Supporting Information

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SI Materials and Methods

Cell Lines. Mouse mammary tumor cell lines 67NR, 168FARN, 4TO7, and 66c14 were from F. Miller (Karmanos Cancer Institute, Detroit, MI). 4T1 breast carcinoma, MDA-MB-231 human breast carcinoma, LLC, and B16F10 mouse melanoma were purchased from ATCC. MDA-MB-231-D3H1 (MDA-MB-231 cells stably expressing luciferase) was from Xenogen. MDA-MB-231-X1.1 cells are GFP-expressing cells previously passaged through mice to generate a highly tumorgenic cell line. They were generated by tranfecting parental MDA-MB-231 cells with vector-expressing GFP and puromycin. Subsequently, GFP-positive cells were injected subcutaneously (in Matrigel) into SCID/bg mice. Visible tumors were harvested, and tumorgenic cells were isolated and expanded. All breast cancer cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) except MDA-MB-231, LLC, and B16F10 cells, which were grown in DMEM (Invitrogen). Both IMDM and DMEM were supplemented with 10% FBS (Sigma). All cells were maintained at 37 °C in a 5% CO_2 and 80% humidity incubator.

Tumor Models. 67NR, 168FARN, 4TO7, 66c14, and 4T1 cells were injected orthotopically into the right fourth mammary fat pad of female BALB/c or CB6F1 at the concentration of 200,000 cells per 10 μL PBS. Anti-Bv8 treatment was performed as described previously (1). Briefly, i.p. anti-Bv8 treatment (5 mg/kg of each 2B9 and 3F1 monoclonal antibodies or 10 mg/kg 2D3 monoclonal antibody) was initiated 2 d after tumor cell inoculation. Antiragweed monoclonal antibody (10 mg/kg) served as control. Antibodies were administered to tumor-bearing mice three times per week. Neutralizing anti-mouse granulocyte-colony stimulating factor (G-CSF) mAb (MAB414), anti-mouse macrophage (M-)CSF (MAB416), anti-mouse GM-CSF (MAB415), and the matching isotype IgG controls (all from R&D Systems) were administered at the dose of 50 μg per mouse every other day. The neutralizing activity of anti–G-CSF, anti–GM-CSF, and anti–M-CSF antibodies was confirmed using appropriate in vitro proliferation assays (Fig. S7).

To deplete myeloid cells, anti-Gr1 antibody (50 μg per mouse, clone RB6-8C5; eBioscience) or anti-Ly6G antibody (50 μg per mouse, clone 1A8; BD Biosciences) was used. Tumor volumes were calculated one time per week using the ellipsoid volume equations ($0.5 \times L \times W^2$ where L is length and W is width) (2). At the end of the study, lungs were perfused with PBS and fixed in formalin, and visible metastases were counted. Authenticity of tumors in lungs was confirmed by H&E staining.

Lewis Lung Carcinoma (LLC) cells were resuspended at a concentration of 1×10^8 cells/mL Matrigel (growth factor-reduced; BD Pharmingen) and injected s.c. (100 μL) into the dorsal flank of C57BL/6 mice. Tissues from LLC-bearing mice were analyzed for the frequency of Cd11b+Gr1+ cells and Bv8 expression 15 d after tumor inoculation, at which point cancer cells were not detected in the lung tissue.

Tissues from female MMTV-PyMT mice were analyzed at ages 4, 8 (lung metastases were not detected), and 12 wk (metastatic nodules were clearly visible) for content of G-CSF and Bv8 in plasma and lung tissue. MMTV-PyMT negative siblings (littermates that lacked the transgene as assessed by genotyping) were used as controls and are referred to as naïve. For anti-Bv8 and anti–G-CSF studies, MMTV-PyMT cells were isolated from primary tumors of 12-wk-old females. All isolated cells (tumor and stroma) were counted and resuspended at a concentration of 2×10^7 cells/mL PBS:Matrigel (1:1 mix, growth factor-reduced; BD Pharmingen), and then, they were injected (50 μL) into the fourth mammary fat pad of FVB mice. Isolated cells were also cultured in DMEM supplemented with FBS. i.p. treatment with anti-Bv8 (10 mg/kg 2D3 monoclonal antibody) or anti-mouse G-CSF (2.5 mg/kg, MAB414; R&D Systems) antibodies was initiated 2 d after tumor inoculation. At the end of the study, lungs were perfused with PBS and fixed in formalin. Subsequently, 7-μm-thick sections were H&E stained, and tumors present in lungs were counted. On average, three sections (separated by 250 μm) per each lung were analyzed.

MDA-MB-231-X1.1 cells were resuspended at a concentration of 4×10^7 cells/mL PBS:Matrigel (1:1 mix; BD Pharmingen) and injected (50 μL) into the fourth mammary fat pad of SCID/bg mice. Treatment with anti-Bv8 (10 mg/kg 2D3 monoclonal antibody) or combination of anti-human (anti–hG-CSF, 2.5 mg/kg, MAB214; R&D Systems) and anti-mouse G-CSF (anti–mG-CSF, 2.5 mg/kg, MAB414; R&D Systems) antibodies was initiated 2 d after tumor cell inoculation. Matching isotype IgG control served as controls. Antibodies were administered i.p. to tumor-bearing mice three times per week. At the end of the study (49 d after inoculation of tumors), lungs were perfused with PBS and fixed in formalin for 16 h. Lung sections were stained with anti-human cytokeratin 18 (CK-18) antibody to detect lung metastases (details in *SI Materials and Methods, CK-18 IHC*). Alternatively, for studies involving injection of recombinant G-CSF into female BALB/c nude mice, hamster anti-mouse Bv8 mAb 2D3 (3) was administered at the dose of 10 mg/kg two times weekly.

Generation of *Bv8* Null Mice. To directly assess the significance of endogenous Bv8 in tumor models, we generated mice lacking Bv8 in bone marrow cells (BMCs) through transplantation of *Bv8* null fetal liver cells. To generate *Bv8* null mice, a BAC clone mVRPA BAC 133 N12 (Research Genetics) containing the mouse gene Bv8 and a targeting vector TNLOX1-3 with three *loxP* sites were used to build a targeting construct designed to generate mice with a knockout and conditional knockout deletion allele of Bv8. ES clones with PGK-Neo cassette correctly targeted at exon 2 were screened by Southern blotting and subjected to electroporation of Cre recombinase to excise both PGK-Neo selection cassette and exon 2 for Bv8 knockout. Six Bv8 KO clones were injected into blastocysts from C57BL/6 mice. Chimeras that transmitted the disruptive mutation through the germline were crossed with C57BL/6 mice. Mice were kept on inbred 129SvEv background and backcrossed to C57BL/6 background.

Fetal Liver Transplantation and Tumor Inoculation into a Lethally Irradiated Host. Fetal liver (FL) cells were isolated from embryos of *Bv8* wild-type (WT) or null mice at embryonic (E) days E13.5–14.5. Pregnant mice were euthanized at days 13.5–14.5 of pregnancy, and FL cells were isolated in a microdissection microscope and immediately placed in a Petri dish containing cold plain DMEM. Single cell suspension was generated by vigorously pipetting the FLs and passing cells through a 40-μm cell strainer (BD Falcon). FL cells were then subjected to red blood cell lysis using ACK lysis buffer (Cambrex), kept in plain DMEM, and subsequently used for transplantation into lethally irradiated mice.

Before transplantation, CB6F1 mice were lethally irradiated with 1,080 Rad in a Cs irradiator to ablate endogenous BMCs in the host followed by i.v. injection of 4×10^5 whole fetal liver cells in 100 μL PBS.

Four weeks after FL transplantation, 4T1 or 66c14 cells were inoculated into the fourth mammary fat pad of CB6F1 mice as

described. Treatment with isotype control or anti-Bv8 antibody (2B9 and 3F1) was performed as described. Lungs were analyzed for the presence of metastases either by microcomputed tomography (micro-CT) or by counting visible tumors on the lungs surface.

Generation of Luc-zsGreen-Hygro Cells Lines. pMSCV vector encoding a fusion protein Luciferase-zsGreen (a functional fusion between firefly luciferase and zsGreen) was generated by inserting a luciferase cDNA in frame with zsGreen cDNA into a pMSCV-zsGreen vector. Hygromycin resistance gene was encoded by pMSCV vector.

To generate 67NR and 4T1 cells stably expressing Luciferase-zsGreen fusion, cells were transfected with pMSCV-Luc-zsGreen-Hygro vector using FuGENE transfection reagent according to the manufacturer's protocol (Roche). Hygromycin-resistant cells were selected by culturing transfected cells in the presence of 200 μg/mL Hygromycin for 10 d. Luciferase-zsGreen–positive cells were sorted by flow cytometry based on their zsGreen expression and further validated to express luciferase by measuring luciferase activity with the Dual-Luciferase Reporter Assay System (Promega). Cells expressing both zsGreen and Luciferase were further expanded in the presence of Hygromycin (100 μg/mL) and used for subsequent experiments.

In Vivo Selection of MDA-MB-231 Cells. To generate MDA-MB-231 clones that show enhanced metastatic potential in lungs, 5×10^5 MDA-MB-231-D3H1 cells in PBS were injected through the tail vein of SCID/bg mice. Six weeks later (when bioluminescence imaging showed presence of growing tumors in lungs), lungs from three mice were harvested, and tumor cells were dissected and expanded in culture. Three independent clones (obtained from three different mice) were generated and named MDA-MB-231-L1.1, -L2.1, and -L3.1. To obtain cells that are tumorigenic at the primary tumor site, 1×10^6 MDA-MB-231-D3H1 cells were inoculated in Matrigel into the fourth mammary fat pad of SCID/bg mice. Primary tumors were harvested, and cells were dissociated and expanded in culture. Three independent cell clones (obtained from three different mice) were generated and named MDA-MB-231-T1.1, -T2.1, and -T3.1. For gene expression analysis, newly established cell lines (MDA-MB-231-L1.1, -L2.1, and -L3.1 and MDA-MB-231-T1.1, -T2.1, and -T3.1) were seeded at a density of 500,000 cells/well (in six-well plates), and RNA was collected 48 h later and used for subsequent quantitative (q)RT-PCR expression analysis. Expression of *G-CSF*, *PKR-1*, *GM-CSF*, *MMP-9*, *Cxcl1*, *MMP-1*, *PKR-2*, *M-CSF*, *SDF1α*, *VEGF-A*, and *PlGF* by MDA-MB-231-L1.1, -L2.1, and -L3.1 or MDA-MB-231-T1.1, -T2.1, and -T3.1 cells was compared with the expression by parental MDA-MB-231-D3H1 cell line (cells that were not injected into mice).

Production of 66c14-shPKR1 and 67NR-PKR1 Cells. 66c14 cells were transfected with shRNA vector-targeting mouse *PKR1* (shPKR1; Open Biosystems), and clones stably expressing shPKR1 were isolated through culturing cells in the presence of puromycin (5 μg/mL). As a control, 66c14 cells were transfected with vector-expressing nontargeting shRNA [sh(Control)]. To generate 67NR cells overexpressing PKR1, 67NR cells were cotransfected with vector containing CMV-PKR1 cassette together with vector carrying the Zeocin-resistance gene. Cells stably expressing PKR1 were selected in the presence of zeocin (200 ng/mL). Subsequently, cells were injected i.v. through the tail vein into BALB/c mice pretreated with vehicle or G-CSF as described. Four weeks later, lungs were removed and checked for the presence of visible tumors. Authenticity of tumors in lungs was confirmed by H&E staining in histologic sections.

Taxotere Treatment. BALB/c mice were inoculated orthotopically with 66c14 or 4T1 cells as described. Taxotere (Sanofi-Aventis) was administered at days 9, 13, and 17 at the dose of 25 mg/kg. G-CSF (Neupogen; Amgen) was administered at 5 μg per mouse for 2 consecutive d after each dose of taxotere. At the end of the study (5.5 wk), mice were analyzed for the presence of visible lung tumors. Lungs were perfused with PBS and fixed in formalin, and visible tumors were counted. Authenticity of tumors in lungs was confirmed by H&E staining in sections. For tail-vein injections, BALB/c mice were pretreated with daily doses of G-CSF (5 μg per mouse) for 5 consecutive d, and taxotere (25 mg/kg) was administered at days 1, 3, and 5. 66c14 or 4T1 cells were injected through the tail vein after the last G-CSF and taxotere injection. Three weeks later, mice were euthanized, lungs were perfused with PBS, and visible tumors present in the lungs were counted. To assess taxotere-associated neutropenia, three mice from each treatment group were euthanized after the last dose of G-CSF (day 20 for tumor-bearing mice and day 6 for mice pretreated with G-CSF), and peripheral blood or lung was analyzed for the presence of Ly6G+Ly6C+ neutrophils by FACS.

RNA Sample Preparation and qRT-PCR Analysis. Total DNA-free RNA was prepared from PBS-perfused lungs with the RNeasy kit (Qiagen) according to the manufacturer's protocol. One-step qRT-PCR was done in a total volume of 50 μL with the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) or TaqMan One-Step RT-PCR Master Mix (Applied Biosystems) and 100 ng of total RNA. The following TaqMan Gene Expression Assay primers and probe mixes were used: *Bv8* (assay ID: Mm00450080_m1), *MMP9* (assay ID: Mm00442991_m1), *S100A8* (assay ID: Mm00496696_g1), *S100A9* (assay ID: Mm00656925_m1), *G-CSF* (assay ID: Hs99999083_m1), *M-CSF* (assay ID: Hs99999084_m1), *GM-CSF* (assay ID: Hs00929873_m1) *VEGF-A* (assay ID: Hs00900054_m1), *PlGF* (assay ID: Hs01119262_m1), *SDF1α* (assay ID: Hs00171022_m1), *Cxcl1* (assay ID: Hs00236937_m1), *MMP1* (assay ID: Hs00899658_m1), *PKR1* (assay ID: Mm00517546_m1 and Hs03044877_m1), *PKR2* (assay ID: Mm00769571_m1 and Hs03046077_m1), *G-CSFR* (assay ID: Mm00432735_m1), *Hprt1* (assay ID: Mm00446968_m1 and Hs99999909_m1), and *GAPDH* (assay ID: Mm99999915_g1 and Hs99999905_m1). *Hygromycin* expression was detected using custom-made primers and TaqMan probe (Applied Biosystems): forward primer: CCGCAAGGAATCGGTCAAT; reverse primer: GATCAGCAATCGCGCATATG; TaqMan probe: 6FAM-CACTACATGGCGTGATT-MGBNFQ.

Reactions were carried out using Applied Biosystems 7500 Real-time PCR System under the following conditions: a reverse transcription step (15 min at 48 °C) followed by denaturation step at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Gene expression levels in each sample were determined and normalized to the expression of endogenous controls (*GAPDH* or *Hprt1*).

Microarray Sample Preparation and Analysis. BALB/c mice ($n = 5$) were injected with nonmetastatic 67NR or metastatic 4T1 cells (200,000 cells in PBS per mouse). Control (naïve) mice received PBS only. Six days after tumor cell inoculation (or when tumors reached about 50 mm^3), mice were euthanized, and lungs were perfused with PBS to completely remove blood from the lung vasculature. Cleaned lung lobes were immersed in RNAlater (Qiagen) to stabilize RNA. Total RNA was isolated as described in *SI Materials and Methods*, *RNA Sample Preparation and qRT-PCR Analysis* and used for subsequent microarray analysis. Affymetrix microarrays (Mouse 430-v2) were used to analyze the expression profile of tissue samples. One sample from the 4T1 group was excluded from the final analysis because of the poor quality of the acquired data. Raw data were processed using a program that implemented the recommendations of Choe et al. (4).

Cloning and Expression of mG-CSF cDNA. To generate a murine G-CSF transient expression construct, a PCR fragment containing the consensus Kozak sequence and the coding region of mG-CSF

with a 6xHis tag at its C terminus was amplified from IMAGE mouse cDNA clone #40129682 (OPEN BIOSYSTEMS) and inserted into the ClaI and EcoRI sites of pRK vector. The resulting construct was then used to transfect CHO cells. The supernatants were harvested, and recombinant mG-CSF protein was purified using nickel nitrilotriacetic acid (Ni-NTA) columns (Qiagen) followed by size exclusion chromatography.

In Vivo G-CSF and Anti–G-CSF Studies. To assess the effects of G-CSF on the mobilization and homing of myeloid cells at distant organs, 8-wk-old female BALB/c or BALB/c nude mice were injected s.c. with 10 μg of recombinant human G-CSF (Neupogen; Amgen) or recombinant mouse G-CSF daily for 5 consecutive d. The G-CSF stock was diluted to the desired concentration with sterile PBS (Invitrogen). Control animals were given PBS as a vehicle. At the end of study (24 h after last G-CSF injection), mice were perfused with PBS, and tissues (lung, bone marrow, whole blood, spleen, liver, and kidney) were harvested for analysis. Cd11b+Gr1+ or Ly6G+Ly6C+ cells were analyzed as described in *SI Materials and Methods*, *Flow Cytometry (FACS)*. Bv8 concentrations in the tissues were measured by ELISA as described. To determine the role of Bv8 in G-CSF–induced mobilization of CD11b+Gr1+ cells, BALB/c nude mice received daily doses (for 5 d) of anti-Bv8 2D3 antibody (2.5 mg/kg) followed by mouse G-CSF 6 h after the treatment. Anti-gp120 antibody (Genentech) was used as an isotype control. In some experiments, mice were treated with rat anti-Gr1 antibody (50 μg per mouse, clone RB6-8C5; eBioscience) or anti-Ly6G antibody (50 μg per mouse, clone 1A8; BD Biosciences). As a positive control, we used a rat anti-mouse G-CSF monoclonal antibody (50 μg per mouse, MAB414; R&D Systems) given at the same interval as anti-Bv8 antibody and followed by mouse G-CSF.

To elucidate the role of G-CSF in metastasis, BALB/c, BALB/c nude, C57BL/6, or SCID/bg mice were pretreated with human or mouse G-CSF (10 μg per mouse) as described above. This was followed by tail-vein injections of 10,000 cells (66c14, 4T1, 67NR, B16F10, or MDA-MB-231-L1.1) in 100 μL PBS. G-CSF administration continued for another 5 d after injection of cancer cells. Mice were analyzed for the presence of lung tumors 3 wk (4 wk for 67NR and 5 wk for MDA-MB-231-L1.1 tumors) after injection of cancer cells. Lungs were perfused with PBS and fixed in formalin, and visible tumors were counted. Authenticity of tumors in lungs was confirmed by H&E staining in tissue sections. To determine the role of Bv8, Gr1+, or Ly6G+ cells in G-CSF–induced metastasis, mice were pretreated with vehicle or mouse G-CSF (2.5 μg per mouse) and further treated with anti-Bv8, anti-Gr1, anti-Ly6G, or anti–G-CSF antibodies for 5 d as described above. Anti-VEGF antibody (G6.31; Genentech) was given at 5 mg/kg every other day. Antibody treatments were discontinued after inoculation of tumor cells.

Migration Assays. Cells were trypsynized, washed with FBS-free Iscove's Modified Dulbecco's Medium (IMDM), and resuspended at the concentration of 100,000 cells/mL; 300 μL of the cell suspension was loaded to the upper well of a FluoroBlok 24-Multiwell Insert System plate (8.0 μm; BD Biosciences) that was coated with collagen type I (BD Biosciences) for 1 h before setting up the assay. The lower well was filled with 1 mL IMDM with the indicated concentration of human Bv8 (Peprotech). IMDM with 1% FCS served as positive control. Plates were incubated for 16 h. Cells that migrated through the membrane were fluorescently labeled with Calcein AM (BD Biosciences). Images of labeled cells were acquired (Zeiss microscope system), and cells were counted using ImageJ software (National Institutes of Health). Numbers of migrated cells were normalized to the Vehicle group (Vehicle = 1).

In Vitro Bioassays for G-CSF, M-CSF, and GM-CSF Activity. Mouse bone marrow NFS-60 cells (5) were maintained in growth media (RPMI 1640 media supplemented with 10% serum, 2 mM glutamine, and antibiotics) with 2 ng/mL recombinant mouse IL-3 (R&D Systems). For inhibition assay, 1×10^4 cells were seeded in 96-well plates in growth media without mouse IL-3. Mouse G-CSF was then added to a final concentration of 0.1 ng/mL After 48 h, cells were treated with Resazurin (R&D Systems) for 4 h. Fluorescence was measured at 544-nm excitation wavelength and 590-nm emission wavelength. For M-NFS60 (6) (ATCC) cell proliferation assay, 5,000 cells were seeded, and assay was performed at the presence of 1 ng/mL of recombinant mouse M-CSF (R&D Systems). For FDC-P1 (7) proliferation assay, 2,500 FDC-P1 cells were plated in a 96-well plate and treated with mouse recombinant GM-CSF (R&D Systems) at a final concentration of 0.1 ng/mL Antibodies [anti–G-CSF (MAB414; R&D Systems), anti–M-CSF (MAB416; R&D Systems), or anti–GM-CSF (MAB415; R&D Systems)] were added at indicated concentrations to triplicate wells first for 10 min.

Collection of Conditioned Media from Tumor Cells. 67NR, 168FARN, 4TO7, 66c14, 4T1, isolated MMTV-PyMT, and MDA-MB-231-X1.1 cells were grown as described above. After reaching 90% confluence, media were changed to serum-free IMDM. The conditioned media were collected after a 2-d incubation, and cell viability and total cell number were measured using Vi-Cell XR (Beckman Coulter). Media were analyzed for the presence of cytokines by ELISA as described. All data were normalized to the total cell number.

In Vivo Extravasation Assay. 66c14 or 4T1 cells were grown until they reached 90% confluence and were fluorescently labeled with 10 μM CellTracker Green CMFDA (Invitrogen) for 30 min according to the manufacturer's protocol. After the labeling, cells were trypsinized, washed with PBS, and counted, and 100,000 labeled cells in 100 μL PBS were injected i.v. through tail vein of BALB/c nude mice that were pretreated with mouse G-CSF (2.5 μg per mouse daily for 5 d) in the presence of anti-Bv8 (2B9+3F1), anti-Gr1, anti–G-CSF, or isotype control (ISO) antibody as described above. As negative controls, unlabeled 66c14 or 4T1 cells were injected into mice pretreated with G-CSF and ISO. Treatment with G-CSF and antibody continued for an additional 1 d after cell inoculation. Mice were euthanized 36 h after cell inoculation, and lungs were perfused with PBS, harvested, and fixed in paraformaldehyde. Fixed lungs were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) and frozen, and 5-μm sections were generated. Sections were counterstained with DAPI and coverslipped with ProLong Gold mounting media (Invitrogen). Images were acquired, and fluorescently labeled cells were manually counted. On average, three mice per group were used, and 15 images (five random sections per mouse) were included into the analysis. The final values were presented as an average number of cells per field.

Histological Analysis (H&E). Formalin-fixed tissues was dehydrated and embedded in paraffin; 5-μm-thick sections were dewaxed, rehydrated, and stained with H&E using standard protocol.

Bv8 Immunohistochemistry. Bv8 immunohistochemistry (IHC) was performed as described previously (3). Briefly, premetastatic lungs from PBS-perfused mice bearing 67NR or 4T1 tumors were harvested and fixed in neutral-buffered formalin; 5-μm-thick paraffin-embedded sections were dewaxed and rehydrated. After antigen retrieval using universal decloaker buffer (Biocare Medical) in a pressure cooker (Biocare Medical), the sections were blocked with peroxidase blocking reagent (DAKO) for 5 min followed by blocking with protein block solution (DAKO) for 30 min. Sections were then incubated with hamster anti-mBv8 mAb 2D3 or hamster IgG1 (BD Pharmingen) at 10 μg/mL for 1 h at room temperature. Next, sections were stained with biotinylated goat anti-hamster antibody (Jackson Immuno Research) for 30

min at room temperature followed by incubation with Vectastain ABC Elite reagents (Vector Laboratories). Sections were then incubated with peroxidase substrate solution (Metal Enhanced DAB; Pierce Chemical) until the desired intensity was developed. Last, sections were light counterstained with H&E, dehydrated, and coverslipped.

CK-18 IHC. To identify MDA-MB-231-X1.1 metastases, lungs from PBS-perfused mice bearing MDA-MB-231-X1.1 tumors were harvested and fixed in neutral-buffered formalin; 5-μm-thick paraffin-embedded sections were dewaxed and rehydrated. After antigen retrieval using 10 mM citrate buffer, pH 6.0 (Thermo Scientific), in a pressure cooker (Biocare Medical), the sections were blocked with peroxidase blocking reagent (DAKO) for 5 min. The following procedures were performed using the Mouse-On-Mouse (M.O.M.) Immunodetection Kit (Vector Laboratories). Sections were blocked with M.O.M. Mouse IgG Blocking reagent for 60 min. Sections were then incubated with mouse anti-human CK-18 mAb DC-10 (Thermo Scientific) or mouse IgG1 (BD Pharmingen) at 2 μg/mL for 30 min at room temperature. Next, sections were stained with biotinylated goat anti-mouse antibody (Vector Laboratories) for 30 min at room temperature followed by incubation with Vectastain ABC Elite reagents (Vector Laboratories). Sections were then incubated with peroxidase substrate solution (metal enhanced DAB; Pierce Chemical) until the desired intensity was developed. Last, sections were light counterstained with H&E, dehydrated, and coverslipped. To detect metastases, CK-18–positive colonies in multiple lung sections were counted.

Ly6G Immunofluorescence Staining. Premetastatic lungs of MMTV-PyMT mice were analyzed for the presence of Ly6G+ cells at weeks 4 and 8 of age. In all other tumor models, such analysis was performed when the primary tumor reached 250 mm^3. Tissues (lungs, liver, spleen, kidney, muscle, and brain) were harvested from naïve mice or mice bearing tumors and immediately fixed in 4% paraformaldehyde for 16 h. Fixed tissues were transferred to 30% sucrose solution for another 2 d. Subsequently, tissues were embedded in OCT; 10-μm sections were generated from frozen tissue blocks. Sections were blocked with 5% normal goat serum in 0.1% tween-20 in PBS for 1 h at room temperature and subsequently stained with 5 μg/mL phycoerythrin (PE)-conjugated anti-Ly6G antibody (1A8; BD Biosciences) for 30 min. Sections were washed three times with PBS and counterstained with DAPI (ProLong Gold mounting media; Invitrogen).

ELISA. In all tumor models, concentration of various proteins in tumors and premetastatic tissues were measured after the primary tumor reached 250 mm^3. Plasma was collected using EDTA-treated tubes. Mice were perfused with PBS, and small fragments of lung, spleen, tumor, kidney, and bone marrow were collected and immediately frozen in liquid nitrogen. Tissues were subsequently lysed in RIPA buffer, and total protein content was measured by the Bradford method according to the manufacturer's protocol (Pierce). Bv8 concentrations in plasma and tissue lysates were measured by ELISA as described (1, 3). Levels of G-CSF, GM-CSF, M-CSF, SDF1α, PlGF, VEGF, and MMP9 were measured by species-specific ELISA kits (R&D System). Total protein concentration in each tissue lysate was measured using the Bradford method. All ELISA values were normalized to the total protein content in each sample.

Flow Cytometry (FACS). Mice were perfused with PBS. Lung, spleen, tumor, kidney, and bone marrow tissues were collected, minced into small pieces, and filtered to generate single cell suspensions. For several experiments, tumor and lung tissue pieces were enzymatically digested with a blend of collagenase and dispase (Roche) followed by mechanical dissociation using a 16-gauge needle. Red blood cells were lysed using ACK lysis buffer (Lonza). Cells were incubated with rat anti-mouse CD16/CD32 mAb (BD Biosciences) to prevent nonspecific antibody binding before staining with the following fluorophore-conjugated antibodies: anti-cd45 (30-F11), anti-cd11b (M1/70), anti-F4/80 (BM8), anti-cd11c (N418), anti-cd117 (2B8; all from eBiosciences), anti-Ly6G (1A8), anti-Ly6C (AL-21), anti-Gr1 (RB6-8C5), anti-SiglecF (E50-2440; all from BD Biosciences), and anti-Flt-1 (141522; R&D Systems). Propidium iodide (Sigma) was used to distinguish viable and dead cells. Data were acquired using the FACSCalibur or BD LSR II instruments (BD Biosciences) and analyzed using FlowJo software (Tree Star) or BD FACSDiva program.

Sorting of Cd11b+Gr1+ or Cd11b+Ly6G+Ly6C+ Cells. Lungs were prepared and stained with Cd11b-APC and Gr1-FITC or Cd11b-FITC, Ly6C-PE, and Ly6G-APC antibodies as described in *SI Materials and Methods, Flow Cytometry (FACS)*. Desired cells populations (Cd11b+Gr1+, Cd11b+Gr1-, Cd11b-Gr1-, Cd11b+Ly6G+Ly6C+, Cd11b+Ly6G-Ly6C+, and Cd11b+Ly6G-Ly6C-) were sorted on FACS instrument, and their purity was confirmed. Total RNA was immediately isolated as described above. On average, the purity of the sorted populations was 98%.

p-ERK Immunoblotting. Cells were seeded at a density of 10,000 per 100 μL 10% FCS/IMDM. Twenty-four hours later, media were changed to serum-free IMDM, and cells were starved for 24 h. Cells were stimulated with 5 ng/mL of human Bv8 (Peprotech) for 5, 10, 15, and 20 min and immediately lysed and boiled in Tris·Glycine SDS Sample Buffer (Invitrogen). Equal volumes of the lysates were subjected to SDS/PAGE electrophoresis and immunoblotted for phosphorylated or total p44/p42 MAP kinase (ERK1 and ERK2) with specific antibodies (Cell Signaling Technology).

Micro-CT Analysis. Lung preparation, imaging, and analysis were previously described in detail (8). Briefly, mice were anesthetized with 5% isoflurane anesthesia. The heart and lungs were exposed, and the left and right atria were removed. A blunt 24-gauge needle was inserted into the right ventricle, and 10 mL PBS were perfused to remove blood from the lung vasculature. Lungs were dissected and inflated with 10% neutral buffered formalin (NBF). Lungs were immersed in 10% NBF for 24 h, then immersed in a 20% solution of an iodine-based X-ray computed tomography contrast agent (Isovue370; Bracco Diagnostics Inc.), and diluted with PBS for 24 h. Lungs were then perfused through the trachea with 20 mL soybean oil (Sigma-Aldrich) to remove excess contrast agent. Lungs were imaged in soybean oil to provide a background media for imaging.

The mouse lungs were imaged ex vivo with a μCT 40 (SCANCO Medical) X-ray micro-CT system. The micro-CT images were generated by operating the X-ray tube at an energy level of 45 kV, a current of 177 μA, and an integration time of 450 ms. Axial images were obtained at an isotropic resolution of 20 μm. The lung tumor estimates (number and volume) were obtained by a semiautomated image analysis algorithm that includes an inspection step by a trained reader. Potential tumor masses were extracted by a series of image processing steps (8). The image analysis software was coded in C++ and used the Analyze (AnalyzeDirect Inc.) image analysis software libraries. The extracted objects were then evaluated by a trained reader with the Analyze 3D visualization software. Individual objects were accepted or rejected as possible tumors based on the appearance of the object and its location within the lung. Tumor count, individual tumor volume, and total tumor volume were determined for each lung.

Bioluminescence Imaging. Mice were injected i.p. with 200 μL of 200 mg/mL D-luciferin (Invitrogen) and were anesthetized during imaging using isoflurane (Henry Schein) through nose cone; body temperature was maintained using a warming pad. Bioluminescence

images (BLIs) were acquired using a cooled intensified charge-coupled device camera fixed to a light-tight imaging chamber (Stanford Photonics). Image acquisition times were typically less than 5 min. Images were processed by coregistering a reference image with the bioluminescence data image.

Statistical Analysis. Unless otherwise stated, Student *t* test was used for all of the statistical analysis, and P value ≤ 0.05 was considered to be significant. Graphs present means ± SEM. All experiments were repeated at least two times, and representative data are presented. For analysis of tumor study in fetal liver transplant experiments, a generalized linear model with Poisson errors and distribution was used.

SI Text

Defining the Premetastatic Lungs. To ensure that the tissues that we examined were truly premetastatic, 4T1 cells expressing *Luciferase-zsGreen* and *hygromycin* (4T1-Luc-zsG-Hyg cells) were inoculated orthotopically, and lungs were harvested every week for the analysis. We could not detect any 4T1-Luc-zsG-Hyg cancer cells in the lungs before week 3 after tumor inoculation as assessed by qRT-PCR detecting *hygromycin* (Fig. S1*B*). In addition, BLI did not detect any luciferase-positive 4T1 cells in the lungs at this stage (Fig. S1*C*). Thus, any signal that we detected in our gene expression analysis was not caused by cancer cells. The period of tumor development without any detectable tumor cells in the lungs is called the premetastatic phase, whereas the stage in which we could detect tumor cells or tumors in the lungs is referred to as the metastatic phase.

Anti-Bv8 Anti–G-CSF Antibodies Inhibit Metastasis. 66c14 or 4T1 tumor cells were orthotopically inoculated in BALB/c or CB6F1 mice. Treatments with anti-Bv8, anti–G-CSF antibodies, or both were initiated 2 d after tumor cell inoculation, and the numbers of metastatic lung nodules were evaluated 5.5 (for 4T1) or 6 (for 66c14) wk after tumor cell injection. Although we could not detect any major changes in primary tumor growth (Fig. S8 *A* and *B*), we observed a significant reduction in the number of metastases in the lungs of mice treated with either anti-Bv8 or anti–G-CSF antibody (Fig. 3 *A* and *B*). In both models, anti-Bv8 antibody decreased the number of visible metastases by 30–60%, anti–G-CSF by 40–70%, and the combination by 40–75% compared with corresponding ISO antibody groups. Fig. 3 *A* and *B* shows representative experiments for 4T1 and 66c14 models. In both cases, the combination therapy (anti-Bv8 plus anti–G-CSF) did not further enhance the effect of anti–G-CSF treatment alone. Similar results were obtained when micro-CT analysis was performed to quantify the numbers of both micro- and macrometastases in lungs from mice bearing 66c14 tumors (Fig. S8*C*).

Subsequently, we analyzed the efficacy of anti-Bv8 and anti–G-CSF antibodies in other breast cancer models, namely human MDA-MB-231 (Fig. 3*D*) and mouse MMTV-PyMT (Fig. S8*G*). In both models, anti-Bv8 or anti–G-CSF treatment resulted in reduction of lung metastasis, but it had little effect on primary tumor growth (Fig. S8 *F* and *H*).

1. Shojaei F, et al. (2007) Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450:825–831.
2. Shojaei F, et al. (2007) Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol* 25:911–920.
3. Shojaei F, et al. (2009) G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proc Natl Acad Sci USA* 106:6742–6747.
4. Choe SE, Boutros M, Michelson AM, Church GM, Halfon MS (2005) Preferred analysis methods for Affymetrix GeneChips revealed by a wholly defined control dataset. *Genome Biol* 6:R16.
5. Holmes KL, Palaszynski E, Fredrickson TN, Morse HC 3rd, Ihle JN (1985) Correlation of cell-surface phenotype with the establishment of interleukin 3-dependent cell lines from wild-mouse murine leukemia virus-induced neoplasms. *Proc Natl Acad Sci USA* 82:6687–6691.
6. Nakoinz I, Lee MT, Weaver JF, Ralph P (1990) Differentiation of the IL-3-dependent NFS-60 cell line and adaption to growth in macrophage colony-stimulating factor. *J Immunol* 145:860–864.
7. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D (1980) Growth of factor-dependent hemopoietic precursor cell lines. *J Exp Med* 152:1036–1047.
8. Caunt M, et al. (2008) Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell* 13:331–342.

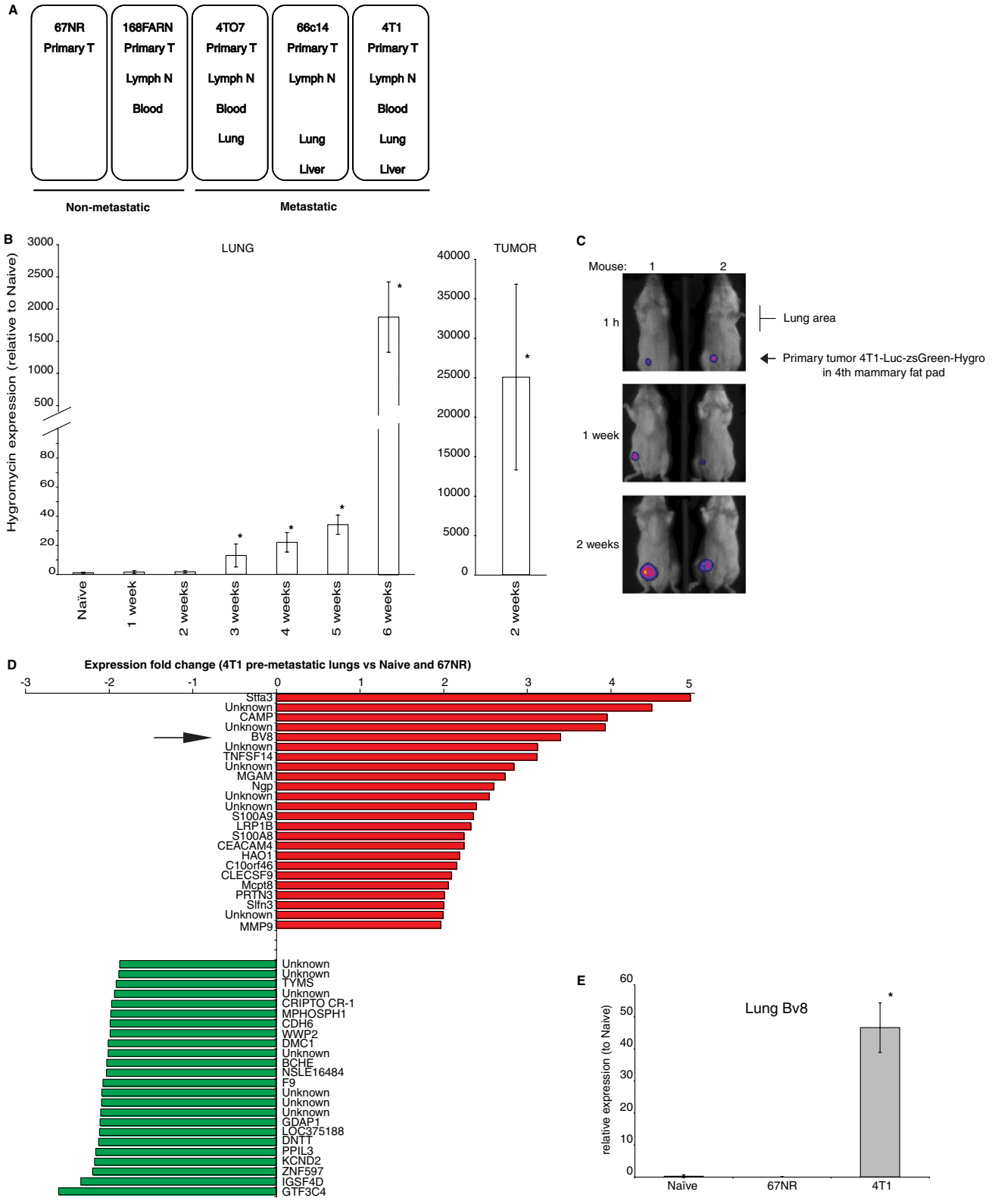

Fig. S1. Premetastatic lungs are tumor-free at the time of tissue harvest. (*A*) The 4T1-related cell lines used in this study with known metastatic sites. (*B*) Timing of development of lung metastasis in 4T1 tumor-bearing mice and qRT-PCR expression analysis of *Hygromycin* gene in lungs or primary tumors showing absence of *Hygromycin* signal in lungs up to 2 wk after inoculation of 4T1-Luc-zsGreen-Hygro tumors. Note the positive signal from the primary tumor. Lungs were harvested every 7 d after tumor inoculation and analyzed for the presence of *Hygromycin* that correlates with the presence of tumor cells in the tissue. (*C*) BLI of mice bearing 4T1-Luc-zsG-Hygro tumors. Note the lack of signal in the lung area and the positive signal in the primary tumor. (*D*) The top 24 up- and down-regulated genes in the premetastatic lungs from mice bearing 4T1 tumors compared with naïve or 67NR-bearing mice. (*E*) *Bv8* qRT-PCR analysis of lung tissues used for microarray analysis. Asterisk indicates significant difference compared with naïve or 67NR tumor-bearing mice.

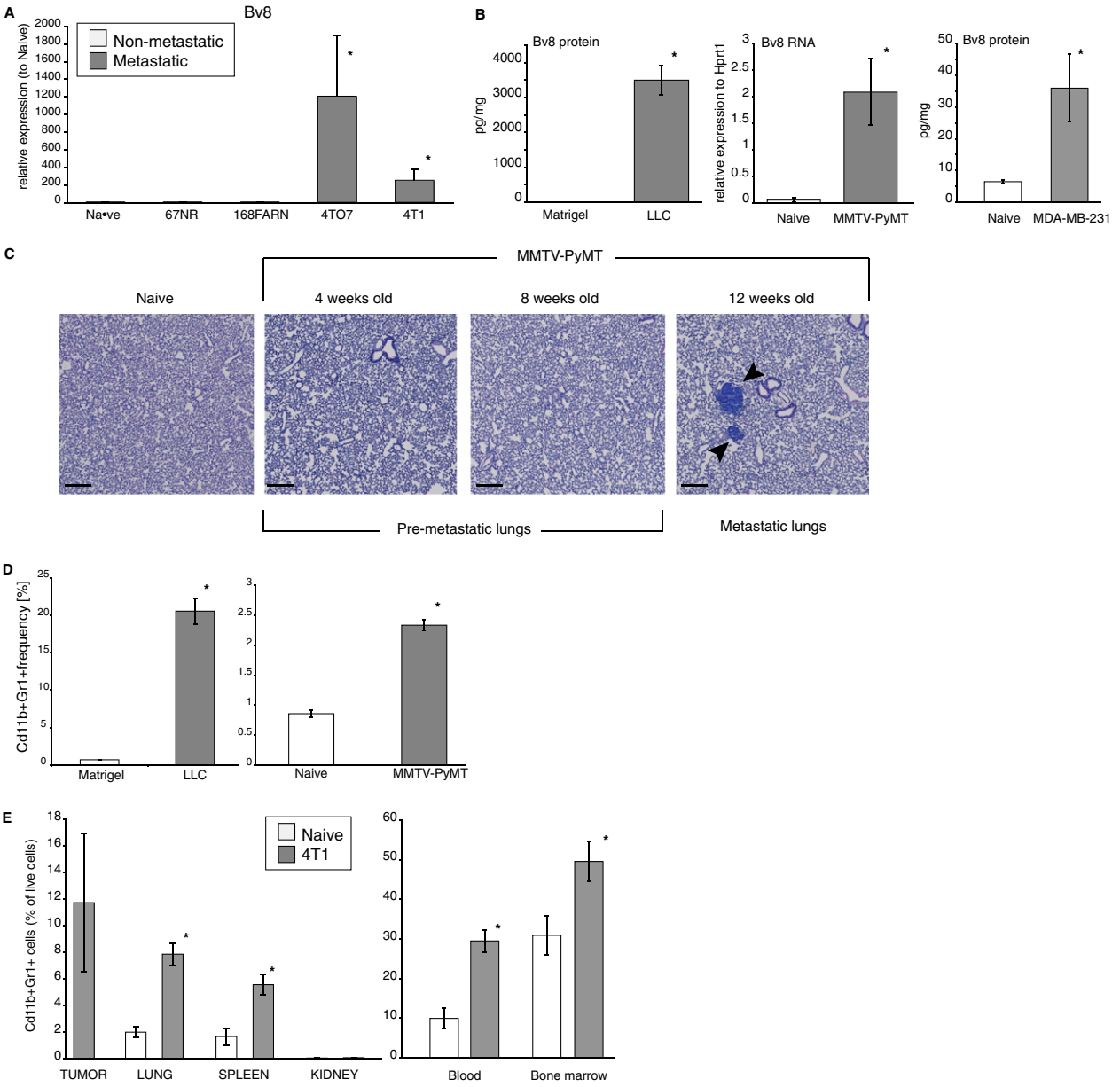

Fig. S2. (Continued)

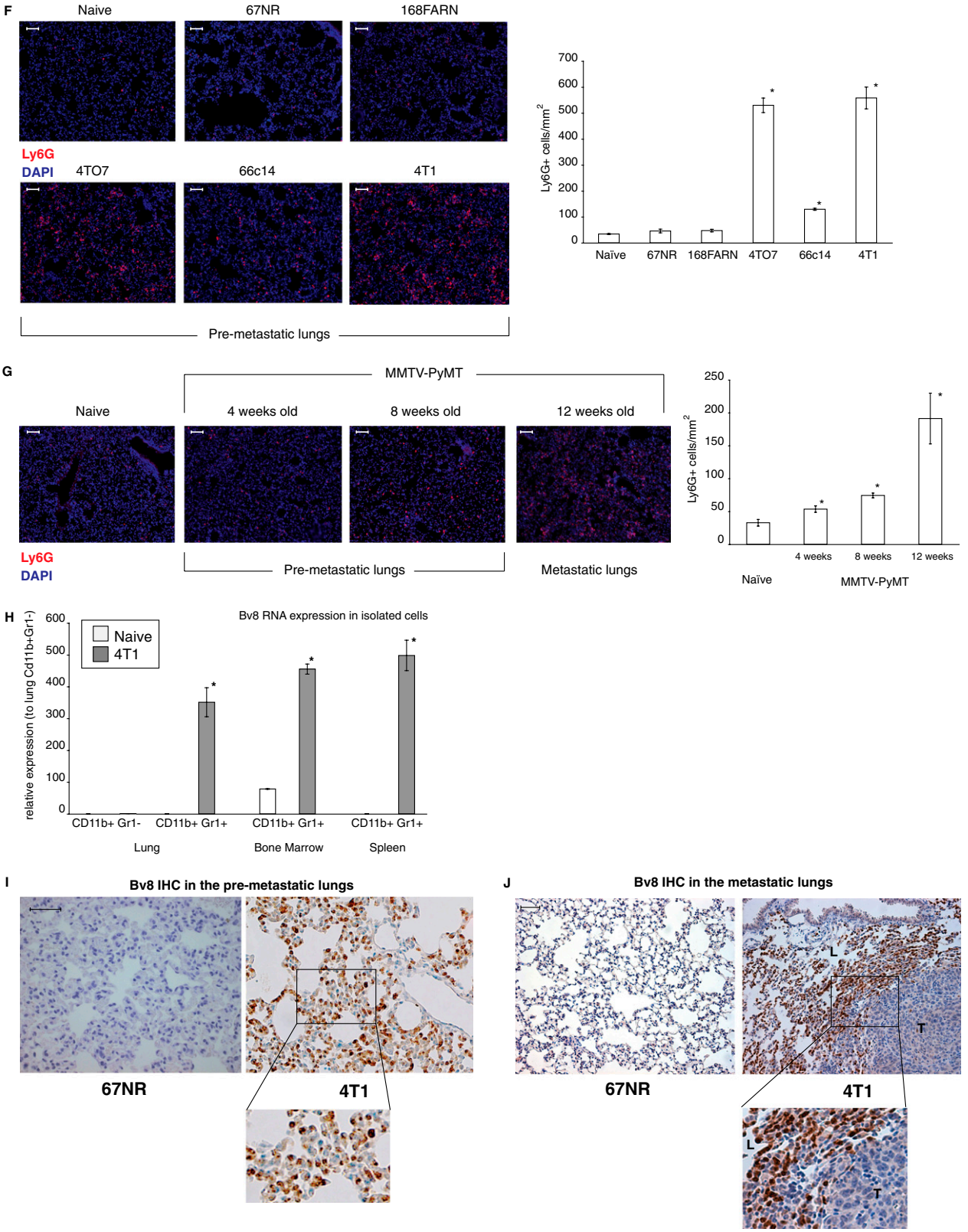

Fig. S2. FACS analysis of Cd11b+Gr1+ cells in premetastatic tissues. Additional analysis of Bv8 expression. (*A*) qRT-PCR analysis of *Bv8* expression in the premetastatic lungs of mice bearing metastatic and nonmetastatic tumors. (*B*) Bv8 expression levels in the premetastatic lungs from mice with LLC, MDA-MB-231-X1.1 (Bv8 protein measured by ELISA), and MMTV-PyMT (*Bv8* RNA measured by qRT-PCR) tumors. Data are shown as expression relative to the naïve tissue. (*C*) Histological analysis of lungs from MMTV-PyMT mice. Note lack of the metastases in 4- and 8-wk-old mice and visible micrometastases (black arrowheads) in 12-wk-old mice. (Scale bar: 250 μm.) (*D*) FACS analysis of Cd11b+Gr1+ in premetastatic lungs from mice bearing LLC tumors (Matrigel-injected mice served as control group) and 8-wk-old MMTV-PyMT mice (PyMT-negative siblings served as control group; $n = 5$). (*E*) FACS analysis of Cd11b+Gr1+ cells in different tissues

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in mice bearing 4T1-Luc-zsGreen-Hygro tumors 7 d after tumor inoculation. The data correspond to the 1-wk point in Fig. S1*B*. (*F*) Examples of Ly6G immunofluorescent staining in lungs collected from mice bearing various tumors in the premetastatic phase. Note increased accumulation of Ly6G+ cells only in mice bearing metastatic tumors (4TO7, 66c14, and 4T1) compared with naïve mice. (Scale bar: 50 μm.) Graph next to the images shows quantification of numbers of Ly6G+ cells in the lungs. (*G*) Ly6G immunofluorescent staining in lungs collected from naïve or MMTV-PyMT mice at 4, 8, and 12 wk of age. Note the increased accumulation of Ly6G+ cells in the premetastatic lungs of 8-wk-old MMTV-PyMT mice. (Scale bar: 50 μm.) Graph next to the images shows quantification of numbers of Ly6G+ cells in the lungs. (*H*) qRT-PCR analysis of *Bv8* transcript levels in Cd11b+Gr1+ and Cd11b+Gr1- cells isolated from lung, bone marrow, or spleen from naïve mice or mice with 4T1 tumors. (*I*) Bv8 IHC staining in the premetastatic lungs from mice bearing 67NR or 4T1 tumors. Brown staining denotes Bv8-positive cells. 67NR or 4T1 cells were orthotopically inoculated; lungs were harvested 2 wk later, and Bv8 was detected by IHC as described in *Materials and Methods*. (Scale bar: 50 μm.) (*J*) Example of Bv8 IHC staining in lungs in the metastatic phase of mice bearing 67NR or 4T1 tumors. L, lung area; T, tumor area. Asterisk indicates significant difference compared with nonmetastatic tumors-bearing mice (*A*) or naïve mice (*B*–*J*). Values shown are means ± SEM.

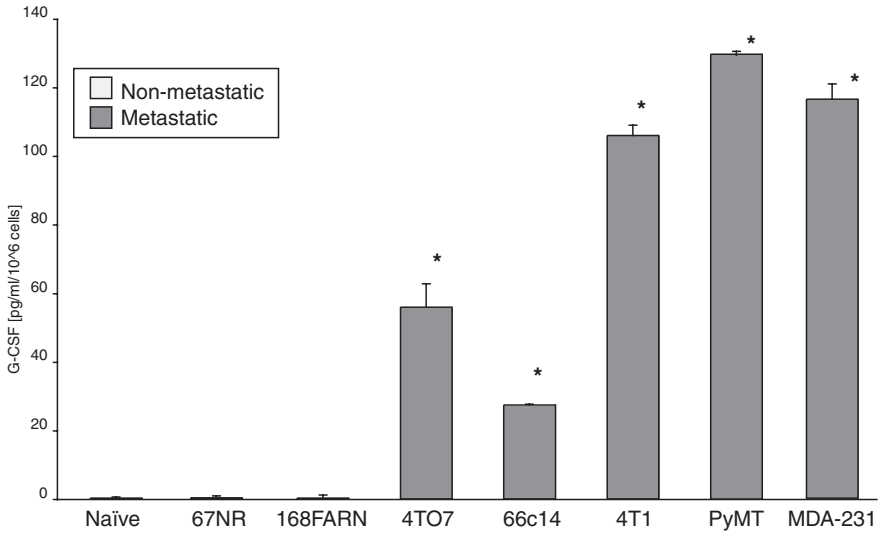


Fig. S3. G-CSF is produced by metastatic tumor cells in vitro. G-CSF concentrations in cell culture supernatants after 48 h incubation as described in *Materials and Methods* (n = 3 per group). Asterisk indicates significant difference compared with naïve and nonmetastatic groups. Values shown are means ± SEM.

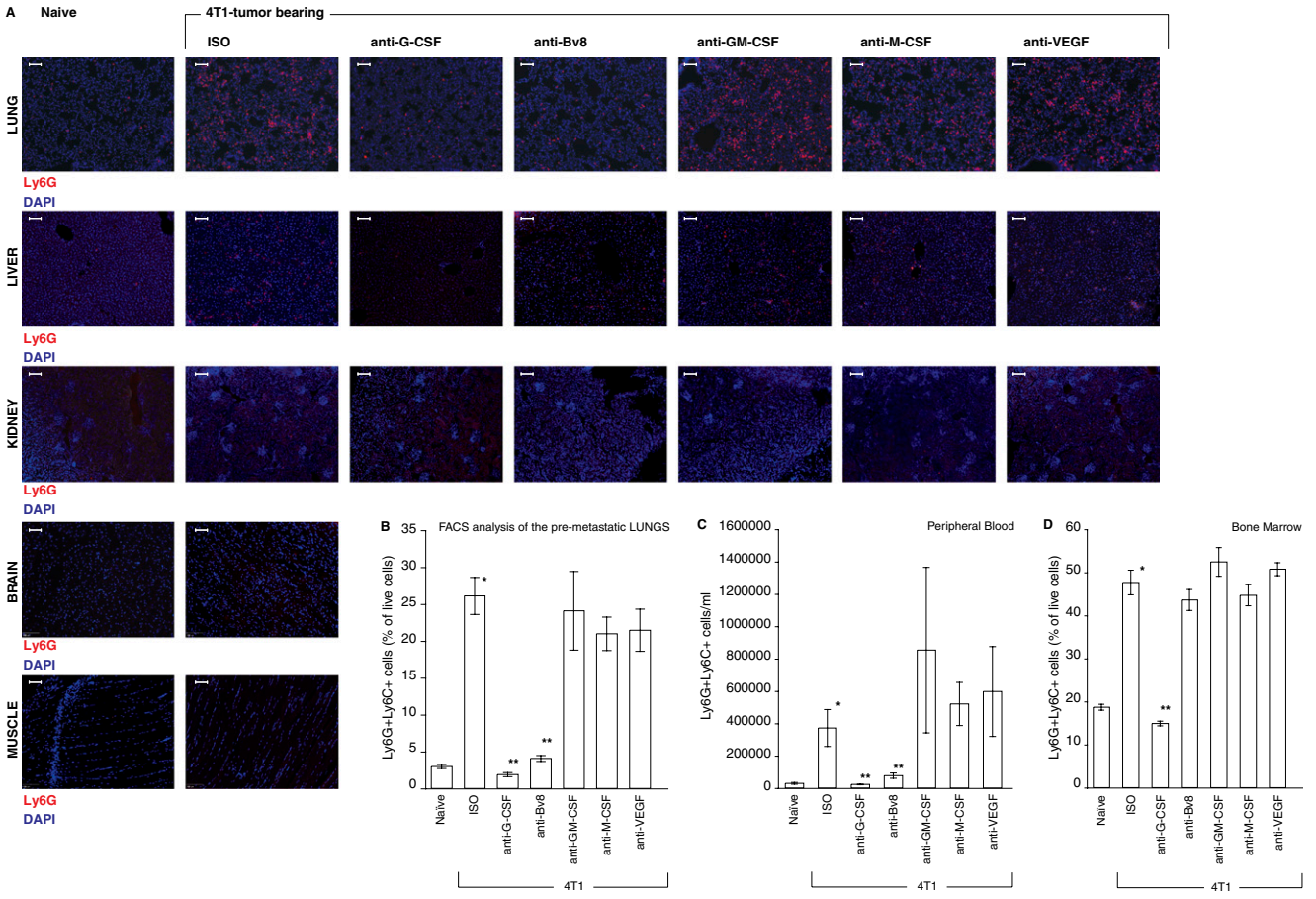


Fig. S4. G-CSF is a major regulator of Ly6G+Ly6C+ cells mobilization in tumor-bearing mice. (*A*) Ly6G immunofluorescent staining in tissues collected from mice bearing 4T1 tumors in the premetastatic phase (2 wk after inoculation of tumors). Mice were treated with indicated antibodies as described in *Materials and Methods*. Note specific accumulation of Ly6G-positive cells only in tissues to which metastasis frequently occurs (lung and liver) but marginal or no staining in tissues that are not common sites of metastasis (brain, kidney, and muscle). Quantification of Ly6G+ cells in the tissues is shown in Fig. 2*C*. (Scale bar: 50 μm.) (*B*) FACS analysis of Ly6G+Ly6C+ cells in lungs from *A*. Note that FACS data correlate with Ly6G staining in *A* and Fig. 2*C*. (*C* and *D*) FACS analysis of Ly6G+Ly6C+ cells in peripheral blood (*C*) and bone marrow (*D*) of mice bearing 4T1 tumors and treated with indicated antibodies as described in *Materials and Methods*. Tissues in *A*–*D* were derived from the same mice. ISO denotes control antibody. In *B*–*D*, asterisk indicates significant difference compared with naïve group, and two asterisks indicate significant difference compared with the 4T1-ISO group. Bv8 protein levels in corresponding tissues from *B*. Values shown are means ± SEM.

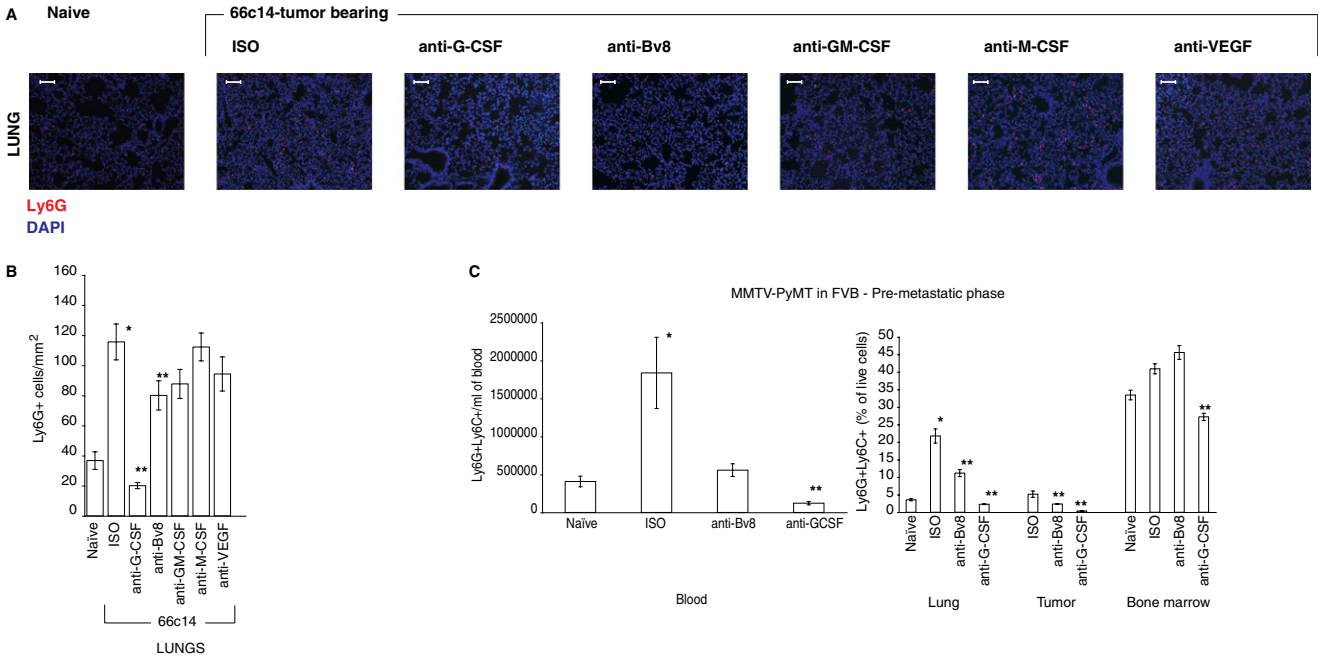


Fig. S5. G-CSF is a major regulator of Ly6G+Ly6C+ cells mobilization in 66c14 and MMTV-PyMT tumor-bearing mice. (*A*) Ly6G immunofluorescent staining in lungs collected from mice bearing 66c14 tumors and treated with indicated antibodies as described in *Materials and Methods*. (Scale bar: 50 μm.) (*B*) Quantification of numbers of Ly6G+ cells from *A*. (*C*) Number of Ly6G+Ly6C+ cells in peripheral blood of naïve or PyMT tumor-bearing FVB mice treated with isotype IgG (ISO), anti-Bv8 (2D3), or anti–G-CSF antibody (*Left*). FACS analysis of Ly6G+Ly6C+ cells in lung, primary tumor, and bone marrow of naïve or PyMT tumor-bearing FVB mice (*Right*). In *B* and *C*, asterisk indicates significant difference compared with naïve group, whereas two asterisks indicate significant difference compared with ISO group.

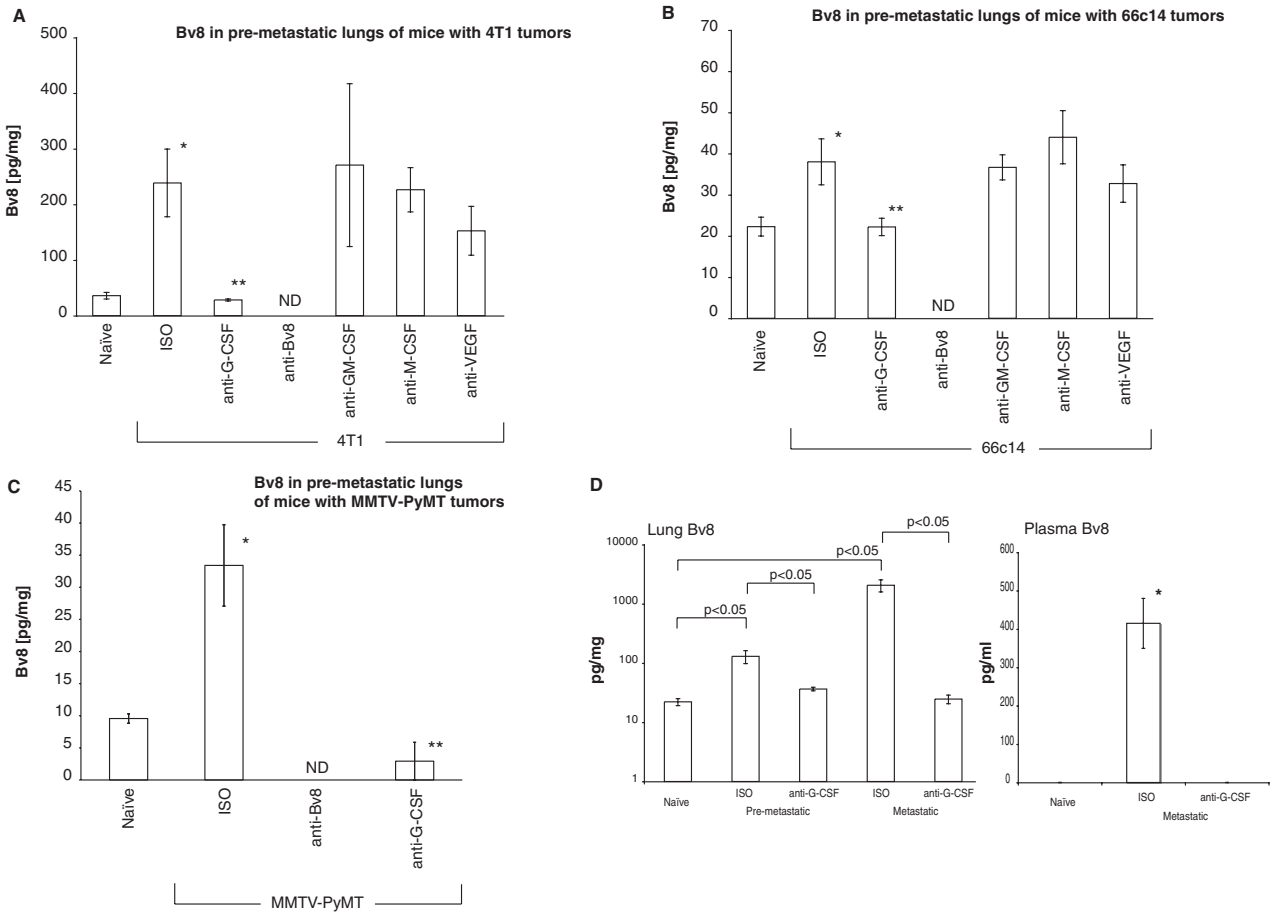


Fig. S6. G-CSF is a major regulator of Bv8 expression in the premetastatic lungs. (*A*) Bv8 concentrations in premetastatic lungs of mice bearing 4T1 tumors from Fig. S4*A*. (*B*) Bv8 concentrations in premetastatic lungs of mice bearing 66c14 tumors from Fig. S5*A*. (*C*) Bv8 concentrations in premetastatic lungs of mice bearing MMTV-PyMT tumors from Fig. S5*C*. (*D*) Bv8 concentrations in lungs (in premetastatic and metastatic phase) and plasma (in metastatic phase) of mice bearing 4T1 tumors and treated with ISO control or anti–G-CSF antibody (n = 5). In all panels, ND is not determined because of interference of anti-Bv8 antibody with Bv8 ELISA. Asterisk indicates significant difference compared with naïve group, whereas two asterisks indicate significant difference compared with ISO group.

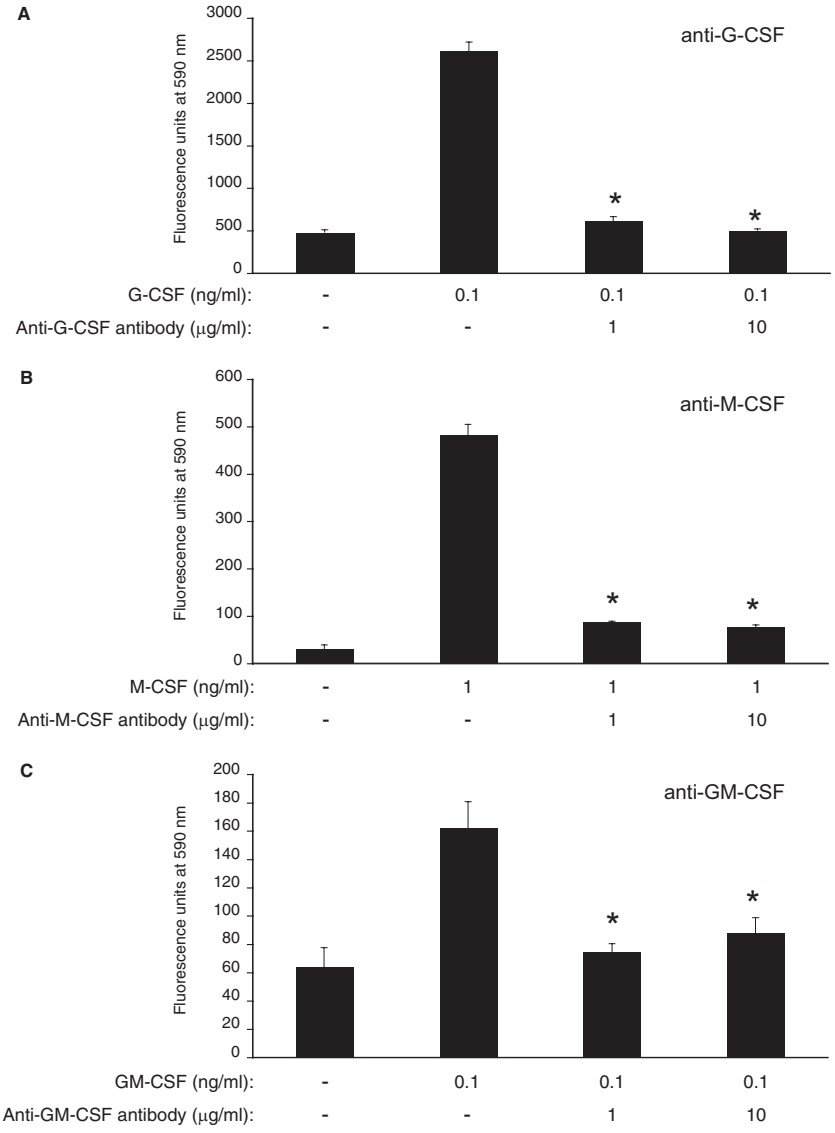


Fig. S7. Anti–G-CSF, anti–M-CSF, and anti–GM-CSF antibodies have neutralizing activity. Dose-dependent inhibition of (*A*) G-CSF–induced proliferation of NFS-60 cells by anti–G-CSF antibody, (*B*) M-CSF–induced proliferation of M-NFS-60 cells by anti–M-CSF antibody, and (*C*) GM-CSF–induced proliferation of FDC-P1 cells by anti–GM-CSF antibody. Cells were pretreated with the indicated antibody before addition of G-CSF (*A*), M-CSF (*B*), or GM-CSF (*C*). In all panels, asterisk indicates significant difference compared with cells treated with growth factor in the absence of neutralizing antibody. Data shown are means ± SEM.

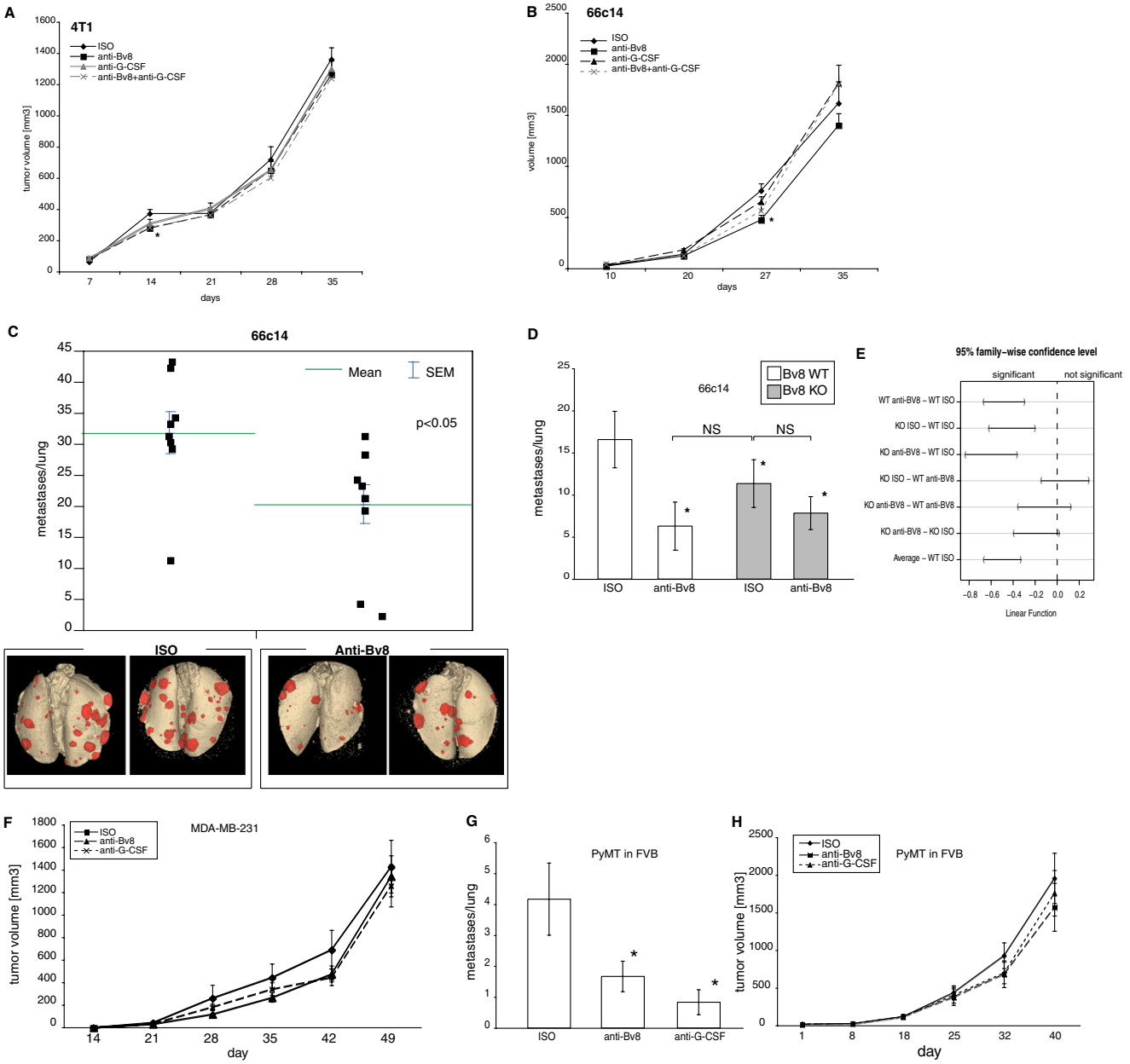
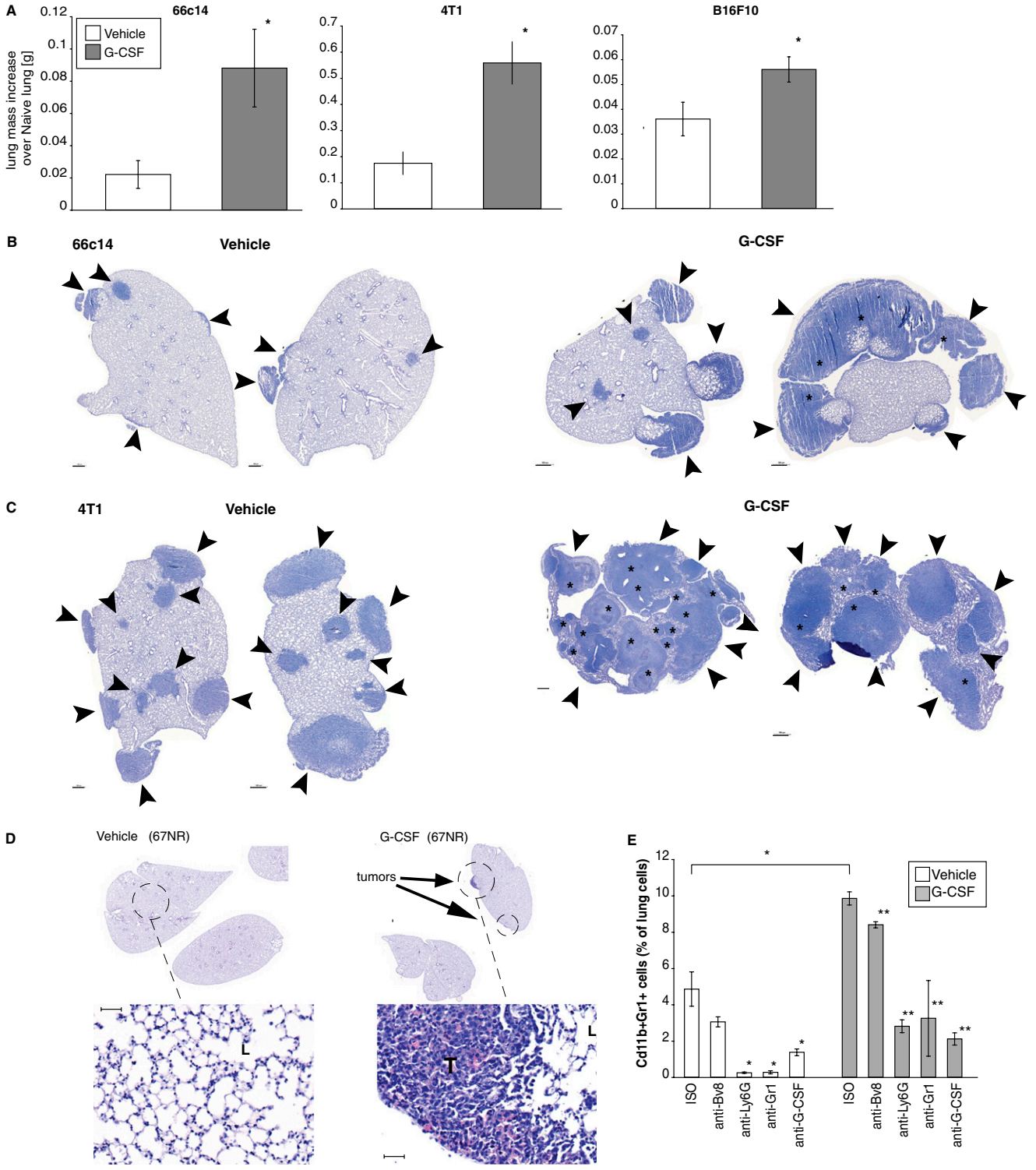


Fig. S8. Analysis of lung tumor burden and primary tumor growth in mice bearing orthotopically inoculated tumors. (*A*) 4T1 primary tumor growth in mice treated with control (ISO), anti-Bv8 (2B, 3F1), anti–G-CSF, or both antibodies (n = 10 per group). 4T1 cells were orthotopically inoculated in female CB6F1 mice. Asterisk next to the anti-Bv8 treatment group indicates significant difference compared with ISO group. (*B*) 66c14 primary tumor growth in mice treated with control (ISO), anti-Bv8 (2B9+3F1), anti–G-CSF, and both antibodies (n = 10 per group). 66c14 cells were orthotopically inoculated in female BALB/c mice. Asterisk next to the anti-Bv8 treatment group indicates significant difference compared with ISO group. (*C*) Numbers of lung tumors analyzed by micro-CT in Balb-c mice bearing 66c14 tumors and treated with control (ISO) or anti-Bv8 (2B9 plus 3F1) antibody (n = 8 for ISO group and n = 10 for anti-Bv8 group). Renderings (two per group) of representative micro-CT scanned lungs showing metastatic nodules (red) in ISO and anti-Bv8 groups are shown below the graph. (*D*) Number of lung metastases in mice with *Bv8* WT or null BMCs obtained by fetal liver transplantation as described in *Materials and Methods*. Mice were inoculated with 66c14 tumors (orthotopic model) and treated with isotype cotrol IgG (ISO) or anti-Bv8 (2B9+3F1) antibody. Asterisk indicates significant difference compared with WT-ISO group. (*E*) Summary of statistical analysis of Bv8 WT and KO experiments from *D* and Fig. 3*E*. Note that comparison is significant only if linear function of Poisson distribution is less than 0. Statistical significance was achieved only for the following comparisons: WT anti-Bv8 vs. WT ISO, KO ISO vs. WT ISO, KO anti-Bv8 vs. WT ISO, and ISO vs. all other groups. (*F*) MDA-MB-231-X1.1 primary tumor growth in SCID/bg mice treated with control (ISO), anti-Bv8 (2D3), or combination of anti–mG-CSF and anti–hG-CSF antibody (n = 10 per group). (*G*) Number of metastases per lung in FVB mice bearing MMTV-PyMT tumors 7 wk after tumor inoculation. Tumors were implanted and treatment performed as described in *Materials and Methods* (n = 6 per group). Asterisk indicates significant difference compared with ISO group. (*H*) MMTV-PyMT primary tumor growth in FVB mice treated with control (ISO), anti-Bv8 (2D3), or anti–G-CSF (n = 10 per group). Values shown are means ± SEM.



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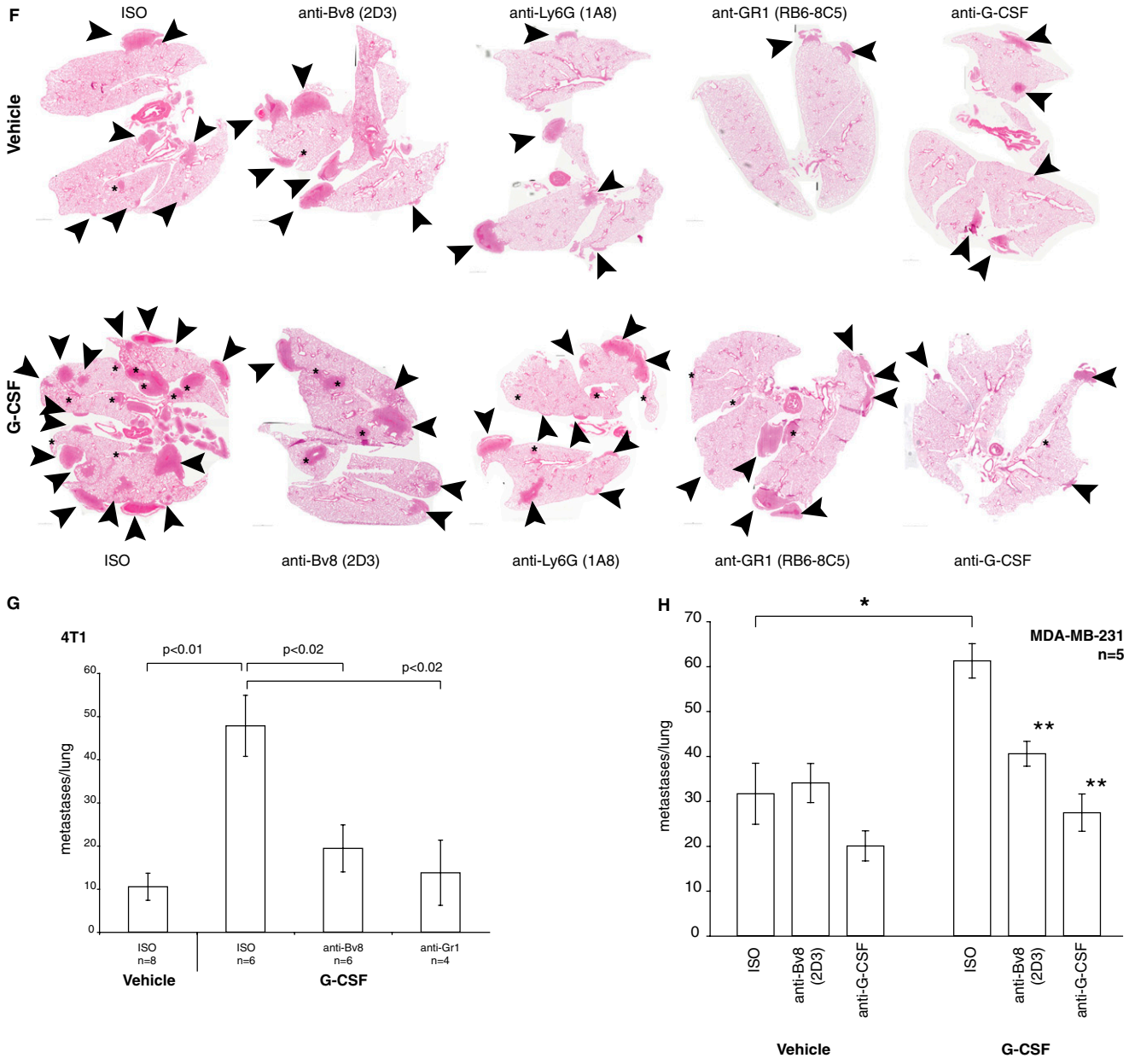

Fig. S9. Analysis of G-CSF–induced metastasis in mice i.v. (tail vein) injected with tumor cells. (*A*) Increases in lung mass (compared with naïve mice) after inoculation of tumor cells and treatment with human recombinant (r)G-CSF or Vehicle. Data correspond to the groups in Fig. 4*D*. Asterisk indicates significant difference (n = 5 per group). (*B*) Representative H&E-stained sections of lungs from mice injected with 66c14 cells and pretreated with either vehicle or rG-CSF (corresponding to Fig. 4*D*). (Scale bar: 500 μm.) (*C*) Representative H&E staining of lungs from mice injected with 4T1 cells and pretreated with either vehicle or rG-CSF as in Fig. 4*D*. In *B* and *C*, note significantly increased number of metastases (black arrowheads and asterisks) in mice pretreated with G-CSF. (Scale bar: 500 μm.) (*D*) H&E staining of lungs from mice injected with 67NR cells and treated with Vehicle or G-CSF. Note presence of tumors in the G-CSF–treated group. (Scale bar in magnified images: 50 μm.) T, tumor area; L, normal lung area. (*E*) FACS analysis of Cd11b+Gr1+ cells in lungs of mice treated daily (for 5 d) with mouse rG-CSF (2 μg/mouse) or vehicle and treated with control antibody (ISO), anti-Bv8 (2D3), anti-Gr1, anti-Ly6G, or anti–G-CSF antibody as described in *Materials and Methods* (n = 5 per group). Asterisk indicates significant difference relative to Vehicle-ISO group, whereas two asterisks indicate significant difference relative to G-CSF–ISO group. Values shown are means ± SEM (*F*) Representative H&E-stained sections of lungs from mice pretreated with vehicle or G-CSF. Mice were i.v. injected with 66c14 cells and treated with indicated antibody. Sections correspond to Fig. 4*F*. Arrowheads and asterisks indicate presence of metastases in the tissue. (*G*) Number of lung metastases in mice 3 wk after inoculation of 4T1 cells. Mice were pretreated (daily) with vehicle or human rG-CSF and treated with control (ISO), anti-Bv8 (2D3), or anti-Gr1 antibody. (*H*) Tumor numbers in lungs of mice injected i.v. with MDA-MB-231-L1.1 cells and treated daily with vehicle or rG-CSF and indicated antibody. Asterisk indicates significant difference relative to Vehicle-ISO group, whereas two asterisks indicate significant difference relative to G-CSF–ISO group. Values shown are means ± SEM.

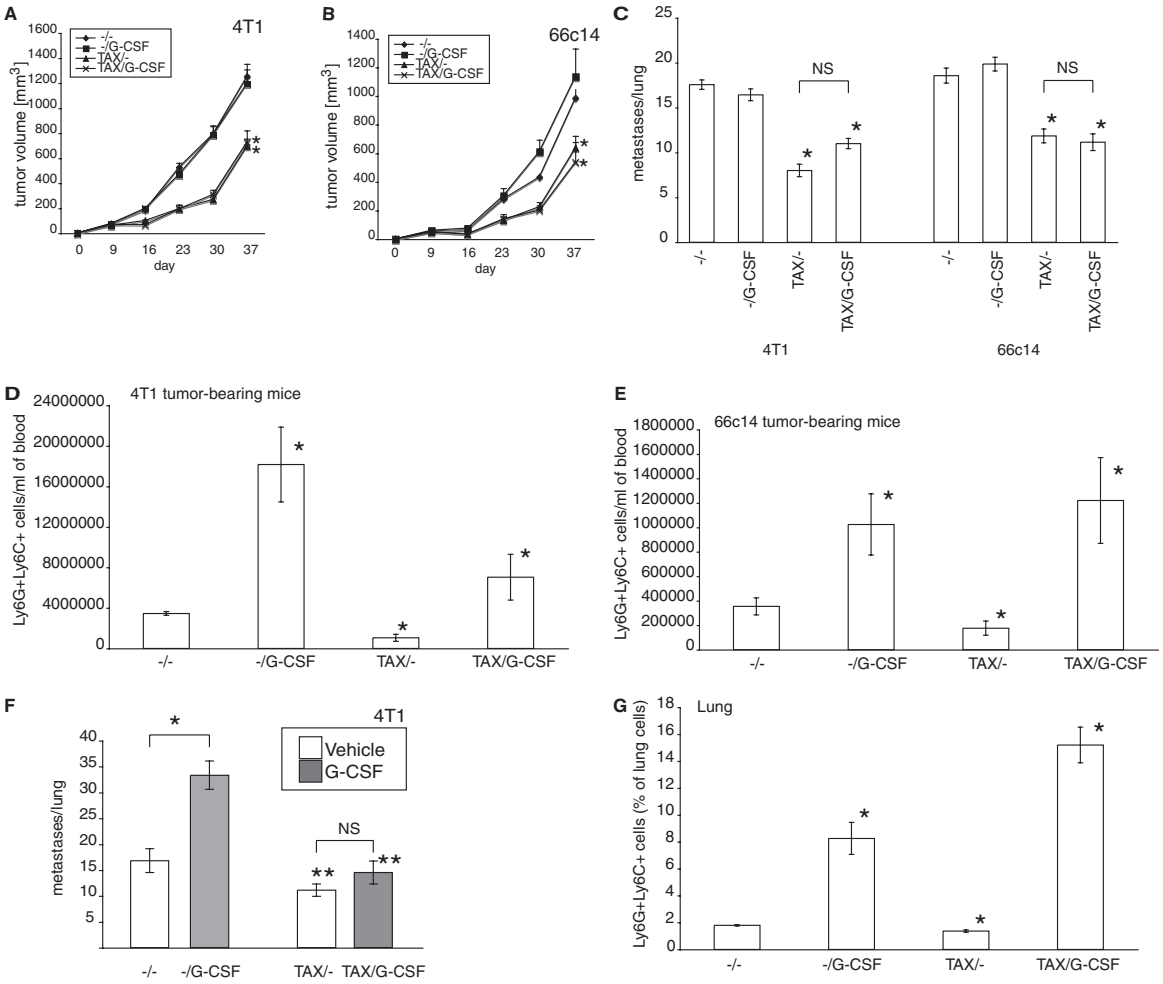

Fig. S10. Taxotere antagonizes the prometastatic effects of G-CSF. (*A*) 4T1 and (*B*) 66c14 primary tumor growth in BALB/c mice treated with vehicle ($^{-/-}$), G-CSF alone (-/G-CSF), taxotere (25 mg/kg) alone (TAX/-), or taxotere (25 mg/kg) and G-CSF (TAX/G-CSF) as described in *Materials and Methods* (n = 10 per group). (*C*) Number of tumors per lung in mice bearing 4T1 or 66c14 tumors treated with G-CSF and taxotere (25 mg/kg) as in *A* and *B* (n = 7 per group). (*D* and *E*) Number of Ly6G+Ly6C+ neutrophils per milliliter of blood in mice bearing 4T1 (*D*) or 66c14 (*E*) tumors and treated as in *A*. Note that G-CSF reduces taxotere-induced neutropenia. (*F*) Number of lung metastases in BALB/c mice administered with vehicle ($^{-/-}$), G-CSF alone (-/G-CSF), taxotere (25 mg/kg) alone (TAX/-), or taxotere (25 mg/kg) and G-CSF (TAX/G-CSF) and injected through the tail vein with 4T1 cells as described in *Materials and Methods*. Asterisk indicates significant difference compared with vehicle ($^{-/-}$) group, whereas two asterisks indicate significant difference compared with (-/G-CSF) group (n = 7 per group). (*G*) Frequency of Ly6G+Ly6C+ cells in lungs of mice pretreated with vehicle or G-CSF in the absence or presence of taxotere as described in *Materials and Methods*. In all panels, asterisk indicates significant difference compared with Vehicle ($^{-/-}$) group. Values shown are means ± SEM.

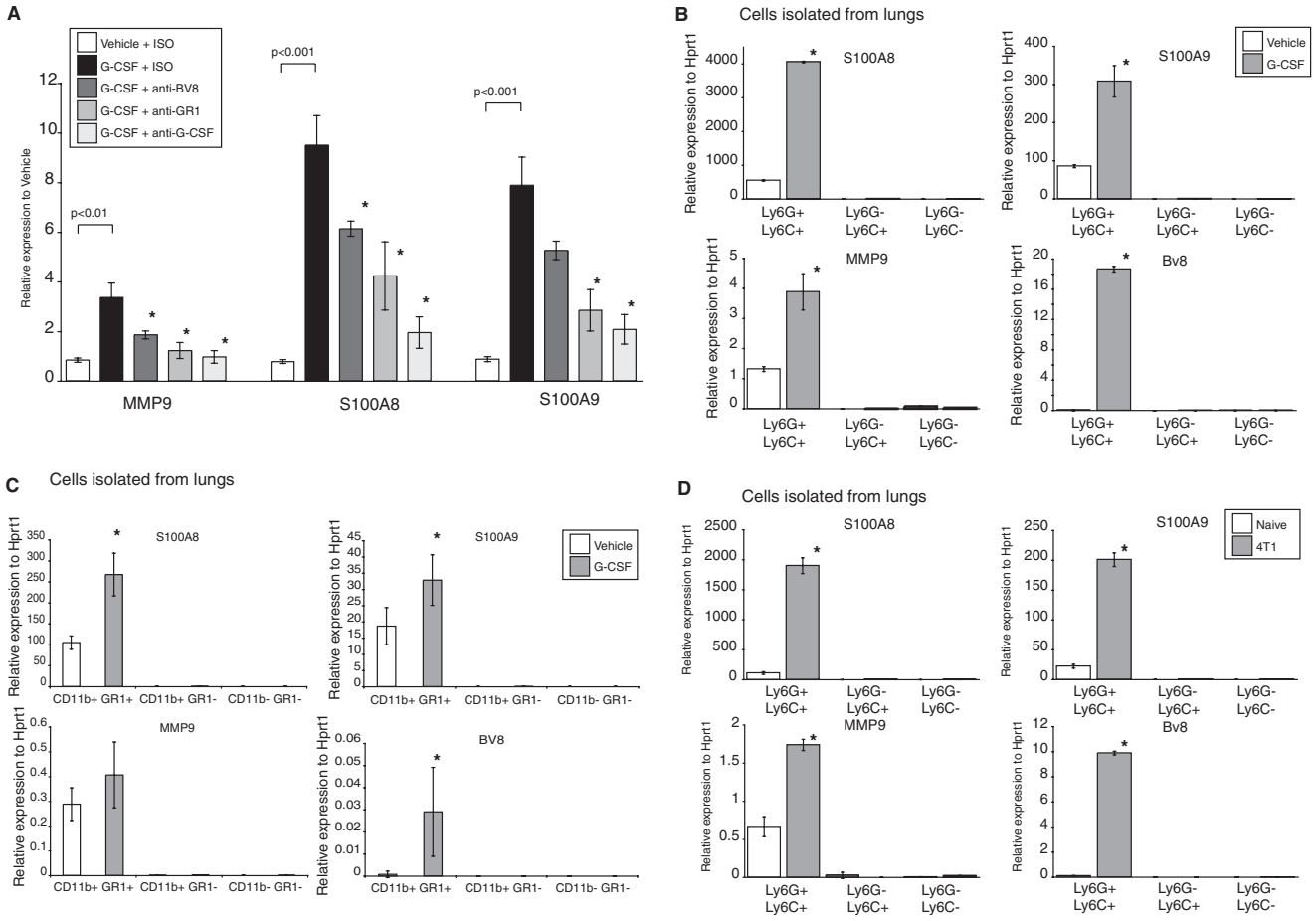

Fig. S11. Mechanisms underlying the G-CSF–initiated premetastatic microenvironment. (*A*) qRT-PCR gene expression analysis of *MMP-9*, *S100A8*, and *S100A9* in total lung tissue from mice pretreated with vehicle or mouse rG-CSF (1 μg/mouse daily for 3 d) and then treated with control (ISO), anti-Bv8 (2D3), anti-Gr1, or anti–G-CSF antibodies. Asterisk indicates significant difference compared with G-CSF+ISO group (n = 5 per group). (*B*) *Bv8*, *S100A8*, *S100A9*, and *MMP9* gene expression measured by qRT-PCR in Ly6G+Ly6C+, Ly6G-Ly6C+, or Ly6G-Ly6C- cells sorted from lungs of mice pretreated with vehicle or rG-CSF. Single cell suspensions were generated from the tissues, cells were stained with specific anti-Cd11b, anti-Ly6G, and anti-Ly6C antibodies, and indicated cell subpopulations were separated by FACS. Asterisk indicates significant difference compared with Vehicle group. (*C*) *Bv8*, *S100A8*, *S100A9*, and *MMP9* gene expression measured by qRT-PCR in Cd11b+Gr1+ (or Gr1-) cells sorted from lungs from mice pretreated with vehicle or rG-CSF. Single cell suspensions were generated from the tissues, cells were stained with specific anti-Cd11b and anti-Gr1 antibodies, and double-positive cell (Cd11b+Gr1+), Cd11b+Gr1-, and double-negative cell (Cd11b-Gr1-) populations were separated by FACS. Total RNA was isolated from the cells, and gene expression was measured by qRT-PCR. Data are presented as relative expression to *Hprt1* (n = 5 per group). Asterisk indicates significant difference compared with Vehicle group. (*D*) *Bv8*, *S100A8*, *S100A9*, and *MMP9* gene expression measured by qRT-PCR in Ly6G+Ly6C+, Ly6G-Ly6C+, or Ly6G-Ly6C- cells sorted from lungs of naïve mice or mice with 4T1 tumors as in *B*. Asterisk indicates significant difference compared with Vehicle group.

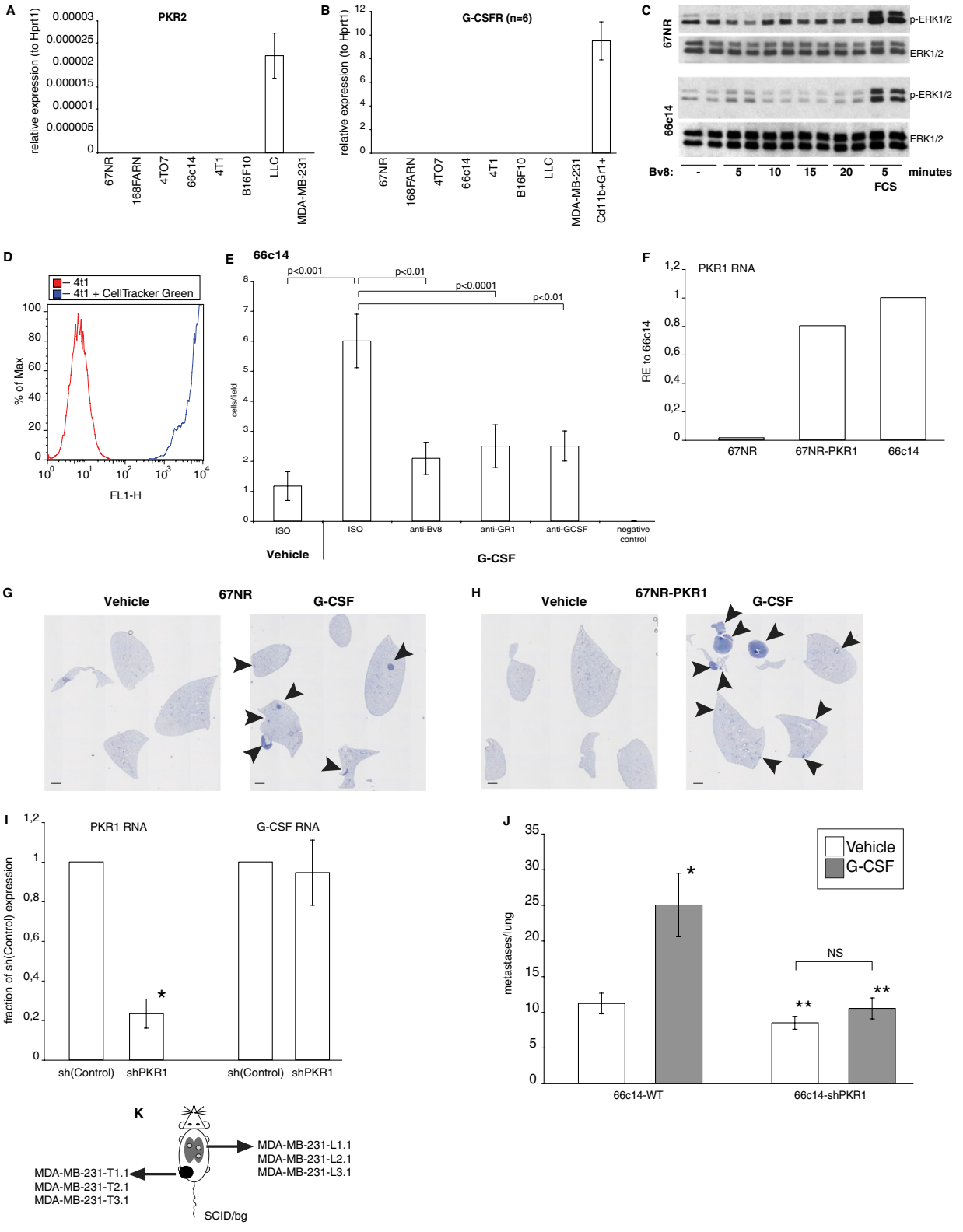

Fig. S12. Additional analysis of the direct effect of Bv8 and PKR-1 on tumor metastasis. (*A*) qRT-PCR analysis of *PKR2* expression by cancer cells in vitro. Note that only LLC cells express detectable levels of *PKR2*. (*B*) qRT-PCR analysis of *G-CSFR* expression by cancer cells in vitro. Cd11b+Gr1+ cells served as a positive control for *G-CSFR* expression. (*C*) Immunoblot analysis showing phosphorylation of ERK1/2 (p-ERK1/2) on stimulation with Bv8 in 67NR and 66c14 cells. Cells were stimulated with Bv8 (5 ng/mL) or 1% FBS for the indicated times. Duplicate samples for each time point are shown. (*D*) FACS analysis of 4T1 cells labeled with CellTracker Green 48 h after labeling. Note that all cells remain labeled at this time point. (*E*) Quantification of in vivo extravasation of 66c14 cells assessed 36 h after i.v. inoculation (tail vein) of cells labeled with CellTracker Green. Mice were pretreated with mouse rG-CSF (2.5 μg/mouse daily) for 5 consecutive d and then treated with control (ISO), anti-Bv8 (2B9+3F1), anti-Gr1, or anti–G-CSF antibodies. (*F*) qRT-PCR analysis of *PKR1* expression in 67NR cells over-expressing PKR1. Note that the expression level is comparable with that in 66c14 cells. (*G* and *H*) Representative sections of lungs from mice pretreated with

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vehicle or G-CSF and i.v. injected with 67NR (*G*) or 67NR-PKR1 (*H*) cells. Sections correspond to Fig. 5*E*. Note the presence of metastases in lungs of mice injected with 67NR-PKR1 and pretreated with G-CSF. (*I*) qRT-PCR analysis of *PKR1* and *G-CSF* expression in 66c14 cells expressing shRNA targeting *PKR1* (shPKR1). Note that *G-CSF* is not affected by shPKR1. sh(Control) is a scrambled shRNA used as control. Asterisk indicates significant difference compared with sh(Control) group. (*J*) Number of metastases in lungs of mice that were pretreated with vehicle or G-CSF and injected with 66c14 or 66c14-shPKR1 cells. Analysis was performed 3 wk after cell inoculation. Asterisk indicates significant difference compared with 66c14-WT ISO group, whereas two asterisks indicate significant difference compared with 66c14-WT G-CSF group (n = 10 per group). (*K*) Schematic presentation of MDA-MB-231 clones used for gene expression analysis in Fig. 5*F*. MDA-MB-231-D3H1 was injected into fourth mammary fat pad or through tail vein of SCID/bg mice. Subsequently, cancer cells from established tumors (either in breast or lung) were isolated and expanded in vitro as described in *Materials and Methods*. From each tissue, three independent cell lines (L1.1, L2.1, and L3.1 from three separate lungs and T1.1, T2.1, and T3.1 from three independent primary tumor) were established. Values shown are means ± SEM.