Supplemental Data for

Bone marrow sinusoidal EphB4 controls hematopoietic progenitor cells mobilization

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Supplemental Methods

Isolation of Primary Sinusoidal Endothelial Cells

Primary bone marrow sinusoidal endothelial cells were isolated by crushing tibias, femurs, hip bones, and back bones of C57BL/6 mice. After red blood cells were lysed, bone marrow cells were incubated in 10% DMEM containing 3mg/ml Collagenase type II (Worthington-Biochem, LS004176) for 45 minutes at 37°C in a CO_2 incubator with occasional shaking. $CD45^+$ cells were removed using Mojosort Mouse CD45 Nanobeads (Biolegend, 480028). Sinusoidal endothelial cells were sorted using FITC-anti-mouse CD31 (Biolegend, 102405) and PE anti-mouse VEGFR3 (Miltenyi Biotech Inc., 130-102-216) antibodies.

Endothelial Permeability Assay

The effects of EphB4 silencing on endothelial permeability were examined using a transwell-based permeability assay as described previously (1). BMECs ($1x10^4$) were grown for 24hrs to form confluent monolayers on the upper chamber of 96 well transwell plates with 5μm pores (Corning, USA,3388). Texas Red-dextran (20μg/ml; 70,000 MW) was added to the upper chamber, and the lower chambers were filled with 200μl medium. Thirty minutes after Texas Red-dextran addition, medium was collected from the lower chamber and the amount of Texas Red-dextran that had gone through the cell monolayer was measured using a plate reader (596nm Excitation/615nm Emission). Values calculated from a Texas Red-dextran-derived standard curve.

Adhesion Assay

The effects of EphB4 silencing on HSPC adhesion to endothelium were examined as described previously with slight modification (2). Briefly, BMECs ($1x10^5$) were plated onto a 96-well flat-bottom gelatin-precoated plate for 24hrs to form confluent monolayers. TdTomato Lin^- cells ($1x10^6$) were added to the wells and incubated for 14 hrs. Unbound cells were removed by washing with PBS. The percent TdTomato cells adhered to BMEC was measured using a plate reader (596nm Excitation/615nm Emission), based on a TdTomato Lin^- cell-derived standard curve.

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed using Dynabeads Protein G Immunoprecipitation kit (ThermoFisher Scientific). Briefly, 2µg of anti-mouse EphB4 antibody (Supplementary Table) was incubated with Dynabeads (10 minutes at RT in 200µl of 0.02% Tween 20/PBS). The antibody-Dynabeads complex was incubated (1 hour) with 500µg protein lysates prepared in lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% triton X-100, 10% glycerol) containing protease inhibitor cocktail (ThermoFisher Scientific). Following washes, immune complexes were eluted in 20µl elution buffer. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, membranes were blocked with 5% skim milk and blotted with primary antibodies (Supplementary Table) at 4°C overnight. Membranes were incubated with secondary antibodies (Supplementary Table) conjugated with horseradish peroxidase, and immune complexes detected using an ECL solution detection system (GE Healthcare, RPN2236). Membranes were stripped and re-stained. Bands were measured by ImageJ software.

Cell proliferation and cell death assays

Cells ($5x10^5$) were seeded in 48-well plates and imaged for 48 hours using Incucyte HD (Essen Bioscience, UK). Cell death was analyzed by flow cytometry using Annexin V/Propidium Iodine (PI) Apoptosis Kit (Invitrogen).

G-CSF ELISAs

G-CSF levels were measured by ELISA (R&D Systems); dilutions of mouse plasma or mouse G-CSF (in 1% BSA/PBS) were added to 96-well plates pre-coated with 0.2µg capture antibody. Detection antibody (20ng/mL, 2 hours), Streptavidin-HRP (1:200, 20 minutes) and the substrate solution was added. When the desired color intensity was reached, the reaction was stopped and absorbance measured at 450nm.

Real Time PCR

Relative mRNA expression was measured by real time PCR using TaqMan Universal PCR Master Mix and TaqMan Probes (Applied Biosystems) for EphrinB2 (Mm00438670_m1), EphB4 (Mm01201157_m1), Sp7 (Mm04209856_m1), and Osteocalcin (Mm03413826_mH). GAPDH (Mm9999991_g1) was used as an internal control. Real time PCR was run on a 7900HT Fast RT PCR System (Applied Biosystems) for 40 cycles with an annealing temperature of 60°C; results were analyzed using SDS 2.4.1 (Applied Biosystems) and Microsoft Excel software.

Homing Assays

Two different homing assays were performed based on previous descriptions (2) with modification: 1) Lin^- cells ($2x10^6$) purified from the bone marrow of pcDNA- or TNYL-RAW-transduced donor TdTomato mice were injected into the tail vein of untreated WT recipients; 2) Lin- cells ($2x10^6$) purified from the bone marrow of untreated TdTomato mice were injected into pcDNA- or TNYL-RAW-transduced WT recipients. In both cases, homing was evaluated by flow cytometry 15 hr after tail vein injection.

Engraftment

Recipient C57BL/6 mice were injected with pcDNA- or TNYL-RAW expression vectors at day -5, and pretreated with Busulfan (4x25mg/kg) for four consecutive days (from day -4 to day -1), followed by transplantation at day 0. TdTomato bone marrow cells ($1x10^7$) were injected into the tail vein of WT recipients. Six weeks after transplantation, the presence of TdTomato donor-derived hematopoietic cell populations was analyzed in blood and bone marrow of the recipient mice.

Bone Marrow Sinusoidal Vessel Leakage

Bone marrow sinusoidal vessel leakage *in vivo* was measured in the calvarium of mice using intravital microscopy as described previously (3). Mice were retro-orbitally injected with 4 mg/kg FITC-conjugated 2,000,000 MW dextran (Sigma-Aldrich) in 100µl saline to visualize calvarial bone marrow sinusoids. After selection of a region of interest, mice received retro-orbital injection of 70,000 MW Texas Red-Dextran (Thermo Fisher Scientific, D1864; 100µl of 5 mg/ml). The area of interest was continuously imaged to

record the leakage from the sinusoidal vessel. For each ROI, Image J was used to measure the Integrated Fluorescence Intensity for the entire time lapse. The values at 30, 60, 120, 180 seconds were normalized for the value at time 0.

Co-injection of B16F10 tumor cells and HSPCs

$Lin^{-}CD150^{+}$ cells ($1x10^{5}$) selected from the bone marrow of TdTomato mice were mixed with B16F10 tumor cells ($2x10^{6}$). The cell mixture was injected s.c. into WT C57BL/6 mice. At day 7 after the injection, the mice were sacrificed and tumor weight was measured. Tumors were dissociated with Tumor Dissociation Kit (Miltenyibiotec, 130-096-730) for flow cytometric analysis and colony forming assays.

References

1. Kobayashi K, Sato K, Kida T, Omori K, Hori M, Ozaki H, and Murata T. Stromal cell-derived factor-1alpha/C-X-C chemokine receptor type 4 axis promotes endothelial cell barrier integrity via phosphoinositide 3-kinase and Rac1 activation. *Arterioscler Thromb Vasc Biol.* 2014;34(8):1716-22.
2. Nguyen TM, Arthur A, Panagopoulos R, Paton S, Hayball JD, Zannettino AC, Purton LE, Matsuo K, and Gronthos S. EphB4 Expressing Stromal Cells Exhibit an Enhanced Capacity for Hematopoietic Stem Cell Maintenance. *Stem Cells.* 2015;33(9):2838-49.

3. Itkin T, Gur-Cohen S, Spencer JA, Schajnovitz A, Ramasamy SK, Kusumbe AP, Ledergor G, Jung Y, Milo I, Poulos MG, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature.* 2016;532(7599):323-8.

Figure S1

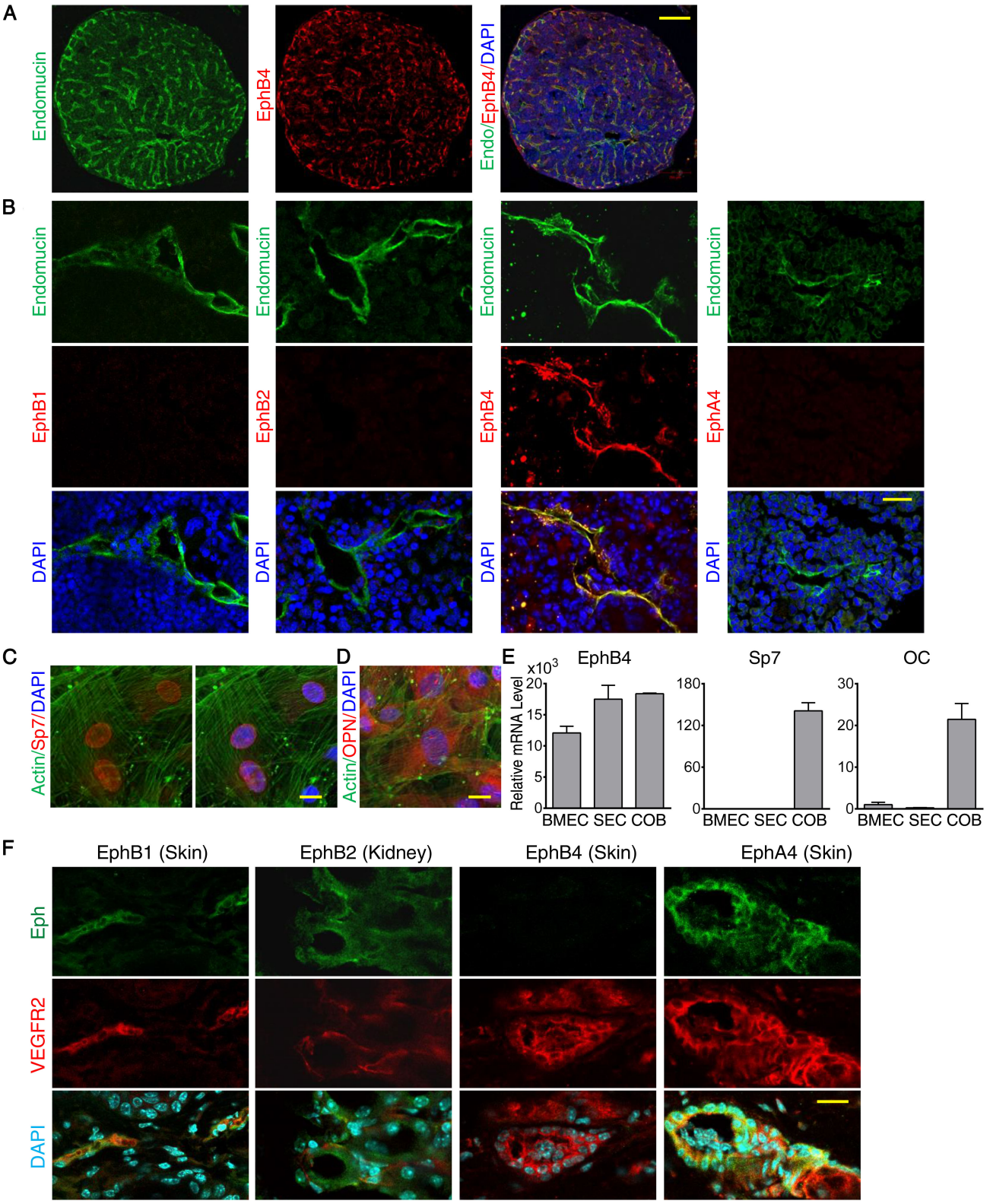


Figure S1, related to Figure 1. EphB4 is visualized in bone marrow sinusoidal vessels. (A) Immunofluorescence staining of a femoral section through the diaphysis showing the distribution of Endomucin$^+$ (green), EphB4$^+$ (red) and Endomucin$^+$/EphB4$^+$ vessels. Nuclei are DAPI$^+$ (blue). Scale bar: 200μm. **(B)** Endomucin$^+$ bone marrow sinusoidal vessels are EphB4$^+$, EphB1$^-$, EphB2$^-$ and EphA4$^-$. Scale bar: 20μm. **(C)** Nuclear Sp7 (red) in primary calvarial osteoblasts co-stained for F-actin (green); nuclei are DAPI$^+$ (blue). Scale bar: 20μm. **(D)** Osteopontin (OPN: red) detected in primary calvarial osteoblasts; nuclei are DAPI$^+$ (blue). Scale bar: 20μm. **(E)** Relative mRNA levels of EphB4, Sp7 and osteocalcin (OC) in BMEC, primary sinusoidal endothelial cells (SEC) and primary calvarial osteoblasts (COB) measured by qPCR; results reflect the means of triplicate measurements; error bars: SDs. **(F)** VEGFR2$^+$ skin vessels are EphB4$^-$, EphB1$^+$, EphA4$^+$; VEGFR2$^+$ epithelium of tubules in the kidney medulla is EphB2$^+$. Scale bar: 20μm.

Figure S2

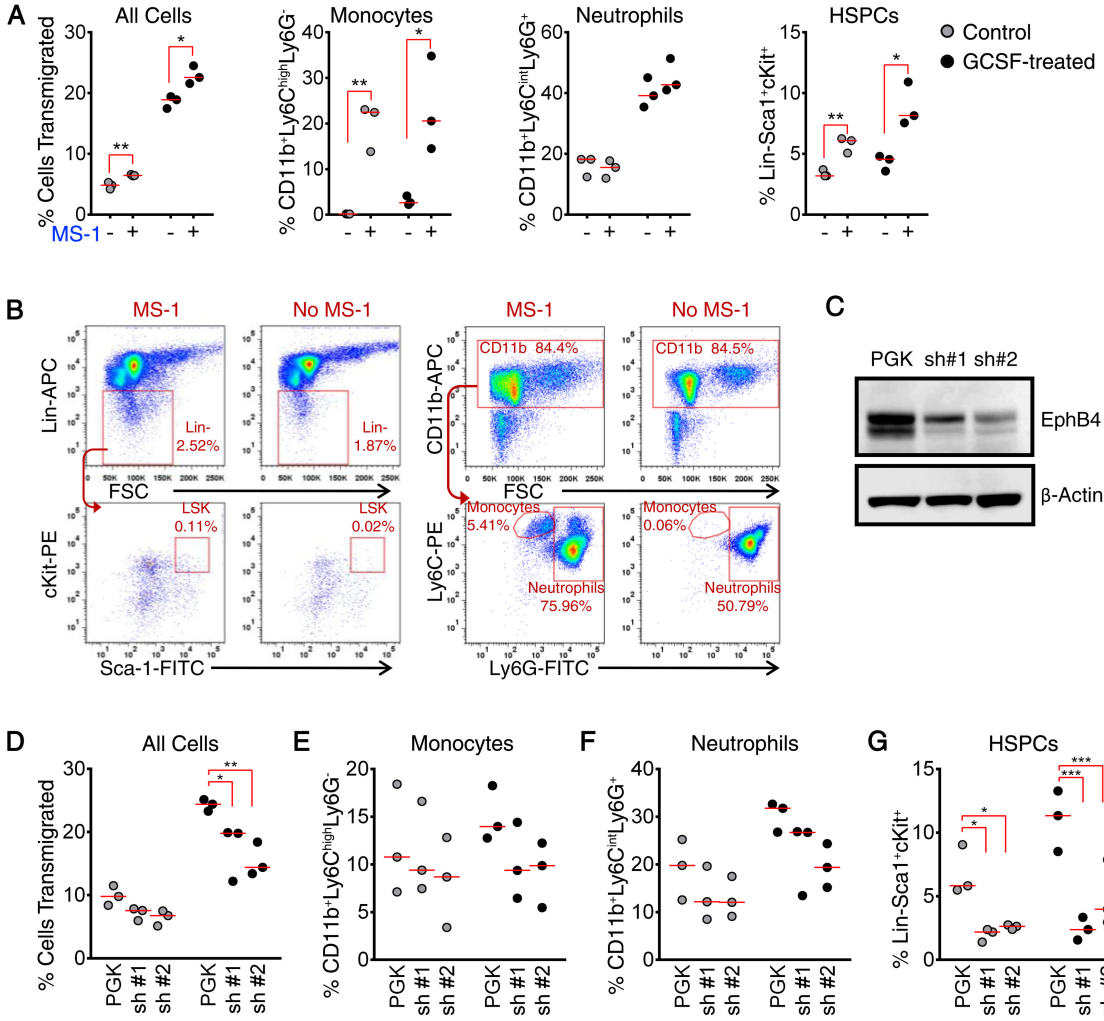


Figure S2, related to Figure 2. EphB4 dependency of hematopoietic cells trans-endothelial migration. (A) Transmigration of bone marrow hematopoietic cells from G-CSF-treated and control mice through membranes coated with a monolayer of MS-1 cells (+) or gelatin only (-). Loaded and transmigrated cells were characterized by flow cytometry as monocytes ($CD11b^+Ly6C^{high}Ly6G^-$), neutrophils ($CD11b^+Ly6C^{int}Ly6G^+$), or HSPCs ($Lin^-cKit^+Sca\text{-}1^+$). The results are expressed as % cells transmigrated/total loaded in the upper chamber. The dot plots reflect individual results from 3 independent experiments (red horizontal line: median). **(B)** Representative flow cytometry profiles of bone marrow cell populations recovered at the bottom of transwells after transmigration. Separating membranes were coated with MS-1 monolayers or gelatin-only. **(C)** EphB4 silencing with lentiviral vectors sh#1 and sh#2 reduces EphB4 protein in MS-1 cells. Immunoblotting results; the membrane was re-probed for β-actin. **(D-G)** Transendothelial migration of bone marrow cells from G-CSF-treated or untreated mice through control (PGK-infected) or EphB4-silenced (sh#1 and sh#2) MS-1 monolayers. Unfractionated bone marrow cells ($1x10^6$) were loaded into the upper chamber of the transwell and the number of cells transmigrated to the bottom chamber was counted. The proportion of monocytes, ($CD11b^+Ly6C^{high}Ly6G^-$), neutrophils ($CD11b^+Ly6C^{int}Ly6G^+$), and HSPCs ($Lin^-cKit^+Sca\text{-}1^+$) in the starting and transmigrated cell populations was measured by flow cytometry. The results are expressed as % cells transmigrated/total loaded in the upper chamber. The dot plots reflect individual results from 3 independent experiments (red line: median). Statistical significance by one-way ANOVA with Dunnett's multiple comparison test. P values: *<0.05; **<0.01; ***<0.001.

Figure S3

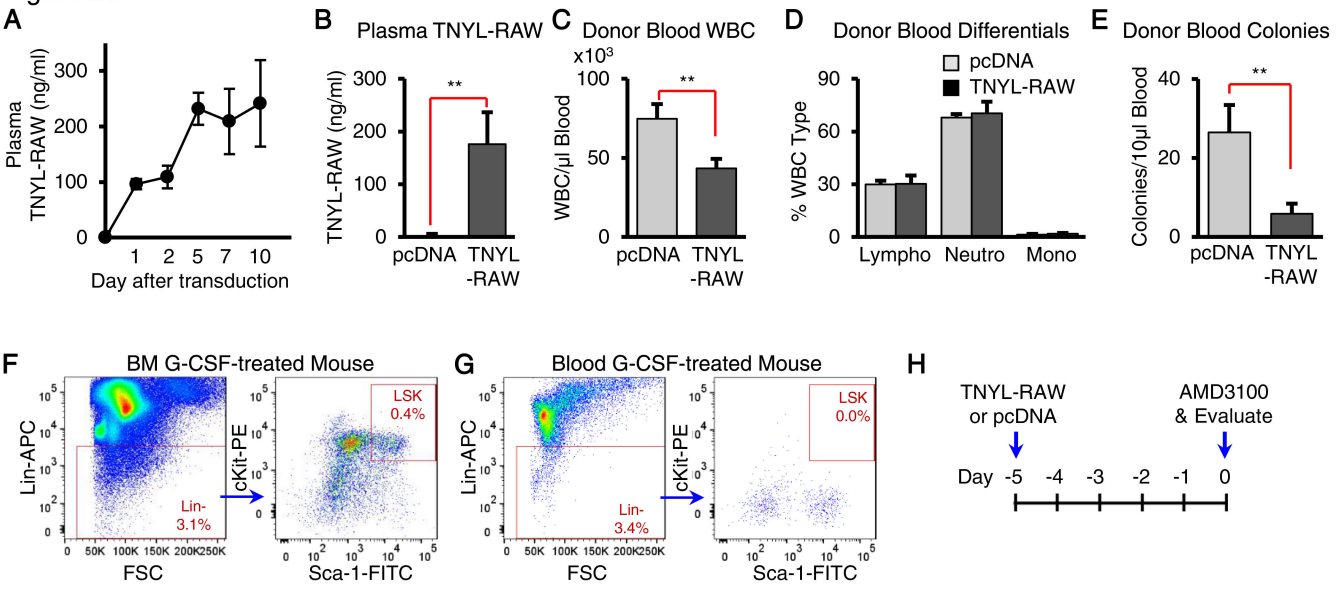


Figure S3, related to Figure 3. EphrinB2/EphB4 blockade reduces hematopoietic cell mobilization from bone marrow to the blood. **(A)** Kinetics of TNYL-RAW production in mice (n=4); results reflect the means (±SD). Error bars reflect SDs. **(B)** Plasma TNYL-RAW levels in Donor TdTomato mice injected 6 days earlier with pcDNA or TNYL-RAW vectors; means (±SD; n=3/group). **(C-E)** Peripheral blood from G-CSF-treated Donor TdTomato mice injected with pcDNA or TNYL-RAW vectors (n=3/group). (C) WBCs; means (±SD); (D) Differential counts; % ±SD. (E) Methylcellulose colonies; means (±SD). **(F and G)** Flow cytometric detection $Lin^{-}cKit^{+}Sca\text{-}1^{+}$ (LSK) cells in bone marrow (F) and blood (G) after G-CSF mobilization. **(H)** Schematic of experiment for AMD3100 mobilization.

Figure S4

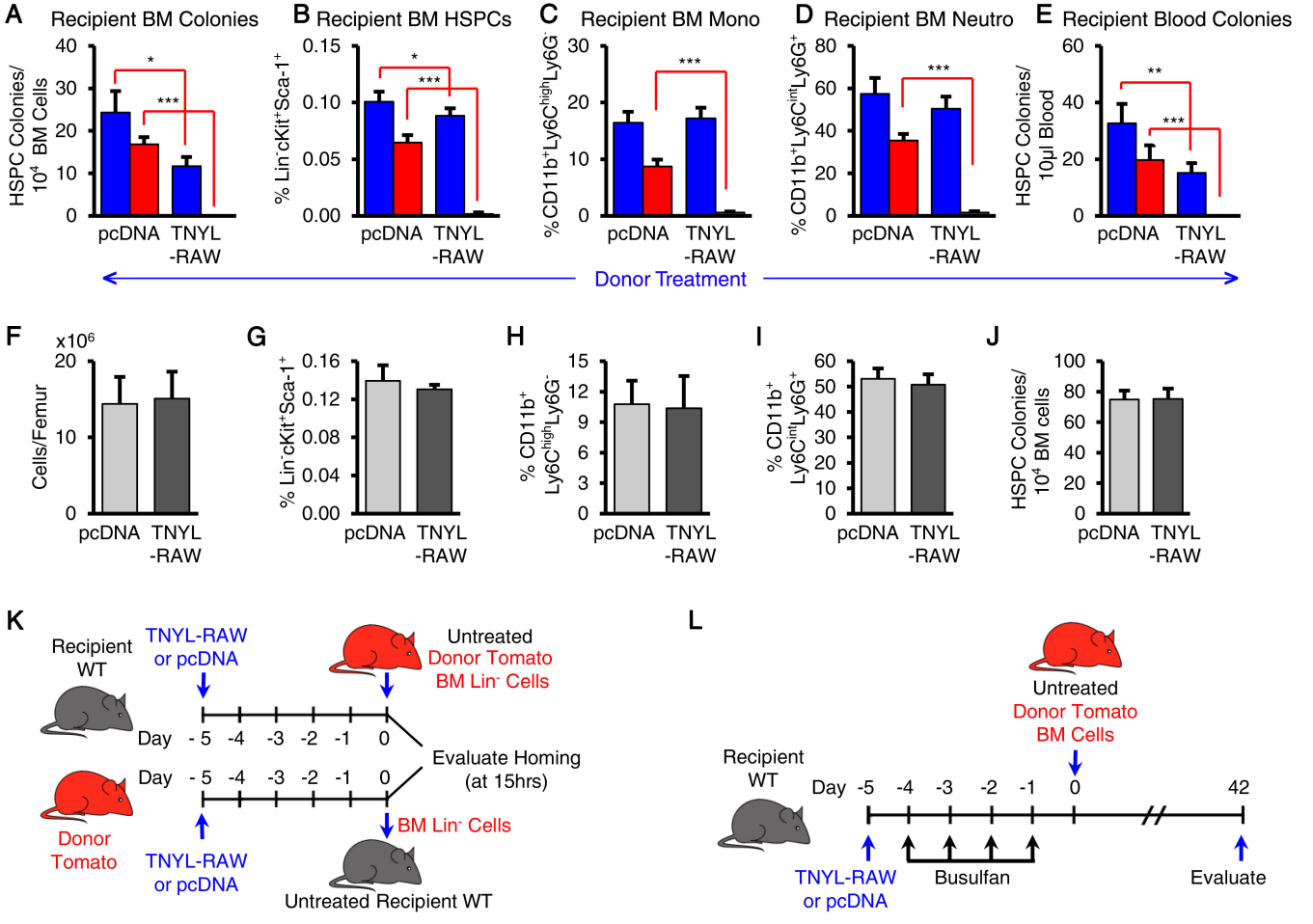


Figure S4, related to Figure 4. EphrinB2/EphB4 blockade reduces mobilization of hematopoietic cells with engraftment potential. **(A-E)** WT recipient mice transplanted with blood from TdTomato G-CSF-mobilized mice. Blue bars: all bone marrow cells (Donor+Recipient); the Red bars: Donor only-derived cells; results from day 42 (A-D) and day 47 (E) post-transplant. P values by Student's t test: *<0.05; **<0.01; ***<0.001. **(F-J)** Bone marrows from femurs of G-CSF-treated mice injected with pcDNA (n=4) or TNYL-RAW (n=4) vectors: cellularity by counting nucleated cells/femur (F); HSPC (G), monocytes (H), and Neutrophils (I) by flow cytometry; methylcellulose colonies (J). Results: means (n=4/group, ±SD). **(K)** Schematic of homing experiments; top: recipient WT mice injected with pcDNA or TNYL-RAW vectors; 5 days later inoculated with TdTomato Lin- bone marrow cells from untreated mice; bottom: Donor Tomato mice injected with pcDNA or TNYL-RAW vectors; after 5 days, bone marrow Lin- cells inoculated into untreated WT Recipients. Homing evaluated 15 hrs after inoculation. **(L)** Schematic of engraftment experiment. Recipient WT mice injected (Day -5) with TNYL-RAW or pcDNA vectors; after 24 hrs mice received 4 daily inoculations of Busulfan (Days -4 to -1). After 24 hrs (Day 0) mice transplanted with donor TdTomato bone marrow cells. Engraftment evaluated on day 42.

Figure S5

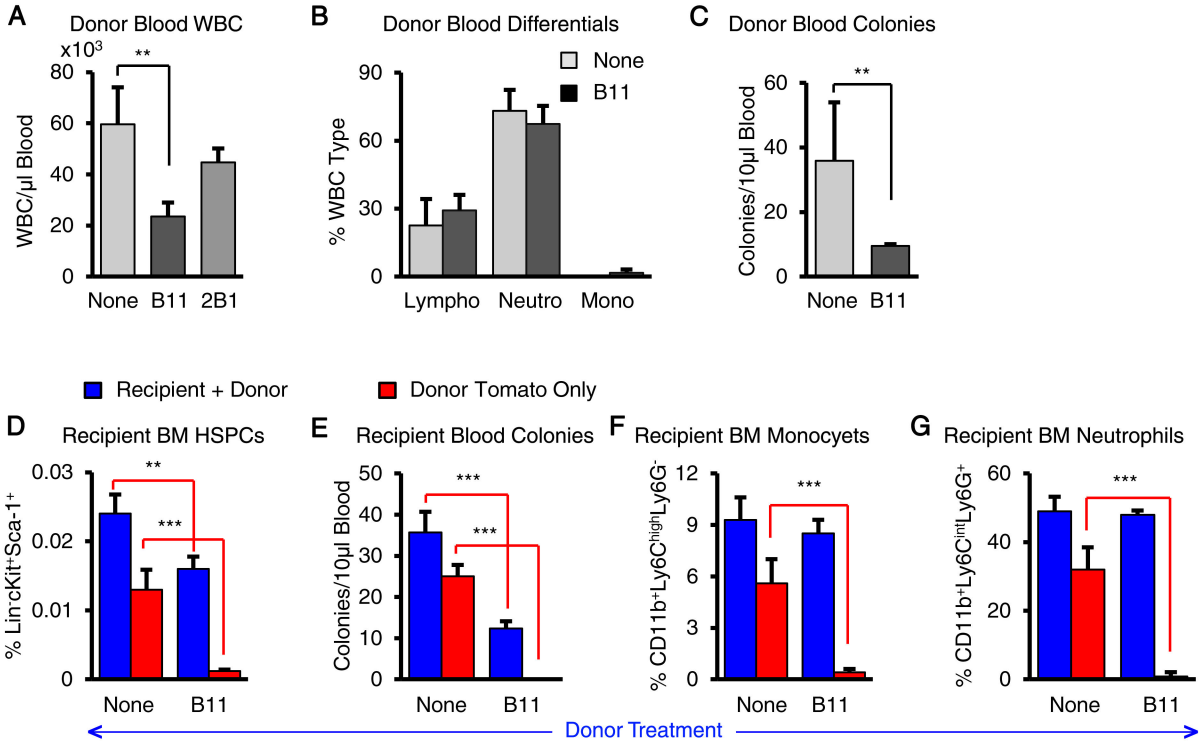


Figure S5, related to Figure 5. EphrinB2 neutralization reduces hematopoietic cell mobilization from bone marrow.
(A-C) Effects of B11 ScFv-antibody treatment on the peripheral blood of Donor TdTomato mice treated with G-CSF. Control mice received PBS (n=3/group). (A) WBCs; means (±SD). (B) Differential counts; % ±SD. (C) Colonies from methylcellulose culture; means (±SD).
(D-G) Evaluation of WT recipient mice transplanted with blood from Donor TdTomato mice (blood from each donor mouse was transplanted into two recipient mice; n=6 recipients/group). Blue bars reflect results from all bone marrow cells (includes cells from Donor and Recipient); the Red bars reflect results from Donor only-derived cells. (D) HSPC ($Lin^-cKit^+Sca\text{-}1^+$) in recipient bone marrows at Day 42 post-transplant; n=3/group; mean % (±SD). (E) Number of colonies from blood of recipient mice at Day 47 post-transplant (after G-CSF treatment on Days 42 through 46); n=3/group; means (±SD); (F) Monocytes ($CD11b^+Ly6C^{high}Ly6G^-$) in recipient bone marrows at Day 42 post-transplant; n=3/group; mean % (±SD). (G) Neutrophils ($CD11b^+Ly6C^{int}Ly6G^+$) in recipient bone marrows at Day 42 post-transplant; n=3/group; mean % (±SD). P values by Student's t test: **<0.01; ***<0.001. Error bars reflect SDs.

Figure S6

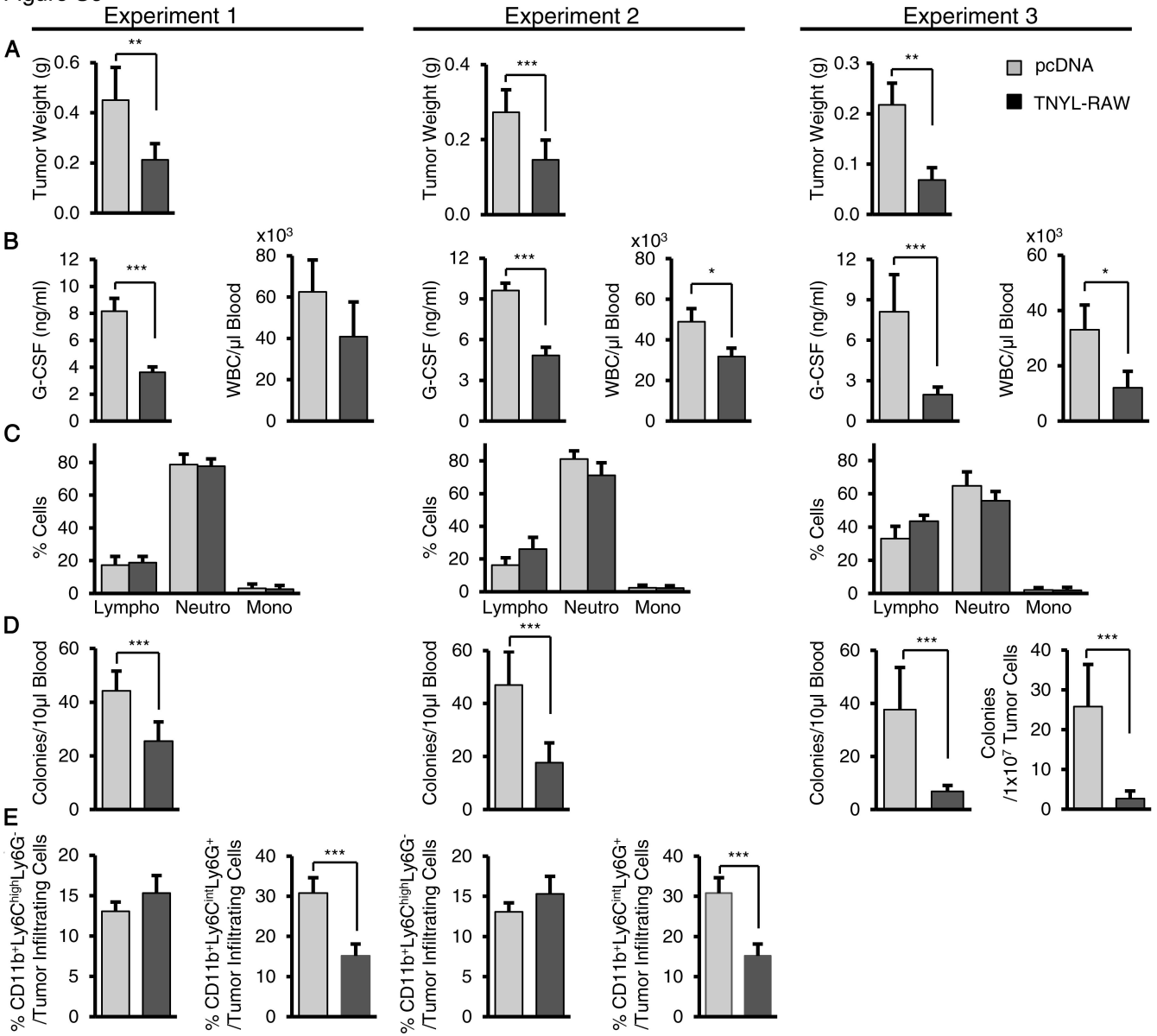


Figure S6, related to Figure 6. TNYL-RAW blocking peptides reproducibly reduce 4T1 tumor growth.
(A) Tumor size in groups of mice transduced with TNYL-RAW or pcDNA vectors. The results are from 3 independent experiments; average tumor weight on day 7 after 4T1 tumor cell injection (n=6; ±SD). Error bars reflect SDs. **(B)** Plasma G-CSF levels and WBCs in blood; mean levels on day 7 after 4T1 tumor cell injection (n=6; ±SD). **(C)** Differential blood counts. Lympho: lymphocytes; Neutro: neutrophils; Mono: monocytes. **(D)** Methylcellulose-supported colonies from precursors in blood of tumor-bearing mice. Results reflect the mean number of colonies/group (n=6; ±SD) in each experiment. The number of hematopoietic colony-forming precursors in the tumor is shown for experiment 3; the results reflect the mean (n=6; ±SD) number of Azurite$^-$ colonies /10^7 Azurite$^+$ tumor cells. **(E)** Proportion of tumor-infiltrating Azurite$^-$ monocytes (CD11b$^+$Ly6C^{high}Ly6G$^-$) and Azurite- neutrophils (CD11b$^+$Ly6C^{int}Ly6G$^+$) measured by flow cytometry of tumor cell suspensions. Results are shown as mean % (n=6; ±SD). Error bars reflects SDs. P values by Student's t test: *<0.05; **<0.01; ***<0.001.

Figure S7

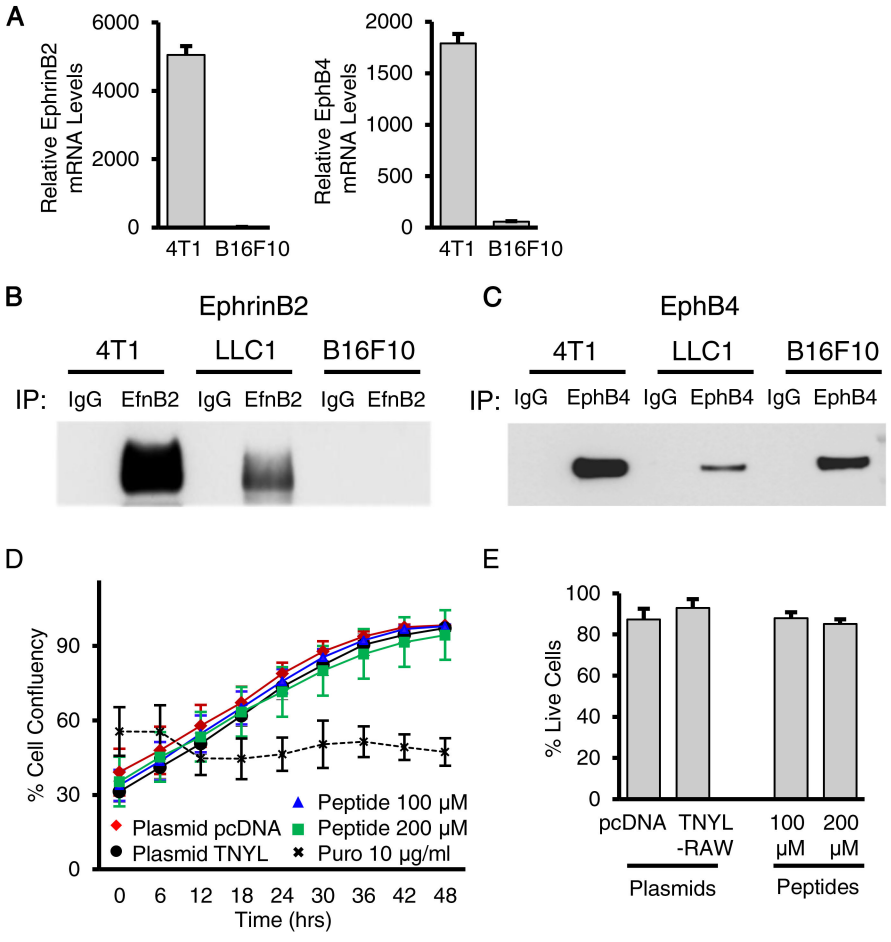


Figure S7, related to Figure 6. Characterization of EphrinB2 and EphB4 expression in 4T1 and B16F10 tumor cell lines. (A) Relative mRNA levels of EphrinB2 and EphB4 measured by qPCR. Means (±SD) of triplicate measurements; error bars reflect SDs. **(B,C)** Detection of EphrinB2 and EphB4 proteins in cell lysates by immunoprecipitation/immunoblotting; rabbit IgG was used as a control for immunoprecipitation. **(D)** 4T1 tumor cells were transfected with pcDNA or TNYL-RAW expression vectors; after 72 hours the cells were cultured in complete culture medium along with parental cells cultured in complete culture medium supplemented with 100mM or 200 mM TNYL-RAW peptides or 10µg/ml Puromycin. Growth curves were measured by Incucyte over 48 observation; the results are expressed as mean % confluency (±SD triplicate samples). **(E)** 4T1 cell viability 5 days after transduction with pcDNA or TNYL-RAW expression vectors, and 48 hours after culture with 100mM or 200mM TNYL-RAW peptides. Means ±SD triplicate samples; error bars: SDs.

Figure S8

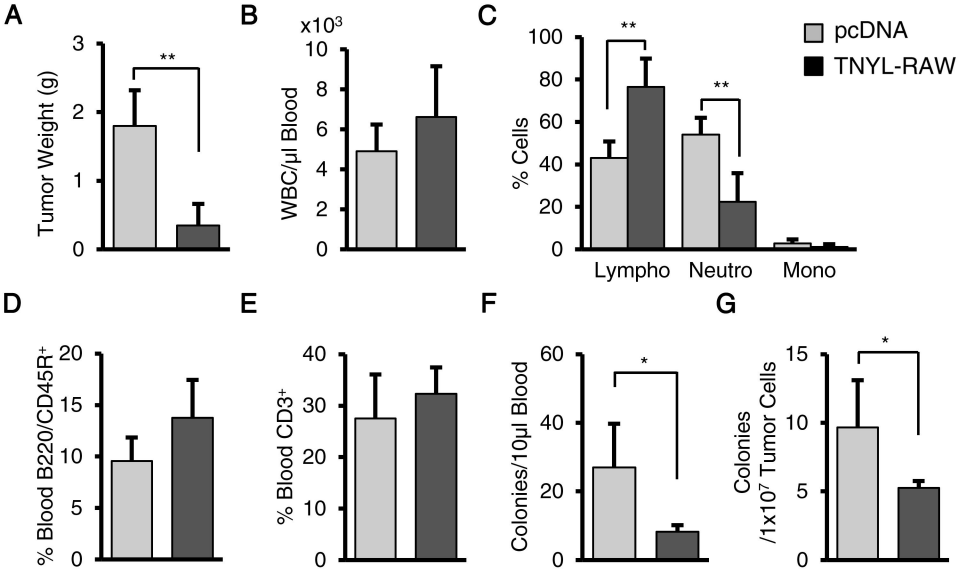


Figure S8, related to Figure 8. TNYL-RAW peptides reduce B16F10 tumor growth. (A) B16F10 tumor weight in pcDNA control and TNYL-RAW expressing mice; tumors were removed 10 days after the mice were injected withpcDNA or TNYL-RAW expression vectors and with Azurite$^+$ tumor cell line; (n=5; ±SD). Error bars: SDs. **(B)** Blood WBCs in pcDNA control and TNYL-RAW expressing mice at Day 10; means (n=5; ±SD). **(C)** Differential blood counts in pcDNA control and TNYL-RAW expressing mice at Day 10; means (n=5; ±SD). Lympho: lymphocytes; Neutro: neutrophils; Mono: monocytes **(D and E)** B-cells (B220$^+$/CD45R$^+$, D) and T-cells (CD3$^+$, E) in blood from pcDNA control and TNYL-RAW expressing mice; flow cytometry results; mean % of blood nucleated cells (n=5; ±SD). **(F)** Methylcellulose-supported colonies from precursors in blood of pcDNA control and TNYL-RAW expressing mice at Day 10; mean number of colonies (n=5; ±SD). **(G)** Colony-forming host (red Tomato$^+$) HSPC from single-cell suspended tumors of pcDNA control and TNYL-RAW expressing mice at Day 10; mean number of colonies (n=5; ±SD). Error bars reflect SDs. P values by Student's t test: * <0.05, **<0.01.

Figure S9

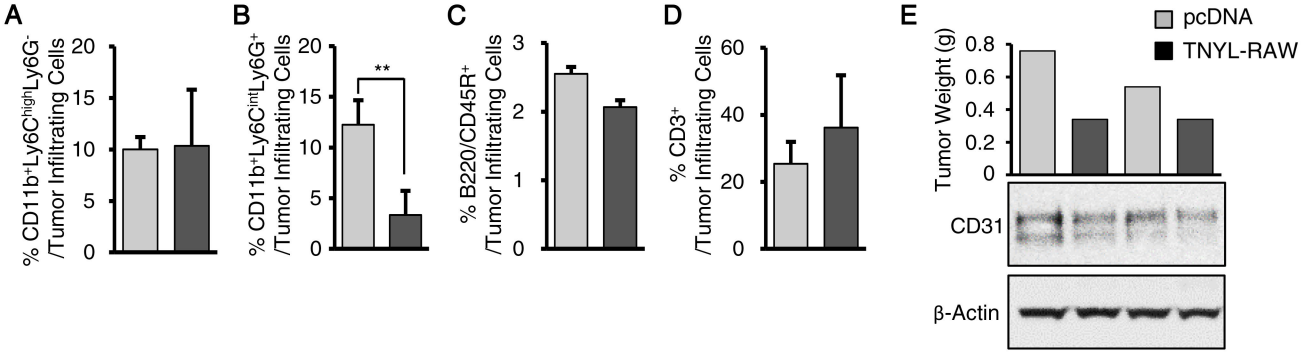
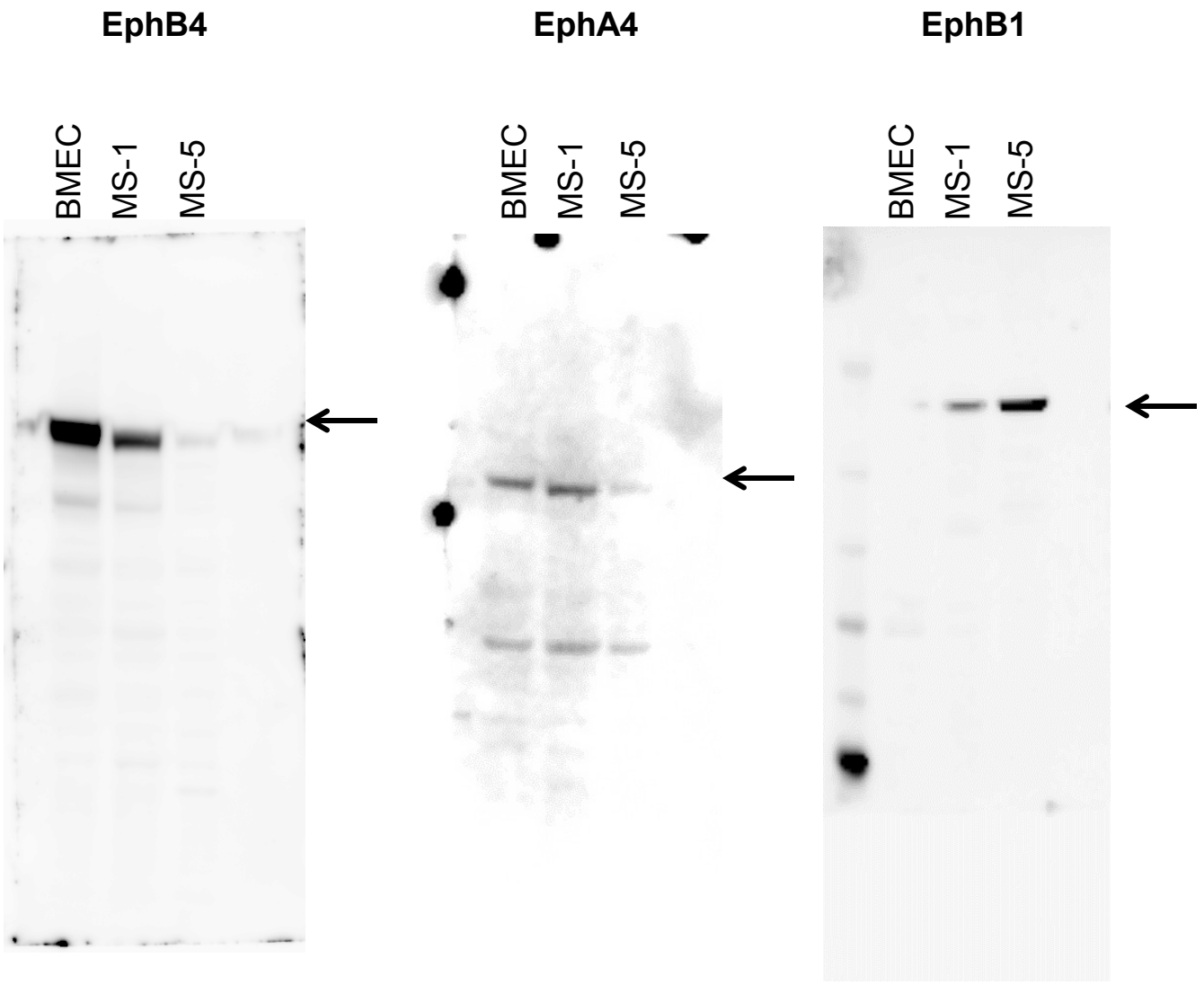
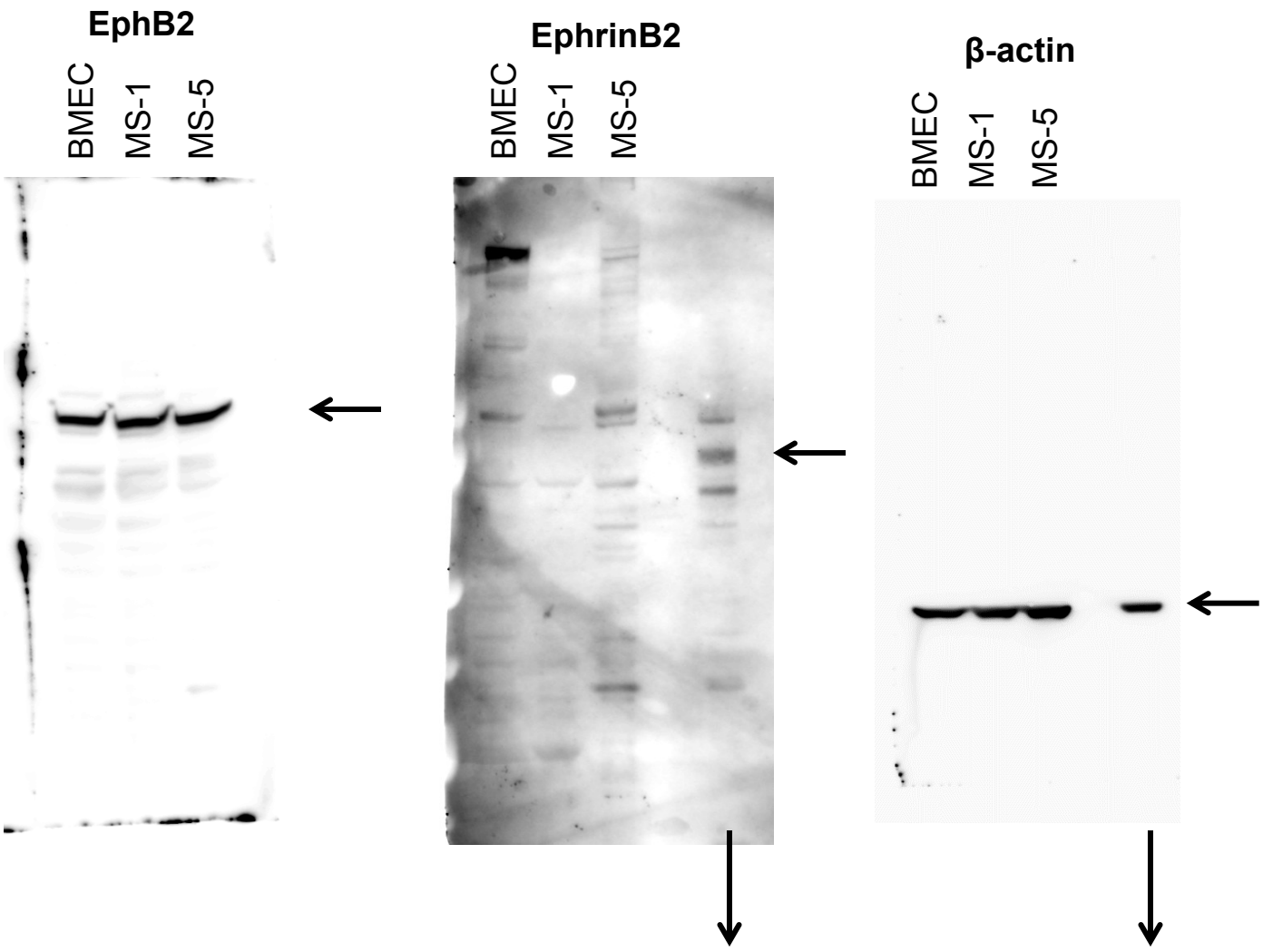


Figure S9, related to Figure 9. EphrinB2/EphB4 blockade reduces myeloid cell infiltration in B16F10 tumors. (A-D) Infiltration of Td-Tomato^{+}/Azurite^{-} monocytes (CD11b^{+}Ly6C^{high}Ly6G^{-}, A); neutrophils (CD11b^{+}Ly6C^{int}Ly6G^{+}, B); B cells (B220^{+}/CD45R^{+}, C) and T cells (CD3^{+}, D) in tumor cell suspensions of B16F10 tumors from pcDNA control and TNYL-RAW expressing mice; flow cytometry results; mean % of infiltrating TdTomato^{+}/Azurite^{-} cells (n=5; ±SD). Error bars reflect SDs. P values by Student's t test: **<0.01. **(E)** Immunoblotting detection of CD31 in cell lysates of B16F10 tumors inoculated in syngeneic mice expressing TNYL-RAW or pcDNA vectors. The blot was re-probed for β-actin. The bar graph depicts the relative weights of tumors used as a source of cell lysates for immunoblotting.

Full unedited gel for Figure 2A



Full unedited gel for Figure 2A, continued



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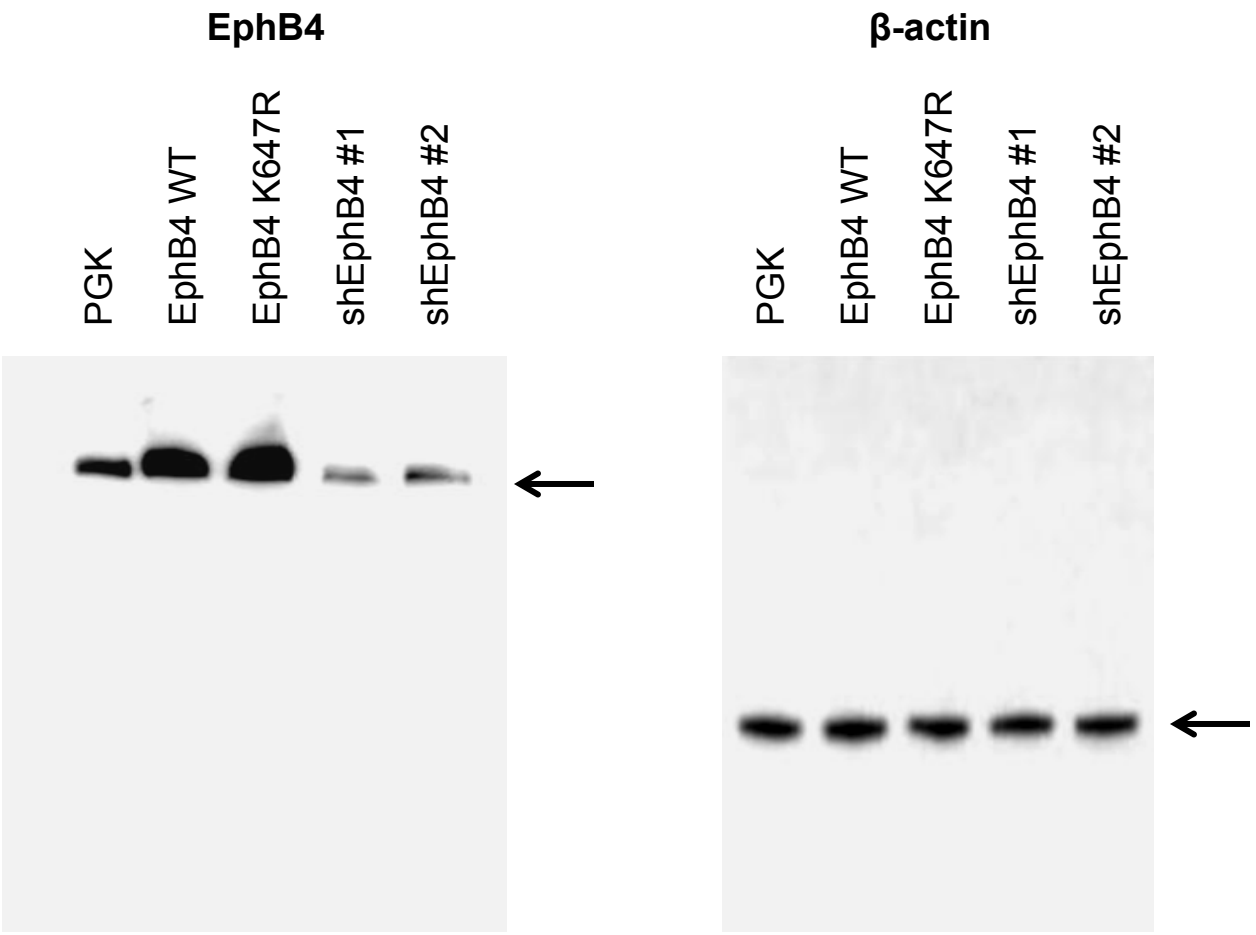

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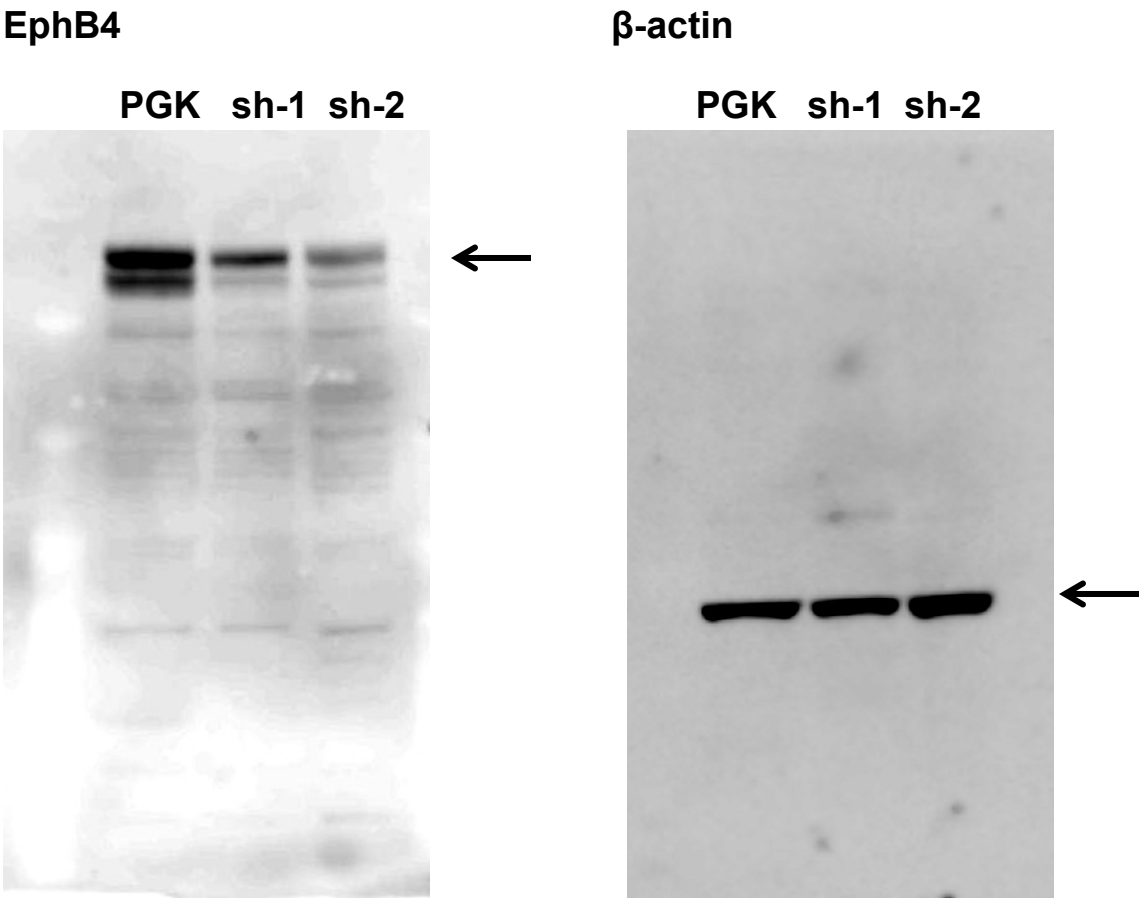
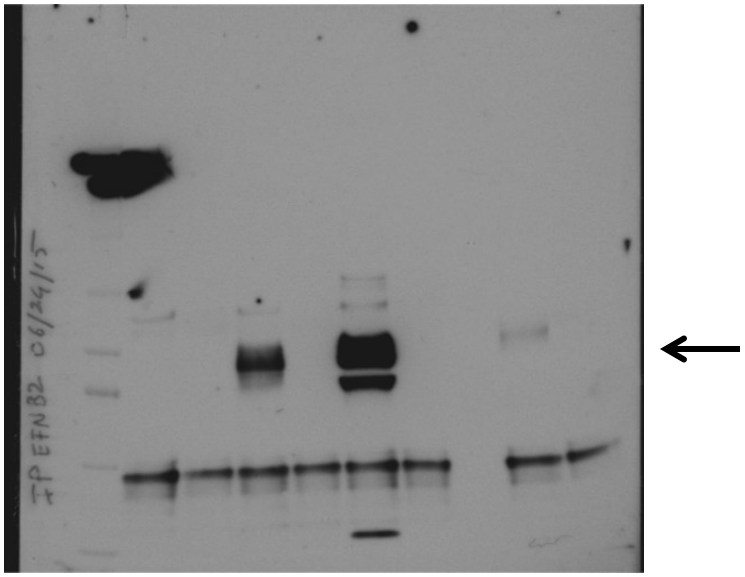


Full unedited gel for Figure S6B.

EphrinB2

B16 LLC1 4T1



Full unedited gel for Figure S6C.

EphB4

B16 LLC1 4T1