehicle and FG-treated mice (n = 3). *p < 0.05; unpaired Student's t-test. (E) IF images of CD31-stained sections of B16F10 tumors on day 16 from vehicle- and FG-treated mice. (F) Quantification of vessel density and vessel lumen area in s.c. B16F10 tumors from vehicle and FG-treated mice (n = 3). *p < 0.05; unpaired Student's *t*-test. (G) IF images of CD31-stained sections of MC38 tumors on day 16 from vehicle and FG-treated mice. (H) Quantification of vessel density and vessel lumen area in s.c. MC38 tumors from vehicle and FG-treated mice (n = 3). *p < 0.05; unpaired Student's *t*-test. Scale bar = 100 μm. Data represent the mean ± SEM. (I) IF images of CD31-stained sections of LLC tumors on day 12 from vehicle- and one-time FG-treated mice. (J) Quantification of vessel density and vessel lumen area in s.c. LLC tumors from vehicle- and FG-treated mice (n = 3). ns, not significant; unpaired Student's *t*-test.

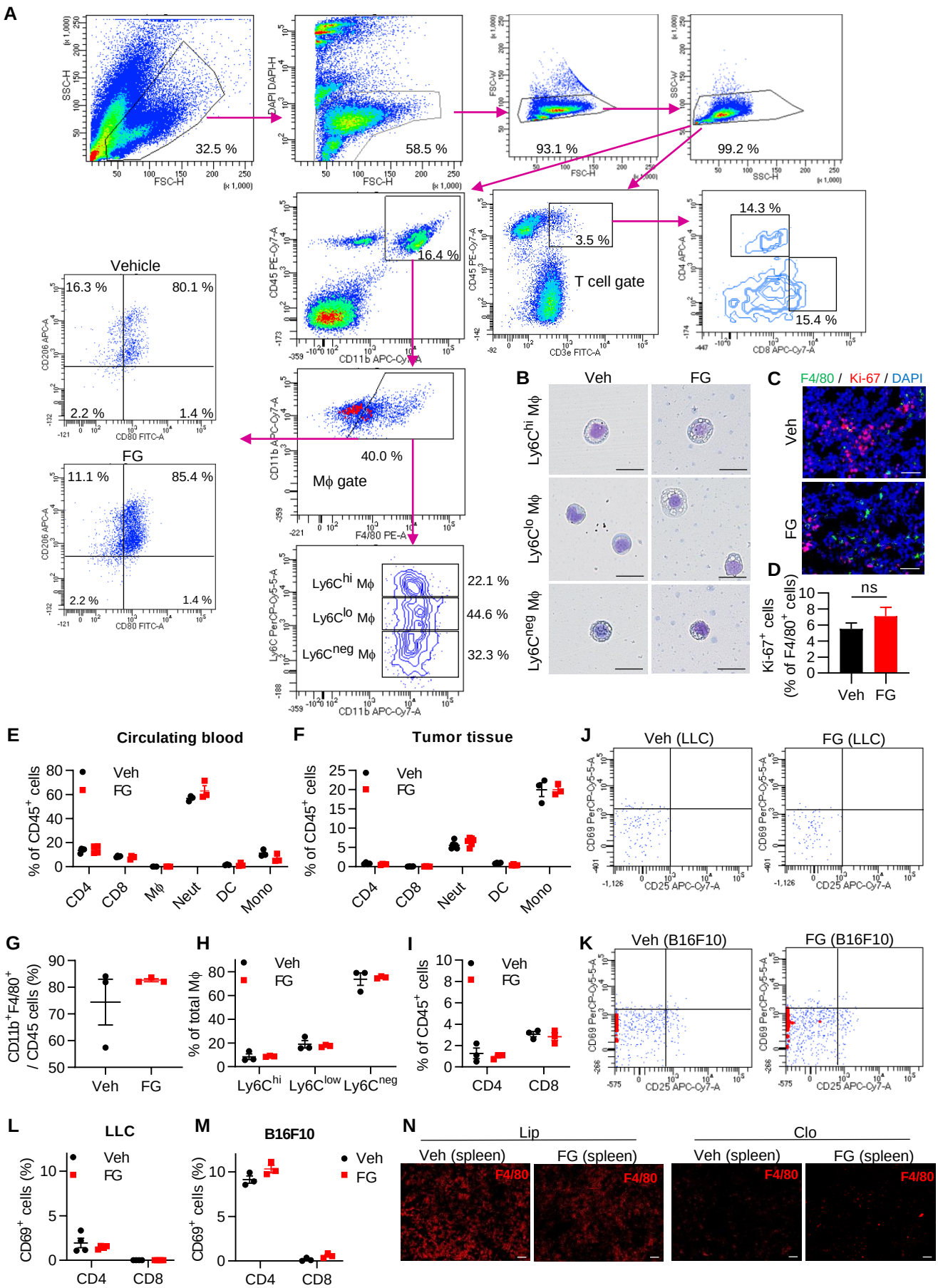
A
32.5 %
58.5 %
93.1 %
99.2 %
16.4 %
3.5 %
T cell gate
14.3 %
15.4 %
Vehicle
16.3 %
80.1 %
2.2 %
1.4 %
FG
11.1 %
85.4 %
2.2 %
1.4 %
40.0 %
Mϕ gate
Ly6C^hi Mϕ
Ly6C^lo Mϕ
Ly6C^neg Mϕ
22.1 %
44.6 %
32.3 %
B
Veh
FG
C
F4/80 / Ki-67 / DAPI
D
ns
Ki-67+ cells (% of F4/80+ cells)
E
Circulating blood
% of CD45+ cells
CD4
CD8
Mϕ
Neut
DC
Mono
F
Tumor tissue
J
Veh (LLC)
FG (LLC)
G
CD11b+F4/80+ / CD45 cells (%)
H
% of total Mϕ
I
K
Veh (B16F10)
FG (B16F10)
L
LLC
CD69+ cells (%)
M
B16F10
N
Lip
Clo
Veh (spleen)
FG (spleen)
F4/80

Figure S3.

Figure S3. FG inhibits tumor growth through Mϕs. Related to Figure 3.
(A) Flow cytometric analysis gate strategy of Mϕs and T cells in s.c. LLC tumors on day 16 (7 days after vehicle/FG treatment). (B) Morphology of Ly6C^{hi}, Ly6C^{lo}, and Ly6C^{neg} Mϕs. Scale bar = 20 μm. (C) IF images of Ki-67 (red)- and F4/80 (green)- stained sections of LLC tumors on day 16. Scale bar = 50 μm. (D) Quantification of the Ki-67+ cell ratio of F4/80^{+} cells on day 16 (n = 3). ns, not significant; unpaired Student's *t*-test. (E) Quantification of the ratio of immune cells in circulating blood of LLC tumor-bearing mice on day 16 by flow cytometry (n = 3–4). (F) Quantification of the ratio of immune cells in LLC tumor tissue on day 16 by flow cytometry (n = 3–6). CD4 lymphocyte; CD45^{+}CD3^{+}CD4^{+}, CD8 lymphocyte; CD45^{+}CD3^{+}CD8^{+}, Mϕ; CD45^{+}CD11b^{+}F4/80^{+}, neutrophil; CD45^{+}CD11b^{+}Ly6G^{+}, dendritic cell; CD45^{+}CD11b^{+}CD11c^{+}, monocyte; CD45^{+}CD11b^{+}F4/80^{-}Ly6G^{-}. (G) Quantification of the ratio of tumor-infiltrating CD11b^{+}F4/80^{+} cells/CD45^{+} cells in MC38 tumors by flow cytometry (n = 3). (H) Quantification of the Ly6C^{hi}, Ly6C^{lo}, and Ly6C^{neg} Mϕ ratio in MC38 tumors by flow cytometric analysis (n = 3). (I) Quantification of CD4^{+} and CD8^{+} lymphocyte cell ratio in MC38 tumors by flow cytometric analysis (n = 3). (J, K) Representative flow cytometric analysis of CD45^{+}CD3^{+} lymphocyte cells of (J) LLC or (K) B16F10 tumor-bearing mice on day 16. (L, M) Quantification of the ratio of CD69^{+} cells in circulating blood of (L) LLC or (M) B16F10 tumor-bearing mice on day 16 by flow cytometry (n = 3–4). (N) IF images of F4/80 (red)-stained spleen sections of LLC tumor-bearing mice treated with liposome control or clodronate liposome and vehicle or 3 mg FG. Scale bar = 50 μm.

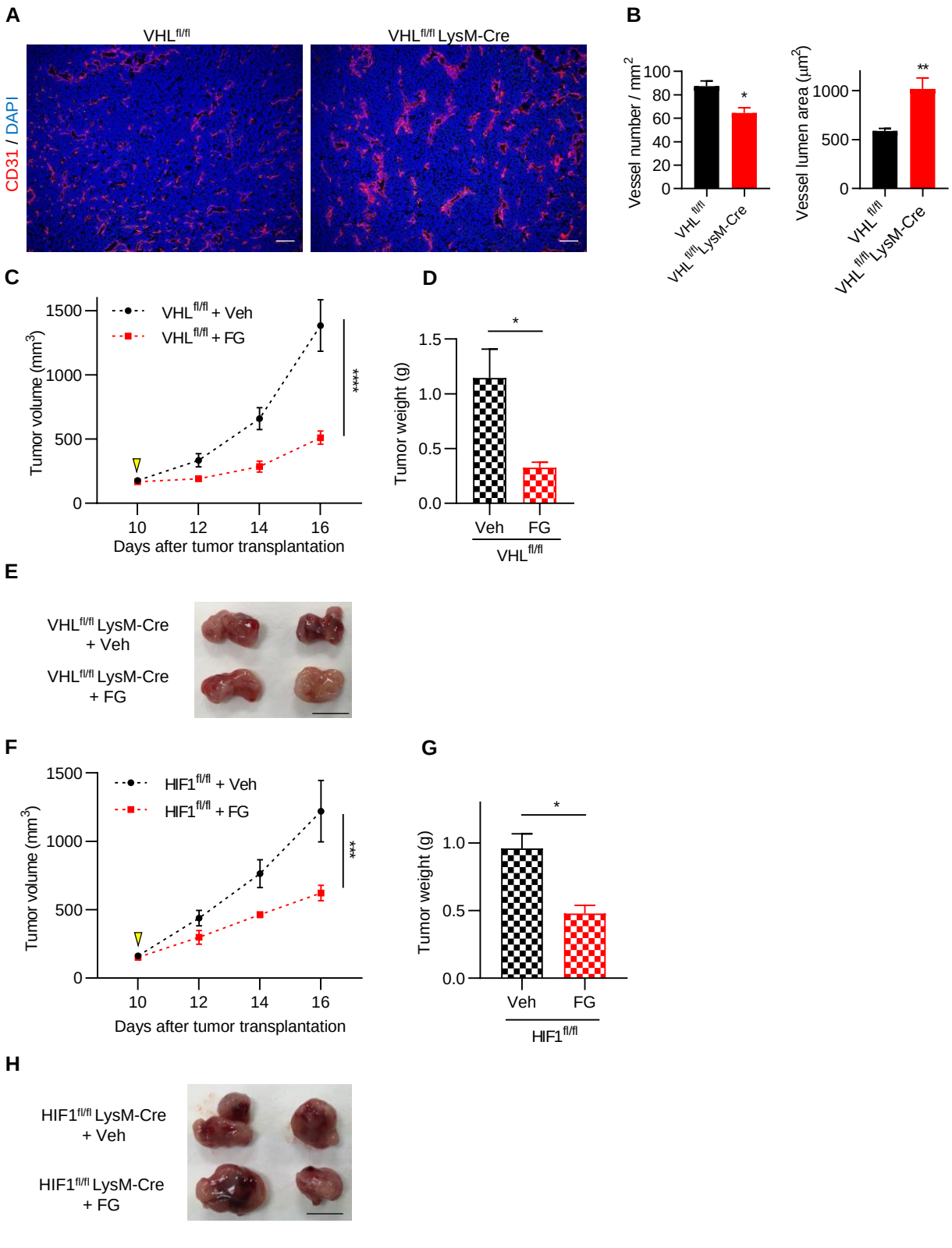
A
VHL fl/fl
VHL fl/fl LysM-Cre
CD31 / DAPI
B
Vessel number / mm2
Vessel lumen area (μm2)
VHL fl/fl
VHL fl/fl LysM-Cre
C
VHL fl/fl + Veh
VHL fl/fl + FG
Tumor volume (mm3)
Days after tumor transplantation
****
D
Tumor weight (g)
Veh
FG
VHL fl/fl
E
VHL fl/fl LysM-Cre + Veh
VHL fl/fl LysM-Cre + FG
F
HIF1 fl/fl + Veh
HIF1 fl/fl + FG
Tumor volume (mm3)
Days after tumor transplantation
***
G
Tumor weight (g)
Veh
FG
HIF1 fl/fl
H
HIF1 fl/fl LysM-Cre + Veh
HIF1 fl/fl LysM-Cre + FG

Figure S4.

Figure S4. FG inhibits tumor growth through Mϕs via the PHD-HIF axis. Related to Figure 4.
(A) IF images of CD31 staining in $VHL^{fl/fl}$ or $VHL^{fl/fl}$ LysM-Cre mouse tumors. Scale bars = 100 μm. (B) Quantification of vessel density and vessel luminal area in $VHL^{fl/fl}$ (n = 5) or $VHL^{fl/fl}$ LysM-Cre mouse tumors (n = 6). *p < 0.05, **p < 0.01; Mann-Whitney test. Data represent the mean ± SEM. (C) Tumor growth curves of the LLC tumor model of $VHL^{fl/fl}$ mice. Treatment with Veh or FG (3 mg; treated on day 10); n = 4. ****p < 0.0001; two-way ANOVA. (D) Tumor weight on day 16 (n = 4). *p < 0.05; Mann-Whitney test. (E) Images of tumors of $VHL^{fl/fl}$ LysM-Cre mice treated with/without FG on day 16. Scale bar = 1 cm. (F) Tumor growth curves of the LLC tumor model of $HIF1^{fl/fl}$ mice. Treatment with Veh (n = 4) or FG (3 mg; treated on day 10; n = 5). ***p < 0.001; two-way ANOVA. (G) Tumor weight on day 16 (n = 4–5). *p < 0.05; Mann-Whitney test. (H) Images of tumors of $HIF1^{fl/fl}$ LysM-Cre mice treated with/without FG on day 16. Scale bar = 1 cm.

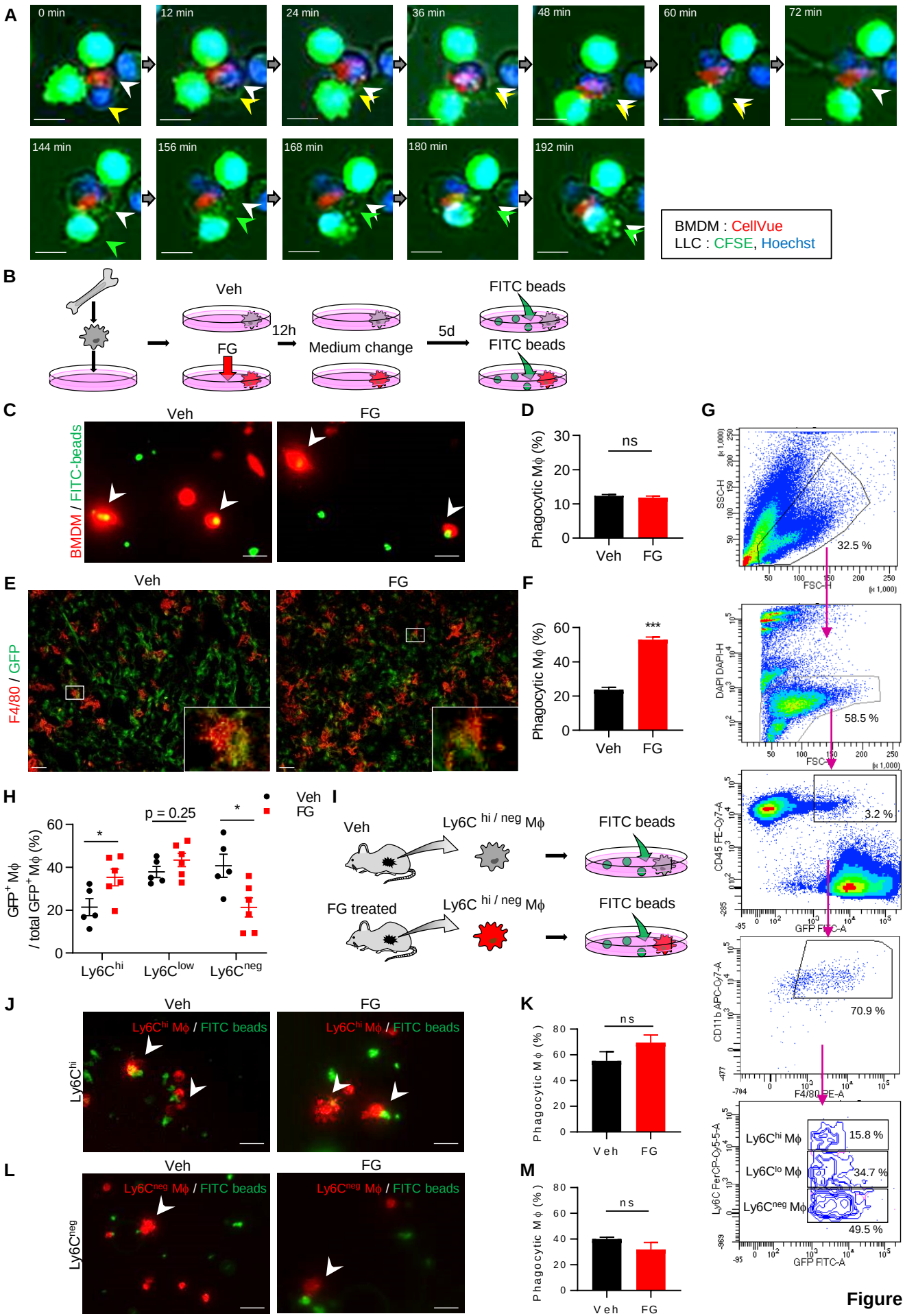
A
0 min
12 min
24 min
36 min
48 min
60 min
72 min
144 min
156 min
168 min
180 min
192 min
BMDM : CellVue
LLC : CFSE, Hoechst
B
Veh
FG
12h
Medium change
5d
FITC beads
FITC beads
C
Veh
FG
BMDM / FITC-beads
D
Phagocytic Mϕ (%)
ns
Veh
FG
E
Veh
FG
F4/80 / GFP
F
Phagocytic Mϕ (%)
***
Veh
FG
G
SSC-H
32.5 %
FSC-H
DAPI DAPI-H
58.5 %
FSC-H
CD45 FE-Cy7-A
3.2 %
GFP FITC-A
CD11b APC-Cy7-A
70.9 %
F4/80 PE-A
Ly6C FerCP-Cy5-5-A
Ly6C^hi Mϕ
15.8 %
Ly6C^lo Mϕ
34.7 %
Ly6C^neg Mϕ
49.5 %
GFP FITC-A
H
Veh
FG
GFP+ Mϕ / total GFP+ Mϕ (%)
p = 0.25
Ly6C^hi
Ly6C^low
Ly6C^neg
I
Veh
Ly6C hi / neg Mϕ
FITC beads
FG treated
Ly6C hi / neg Mϕ
FITC beads
J
Veh
FG
Ly6C^hi
Ly6C^hi Mϕ / FITC beads
Ly6C^hi Mϕ / FITC beads
K
Phagocytic Mϕ (%)
ns
Veh
FG
L
Veh
FG
Ly6C^neg
Ly6C^neg Mϕ / FITC beads
Ly6C^neg Mϕ / FITC beads
M
Phagocytic Mϕ (%)
ns
Veh
FG

Figure S5.

Figure S5. FG activates phagocytosis of Ly6C-positive macrophages while TME improvement prolongs the anti-tumor effect of Mϕs. Related to Figure 5.
(A) In vitro live-cell microscopy of CellVue-labeled (red) BMDM as well as CFSE (green)- and Hoechst (blue)-labeled LLC cells. BMDMs were treated with FG. Upper row, activated BMDM phagocytosed first-encountered LLC cells; lower row, activated BMDM phagocytosed second-encountered LLC cells continuously. (B) Schematic diagram of in vitro bead phagocytosis assay. (C) Images of BMDM bead phagocytosis assay. BMDM, red; FITC-beads, green. Scale bar = 20 μm. (D) Quantification of phagocytic BMDM ratio. ns, not significant; Mann-Whitney test. Two independent experiments were performed. (E) Images of IF staining of tumor phagocytic macrophages. C57BL/6 mice were transplanted with GFP-expressing LLC and treated with vehicle or 3 mg FG. Tumor sections were stained with anti-GFP (green) and anti-F4/80 (red) antibodies. Scale bar = 20 μm. (F) Quantification of phagocytic ($GFP^+F4/80^+$) cell number in the tumors (n = 3). ***p < 0.001; unpaired Student's *t*-test. (G) Flow cytometric analysis gate strategy of GFP^+Mϕs in s.c. LLC tumors on day 16 (7 days after vehicle/FG treatment). (H) Quantification of GFP^+ Mϕ/total GFP^+ Mϕ ratio subdivided by $Ly6C^{hi}$, $Ly6C^{lo}$, and $Ly6C^{neg}$ in LLC tumors by flow cytometric analysis (n = 5–6). ns, not significant (p = 0.25 vs. vehicle); *p < 0.05, **p < 0.01 vs. vehicle; Mann-Whitney test. (I) Schematic diagram of ex vivo bead phagocytosis assay. (J) Images of ex vivo phagocytosis assay. $Ly6C^{hi}$ Mϕ, red; FITC-beads, green. Scale bar = 20 μm. (K) Quantification of phagocytic $Ly6C^{hi}$ Mϕ ratio (n = 4–5). ns, not significant (p = 0.19); Mann-Whitney test. (L) Images of ex vivo phagocytosis assay. $Ly6C^{neg}$ Mϕ, red; FITC-beads, green. Scale bar = 20 μm. (M) Quantification of phagocytic $Ly6C^{neg}$ Mϕ ratio (n = 3–4). ns, not significant; Mann-Whitney test. Data represent the mean ± SEM.

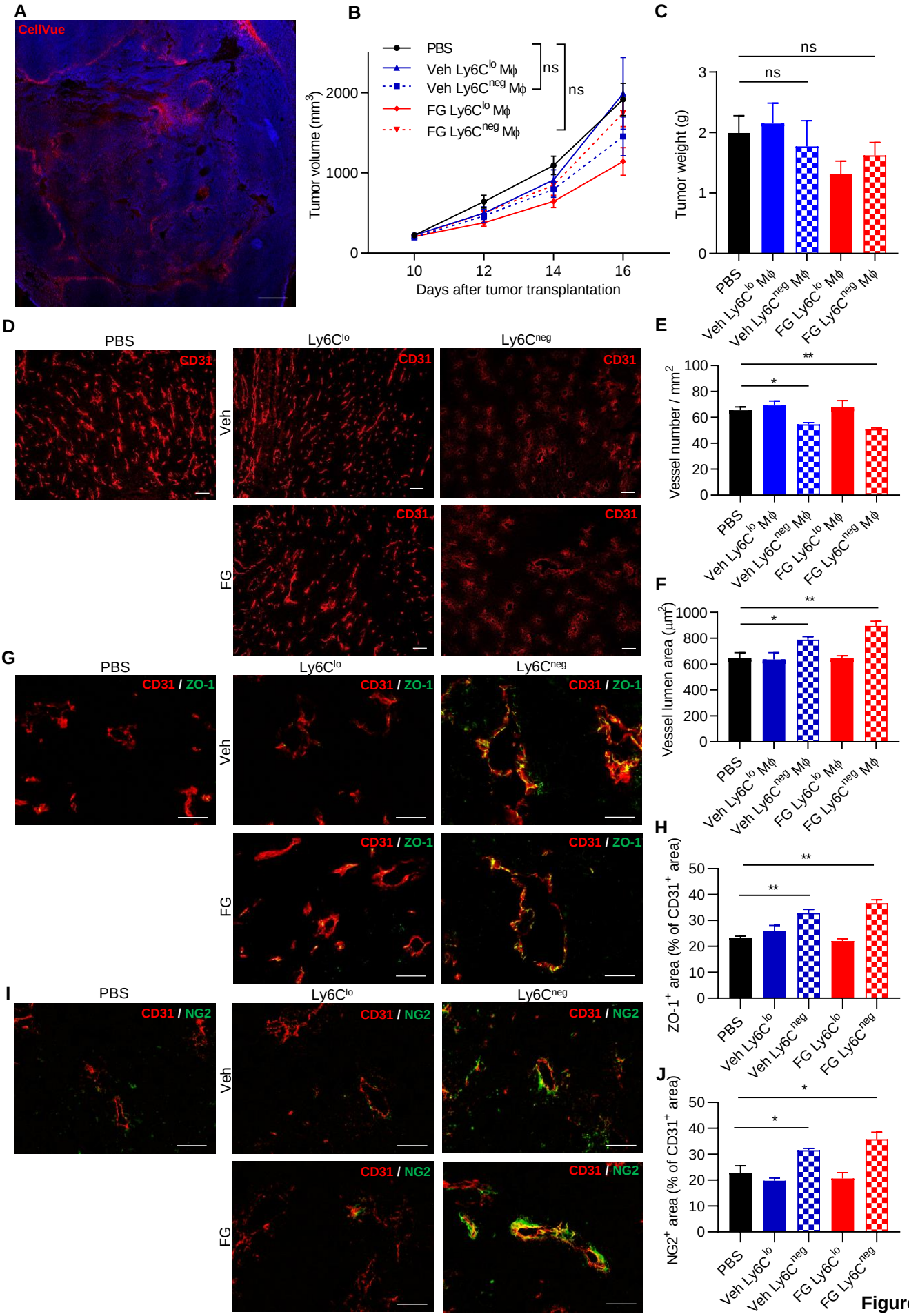
A
CellVue
B
PBS
Veh Ly6C^lo Mϕ
Veh Ly6C^neg Mϕ
FG Ly6C^lo Mϕ
FG Ly6C^neg Mϕ
ns
ns
Tumor volume (mm^3)
2000
1000
0
10
12
14
16
Days after tumor transplantation
C
ns
ns
Tumor weight (g)
3
2
1
0
PBS
Veh Ly6C^lo Mϕ
Veh Ly6C^neg Mϕ
FG Ly6C^lo Mϕ
FG Ly6C^neg Mϕ
D
PBS
Ly6C^lo
Ly6C^neg
Veh
FG
CD31
E
**
*
Vessel number / mm^2
100
80
60
40
20
0
PBS
Veh Ly6C^lo Mϕ
Veh Ly6C^neg Mϕ
FG Ly6C^lo Mϕ
FG Ly6C^neg Mϕ
F
**
*
Vessel lumen area (μm^2)
1000
800
600
400
200
0
PBS
Veh Ly6C^lo Mϕ
Veh Ly6C^neg Mϕ
FG Ly6C^lo Mϕ
FG Ly6C^neg Mϕ
G
PBS
Ly6C^lo
Ly6C^neg
Veh
FG
CD31 / ZO-1
H
**
**
ZO-1^+ area (% of CD31^+ area)
50
40
30
20
10
0
PBS
Veh Ly6C^lo
Veh Ly6C^neg
FG Ly6C^lo
FG Ly6C^neg
I
PBS
Ly6C^lo
Ly6C^neg
Veh
FG
CD31 / NG2
J
*
*
NG2^+ area (% of CD31^+ area)
50
40
30
20
10
0
PBS
Veh Ly6C^lo
Veh Ly6C^neg
FG Ly6C^lo
FG Ly6C^neg

Figure S6.

Figure S6. Ly6C^{neg} Mϕs normalize tumor vessel structure. Related to Figure 6.
(A) Histological identification of CellVue-labeled (red) transplanted Mϕs. Scale bar = 1 mm. (B) Tumor growth curves of LLC tumor mouse models under conditions of PBS injection or transplantation with Ly6C^{lo} or Ly6C^{neg} Mϕs sorted from vehicle/FG-treated mouse tumors (n = 5–6 per group). ns, not significant; two-way ANOVA. (C) Tumor weight was measured at the end of the experiment on day 16 (n = 5–6). ns, not significant; Mann-Whitney test. (D) IF images of CD31-stained sections of LLC tumor mouse models that were injected with PBS or received transplants of Ly6C^{lo} or Ly6C^{neg} Mϕs sorted from vehicle/FG-treated mouse tumors. Scale bar = 100 μm. (E, F) Quantification of (E) vessel density and (F) vessel lumen area of s.c. LLC tumors (n = 3 per group). *p < 0.05, **p < 0.01 vs. PBS; unpaired Student's *t*-test. (G) IF images of ZO-1 (green) and CD31 (red)-stained sections of LLC tumors. Scale bar = 50 μm. (H) Quantification of the ZO-1^{+} area ratio in the CD31^{+} area (n = 3). **p < 0.01 vs. PBS; unpaired Student's *t*-test. (I) IF images of NG2 (green) and CD31 (red)-stained sections of LLC tumors. Scale bar = 50 μm. (J) Quantification of the NG2^{+} area ratio in the CD31^{+} area (n = 3). Data represent the mean ± SEM. *p < 0.05, vs. PBS; unpaired Student's *t*-test.

Transparent Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka City University (approval number: 16022, 16021), which was approved by the Japanese Association for Accreditation for Laboratory Animal Care. Furthermore, animal research and handling were performed in strict conformance with the Institutional Animal Care and Use Committee guidelines.

C57BL/6 male mice were obtained from SLC Japan, Inc. *LysM Cre*, *VHL* floxed and *HIF1* floxed mice were obtained from Jackson Laboratories and bred in our facility. Mice were housed in cages with food and water available *ad libitum* in a 12-h light-dark cycle at 22 ± 1 °C.

Cell cultures, tumor transplant model, and PHD inhibitor treatment

LLC and B16F10 cells (RIKEN BRC) were maintained in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI1640). These media contained 10% fetal bovine serum (FBS) and penicillin/streptomycin in 5% CO_2 and 95% room air at 37 °C. MC38 cells were maintained in DMEM containing 10% FBS, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and penicillin/streptomycin in 5% CO_2 and 95% room air at 37 °C. These cells were harvested and re-suspended (at 1×10^7 cells/mL) in phosphate-buffered saline (PBS). Some of the cells (1×10^6 cells) were subcutaneously transplanted into the right flanks of 8–12-week-old mice. The mice were treated with 3 mg FG-4592 (Selleck Chemicals, Housten, TX) intraperitoneally 10 days after the tumor transplant (eligible tumor size for this study was 100–350 mm^3). Once every two days, the tumors were measured in two dimensions using a caliper. The tumor tissue volume was calculated using the formula $V = (\text{length} \times \text{width}^2)/2$. The mice were sacrificed at a defined time point or when the tumor volume exceeded 4500 mm^3 or ruptured.

MTT assay

LLC cells were plated at 2×10^4 cells per well in a 96-well plate. Subsequently, FG was added to the medium at the indicated concentration (0, 50, 100, 200, and 400 μM). Cell viability was evaluated 72 h after FG treatment. Culture medium was exchanged 6 h after FG treatment. After 72 h, AlamarBlue (Bio-Rad Laboratories, Hercules, CA) reagent was added at a 1:10 volume. Following this, the plate was incubated for another 6 h at 37 °C in a 5% CO_2 incubator. Finally, optical density (OD) was measured at 570 and 600 nm, and cell viability was calculated. The experiment was performed three times with three replicates per experiment.

Establishment of stable GFP-expressing LLC

cDNAs encoding GFP1 were double digested with SalI and NotI from pAcGFP1 (Clontech, Mountain View, CA) and inserted into the SalI and NotI sites of the pEBMulti-Hyg vector (Wako Pure Chemical Industries Ltd., Osaka, Japan). This expression vector was transfected into LLC using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA), and GFP stably expressing LLC was selected by Hygromycin B (250 μg/mL) for 7 days.

Immunofluorescence staining

Tumor tissues were sliced into frozen sections of 8-μm thickness at −20 °C and air-dried. The sectioned tissue samples were rehydrated in PBS for 10 min and fixed with 4% (w/v) cold paraformaldehyde for 10 min. The sections were then washed with PBS and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min, after which they were blocked in 5% normal goat serum for 30 min at room temperature (20–25 °C). The sections were incubated with the following primary antibodies, anti-CD31 (1:1000; eBioscience, San Diego, CA), anti-NG2 (1:400; Merck Millipore, Burlington, MA), anti-ZO-1 (1:400; Thermo Fisher Scientific, Waltham, MA), and anti-CC3 (1:400; Cell Signaling Technology, Danvers, MA) overnight at 4 °C. The sections were then washed with 0.1% Tween 20 in PBS and incubated for 1 h at room temperature with the appropriate fluorophore secondary antibody (AlexaFluor 488 or Cy3, goat anti-rat, or goat anti-rabbit IgG; BioLegend, San Diego CA). Finally, the sections were washed with 0.1%

Tween 20 in PBS, dehydrated with ethanol, and air-dried, after which they were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindole stain (1:5000) and a cover slip.

Quantification of immunofluorescence images

Quantification of the immunofluorescent images, which were taken using a microscope (BZX-710; Keyence, Osaka, Japan), was performed using the microscope software. At least 15 fields of images at 100, 200, or 400× magnification were analyzed for each sample. Each experiment used at least three animals from each group.

Tumor vessel perfusion and permeability analysis

Dextran-FITC (150 kDa; Sigma-Aldrich, St. Louis, MO) was intravenously administered into the tail vein 10 min prior to sacrifice. The tumor tissues were excised and immediately frozen in liquid nitrogen. The tumor samples were stored at −80 °C until sectioning. The tumor tissues were sectioned into 8 and 20-μm thick sections and then observed using the BZX-710 microscope.

Detection of tumor hypoxic regions and analysis

Hypoxic regions of the tumor tissues were detected using the Hypoxyprobe™ Kit (HP3-100; Hypoxyprobe Inc., Burlington, MA), which included pimonidazole, according to manufacturer's instructions. In brief, pimonidazole was injected at 60 mg/kg into the tumor-bearing mice. Ninety minutes later, the mice were sacrificed, after which the tumors were collected and cryosectioned into 4-μm thick sections. The sections were then fixed with ice-cold acetone for 10 min, washed with PBS, and incubated with rabbit anti-pimonidazole anti-sera (1:20) overnight at 4 °C. Subsequently, the sections were incubated with AlexaFluor 488 conjugated goat anti-rabbit antibody (1:1000) for 1 h. Images were acquired using the BZX-710 microscope, and the ratio of the pimonidazole-positive area was quantified using the equipped software.

In vivo macrophage depletion

Macrophage depletion was performed according to manufacturer's instructions. In brief, mice were intraperitoneally administered 100 µL clodronate-loaded liposome (F70101C-A; FormuMax Scientific Inc., Sunnyvale, CA) or 100 µL plain control liposomes (F70101-A; FormuMax Scientific Inc.) 24 h prior to FG injection. The second administration contained 70 µL of each liposome and was administered 72 h following FG injection.

Quantitative RT-PCR (qRT-PCR)

BMDMs generated from separate mice were treated with 100 µM FG-4592 or an equal volume of DMSO for 12 h before harvesting. RNA was extracted from BMDMs with ISOGEN II (Nippon Gene, Tokyo, Japan) according to manufacturer's instructions. cDNA was reverse transcribed from 1 µg total RNA using the Prime Script RT Reagent Kit (TaKaRa Bio, Kusatsu, Japan). qPCR was then performed on the 7500 Fast Real-Time PCR system using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). Five independent qRT-PCR assays were performed for each sample pair in triplicate. Relative transcript levels were normalized to 18S ribosomal RNA and analyzed using 7500 software v2.3 (Applied Biosystems, Foster City, CA).

Tumor dissociation

Perfused tumors were minced and digested with 1 mg/mL collagenase IV with 50 µg/mL DNase I for 45 min at 37 °C with shaking. Cells were filtered through a 100-µm nylon mesh and washed in isolation buffer (PBS containing 2% FBS and 2 mM EDTA). Erythrocytes were lysed using RBC lysis buffer (BioLegend). Cells were resuspended in isolation buffer, after which Fc receptors were blocked with CD16/32 blocking antibody (BioLegend) for 15 min on ice.

Flow cytometry and cell sorting

Dissociated single tumor cells were stained with the following fluorochrome-conjugated antibodies at the manufacturer's (BioLegend) recommended dilution for 30 min on ice in the dark for detecting macrophages, anti-mouse F4/80-PE (clone BM8), anti-mouse CD45 PE-Cy7 (clone 30-F11), anti-mouse Ly6C-PerCP-Cy5.5 (clone HK1.4), anti-mouse CD11b-APC-Cy7 (clone M1/70), anti-mouse CD206-APC (clone C068C2), and anti-mouse CD80 FITC (clone 16-10A1); for T cells, anti-mouse CD3ε-FITC or -PE (clone 145-2C11), anti-mouse CD45 PE-Cy7 (clone 30-F11), anti-mouse CD4-APC (clone GK1.5), and anti-mouse CD8-APC-Cy7 (clone 53-6.7) antibodies were used. To exclude dead cells, DAPI was added just before analysis. Flow cytometric analysis and cell sorting were performed on the BD LSR II or BD FACSAria III. Briefly, 100,000 cells were analyzed per sample per mouse using BD FACSDiva software ver. 8.0 (BD Biosciences). The sequential gating strategy is outlined in Supplementary Figure 3a.

Purification of CD11b-positive cells and sorting of Ly6C-positive macrophages

CD11b-positive cells were purified using the Dynabeads FlowComp Flexi Kit (Invitrogen) according to manufacturer's instructions. In brief, FcR-blocked tumor single cell suspensions were added to biotin-labelled CD11b antibody (clone M1/70; #101231; BioLegend) and incubated for 20 min at 4 °C. Cell suspensions were added to FlowComp Dynabeads, after which CD11b-positive cells were magnetically separated. FlowComp Dynabeads were released from the cells using the FlowComp Release Buffer. Bead-free cells were stained with anti-rat IgG-AlexaFluor 488 at first and subsequently stained with anti-mouse F4/80-PE, anti-mouse CD45 PE-Cy7, or anti-mouse Ly6C-PerCP-Cy5.5. To exclude dead cells, DAPI was added just before cell sorting. Cell sorting was performed using a 100-µm nozzle on a BD FACSAria III and analyzed using BD FACSDiva software.

Macrophage morphology and staining

Sorted macrophages were washed with PBS and resuspended at 5×10^4 cells/mL in PBS. Cells were attached to slides using cytospin3 and centrifuged at 200 × g for 3 min. Cells were stained with the Diff-Quick Staining Kit according to manufacturer's instructions, after which the cells were observed and images captured using a microscope.

Isolation of BMDMs

Bone marrow was isolated from the tibia and femur of 8–12-week-old C57BL/6 mice and incubated in DMEM supplemented with L929 cell-conditioned medium (20%). Cells were cultured for 7 days, and the medium was replaced every 2 days. Differentiated macrophages were identified via staining for F4/80 antibody and flow cytometry.

In vitro and ex vivo beads phagocytosis assay

BMDMs and sorted macrophages were stained with CellVue Claret® (Sigma-Aldrich) according to manufacturer's instructions before seeding in a 96-well plate. Sorted cells were incubated for 2 h at 37 °C to allow them to rest after sorting. Subsequently, 24 h later, 100 μM FG or equal volume DMSO were added to the culture media. After 12 h, Latex Beads-Rabbit IgG-FITC Complex (Cayman Chemical, Ann Arbor, MI) was added to the culture media (1:400). After 45 min, phagocytic macrophages were imaged, counted using the BZX-710 microscope, and analyzed with the equipped software. Over 800 cells were analyzed per group.

Coculture phagocytosis assay

BMDMs were stained with CellVue Claret® (Sigma-Aldrich) according to manufacturer's instructions before seeding 5×10^4 cells/well in a 24-well plate. After 24 h, 100 μM FG or equal volume DMSO were added to the culture media. After 12 h, 5×10^4 LLC were seeded in each well. Before seeding, 1 μg/mL Hoechst 33342 was added to the LLC culture media and incubated for 1 h, after which the LLC cells were trypsinized and stained with CFSE according to manufacturer's instructions. After LLC seeding, images

were immediately acquired using the IN Cell Analyzer 2500HS (GE Healthcare, Chicago, IL) or the BZX-710 microscope.

Transplantation of sorted macrophages into tumor tissues

Sorted macrophages were spun down in PBS and resuspended in 20 μL PBS. For FG treatment, sorted macrophages were seeded in 10-cm dishes and incubated with/without 100 μM FG for 12 h. Subsequently, macrophages were harvested and resuspended in 20 μL PBS. The mice were anaesthetized on day 10 after tumor transplantation, after which 1×10^5 cells were injected into the tumor using a 26-gauge needle. After intra-tumor injection of the macrophages, the tumors were measured once every 2 days. The mice were then sacrificed on day 16.

Statistical analysis

Statistical analyses were performed using the unpaired *t*-test followed by a Bonferroni's post-hoc test via GraphPad Prism software (ver 7.02; GraphPad Software Inc., La Jolla, CA). P values < 0.05 were considered statistically significant. All analysis was performed using a two-tailed analysis.