Supporting Information

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SI Methods

Generation of Granulocyte Colony Stimulating Factor (G-CSF)-Expressing B16F1 Cells. A PCR fragment containing the consensus Kozak sequence and the coding region of murine (m) G-CSF with a 6× polyHis tag at the C terminus was amplified from IMAGE mouse cDNA clone no. 40129682 (Open Biosystems) and inserted into the ClaI and EcoRI sites of a pRK vector. To generate B16F1 cells stably expressing G-CSF, B16F1 cells were cotransfected with pRK-mG-CSF vector (or empty pRK5 vector) and a vector providing Zeocin resistance using FuGENE transfection reagent, according to the manufacturer's instructions (Roche). Zeocin-resistant cells were selected by culturing transfected cells in the presence of 100 μg/mL of Zeocin for 10 days. To identify G-CSF expressing clones, cells were plated in 96-well plates at density of 1 cell per well, and allowed to proliferate for about 2 weeks. As soon as cells reached 90% confluence, growth media were replaced with serum-free media, and cells were incubated for 48 h. Supernatants were then collected, and mG-CSF levels were measured by ELISA. Five clones with the highest expression of G-CSF (over 200 pg/mL/10^6 cells) were pooled and used for subsequent in vivo studies. G-CSF was undetectable in media from parental or control vector-transfected B16F1 cells.

Flow Cytometry. Single cells from tumors, peripheral blood (PB) and bone marrow (BM) were stained with fluorochrome conjugated CD11b and Gr1 antibodies as described (1). All of the samples were analyzed in a LSRII machine (Genentech, Flow cytometry Department), and the data were analyzed by using Flowjo software (Flowjo).

Immunohistochemistry (IHC) and Vascular Surface Area Measurements (VSA). Sections of tumors (6 μm) were obtained from OCT (Sakura Finetek) embedded frozen tissues using a cryostat (Leica Microsystem), and were kept at −20 °C. Sections were then stained with anti-CD31-PE (#553373), anti-Gr-1-APC (#553124), and anti-CD11b-FITC (#553310) conjugated antibodies (BD BioSciences) as described (1). Immunofluorescent images were collected on an AxioPhot microscope equipped with a Plan-Apochromat 20× objective and digitally merged. Tumor VSA was quantified from digital images of CD31-stained sections using a 20× objective as described. The pixels corresponding to stained vessels were selected by using ImageJ Software, on adjusting the threshold, and excluding contaminating (non-vessel) stray pixels.

For Bv8 IHC, neutral-buffered fixed, 5-μm-thick, paraffin-embedded sections from tumors or adult BALB/c mouse testes 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 blocked with protein block solution (DAKO) for 30 min. Sections were then incubated with hamster anti-mBv8 Mab 2D3 (Genentech), or with 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 hematoxylin, dehydrated, and coverslipped. Testes from adult BALB/c mice were used as positive control for Bv8 IHC, because spermatocytes are known to be a major site of Bv8 mRNA expression (2, 3).

For mBv8 and myeloperoxidase (MPO) double staining, acetone-fixed 5-μm-thick tumor sections were blocked with protein block solution (DAKO) for 30 min at room temperature. Sections were incubated with Mab 2D3 at 10 μg/mL and rabbit anti-MPO (Thermo Scientific) at 5 μg/mL for 1 h at room temperature, followed by Cy3-conjugated goat anti-hamster (Jackson Immuno Research) at 2 μg/mL and Alexa Fluor 488 conjugated donkey anti-rabbit (Invitrogen) at 2.5 μg/mL for 2 h at room temperature. Sections were coverslipped with flurecent mounting medium with DAPI (DAKO).

ELISA. Levels of Bv8 protein in plasma, BM mononuclear cells (MNCs), and tumors were measured by ELISA essentially as described (4). However, we recalibrated the standards against a new preparation of mouse Bv8, and corrected the concentrations of the standards from 0.0195–2.50 ng/mL to 1.77–227 pg/mL. Briefly, mouse Bv8 standards and samples (minimum 1:10 dilution) were serially diluted, and were added to coated-MaxiSorp 96-well microwell plates (Nalge Nunc), followed by RT incubation for 2 h. After a complete washing step, the secondary antibody, a biotinylated hamster anti-mouse Bv8 antibody clone 4E10 (Genentech) was added to each well followed by adding streptavidin-HRP (GE Healthcare) and substrate (3,3′,5,5′-tetramethyl benzidine, Kirkegaard & Perry Laboratories). The colorimetric reaction was stopped by adding 1 M phosphoric acid, and the absorbances were read at 450 nm on a microplate reader (multiskan ascent; Thermo Scientific). Levels of other cytokines, G-CSF, GM-CSF, stromal derived factor (SDF)1a, and placenta growth factor (PlGF) were measured by using species–specific ELISA kits (R&D system), according to the protocols provided by the manufacturer.

Tumor Cell Proliferation Assay. LLC, TiB6, EL4, or B16F1 cells were plated at the density of 1.5×10^4 cells per well in 24-multiwell plates in high-glucose DMEM supplemented with 2% FBS; 100 ng/mL human G-CSF (Amgen), or 10 μg/mL anti-G-CSF Mab and the matching isotype IgG control (R & D Systems), or 100 ng/mL human Bv8 (Peprotech), or 10 μg/mL anti-Bv8 Mab (2D3) (Genentech) and anti-GP120 (Genentech) was added after plating cells. After 3 days, cells were trypsinized, and the cell numbers were determined by using a Coulter counter (BD Biosciences).

Statistical Analysis. Student's t test was used for all of the statistical analysis, and $P <= 0.05$ was considered to be significant.

1. Shojaei F, Singh M, Thompson JD, Ferrara N (2008) Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc Natl Acad Sci USA* 105:2640–2645.
2. Wechselberger C, et al. (1999) The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes. *FEBS Lett* 462:177–181.
3. LeCouter J, et al. (2003) The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. *Proc Natl Acad Sci USA* 100:2685–2690.
4. Shojaei F, et al. (2007) Bv8 regulates myeloid cell-dependent tumour angiogenesis. *Nature* 450:825–831.

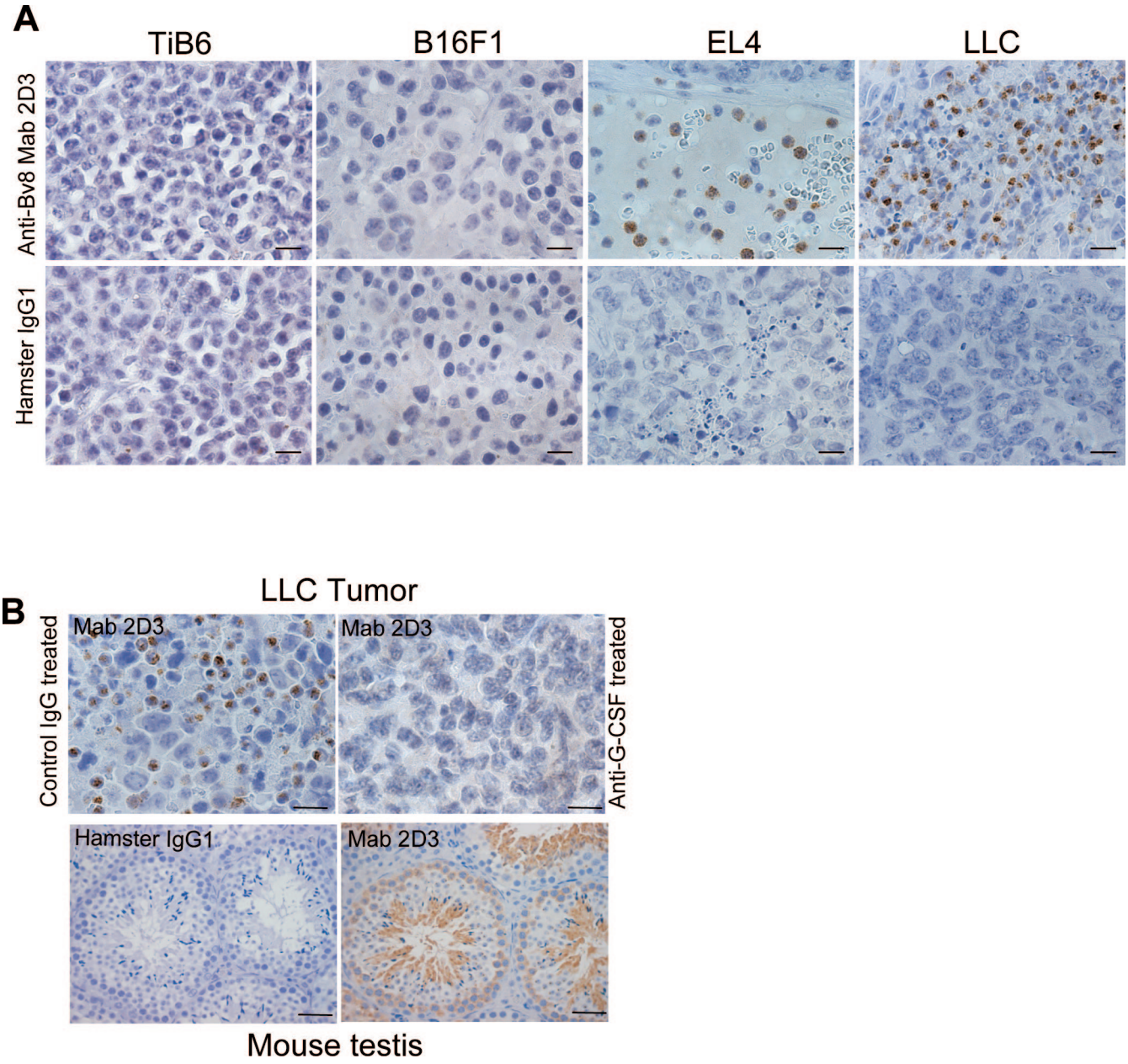

Fig. S1. (*A*) Bv8 is expressed in refractory tumors. BALB/c nude mice were implanted with sensitive (B16F1, Tib6) or refractory (EL4, LLC) tumors, and were then treated with control or anti-G-CSF Mab as described in *Methods*. Mice were euthanized when tumors reached ≈2,000 mm^3. Paraffin-embedded sections of sensitive or refractory tumors were stained with a hamster anti-Bv8 Mab (2D3) or isotype control as described in *Methods*. Bv8 is detected in neutrophils in refractory tumors. (*B*) Paraffin-embedded sections from LLC tumor-bearing mice treated with anti-G-CSF or control Mab were stained with anti-Bv8 Mab 2D3. Bv8 was strongly expressed in sections from control, but not anti-G-CSF treated mice, further confirming the critical role of G-CSF in regulating Bv8 expression. Paraffin-embedded sections from adult BALB/c mouse testis were used as positive control for Bv8 IHC (*Methods*). (Scale bar, 20 μm.)

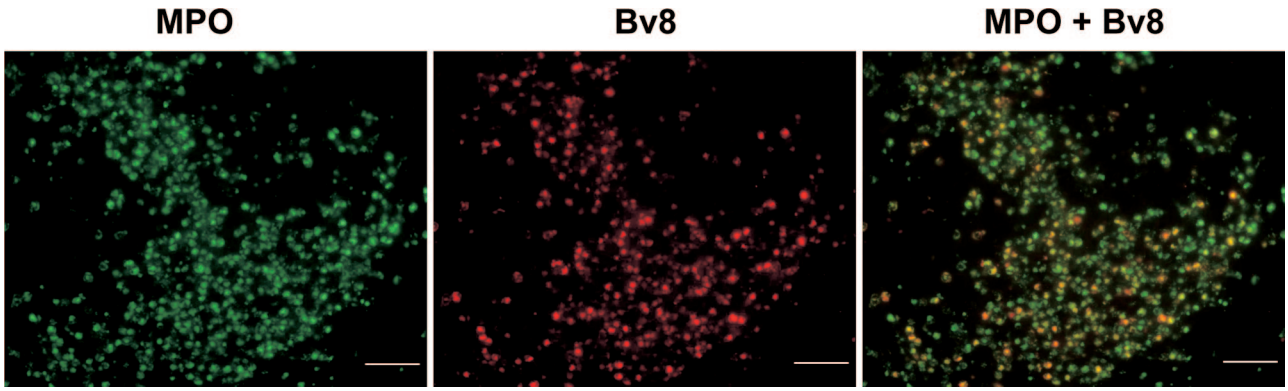

Fig. S2. Bv8 is predominantly expressed in neutrophils. Frozen LLC tumor sections from mice treated with control antibody for 21 days were stained with anti-mBv8 Mab 2D3 (shown in red) and an anti-MPO antibody (shown in green) as described in *Methods*. (Scale bar, 50 μm.) Bv8 is predominantly expressed in neutrophils.

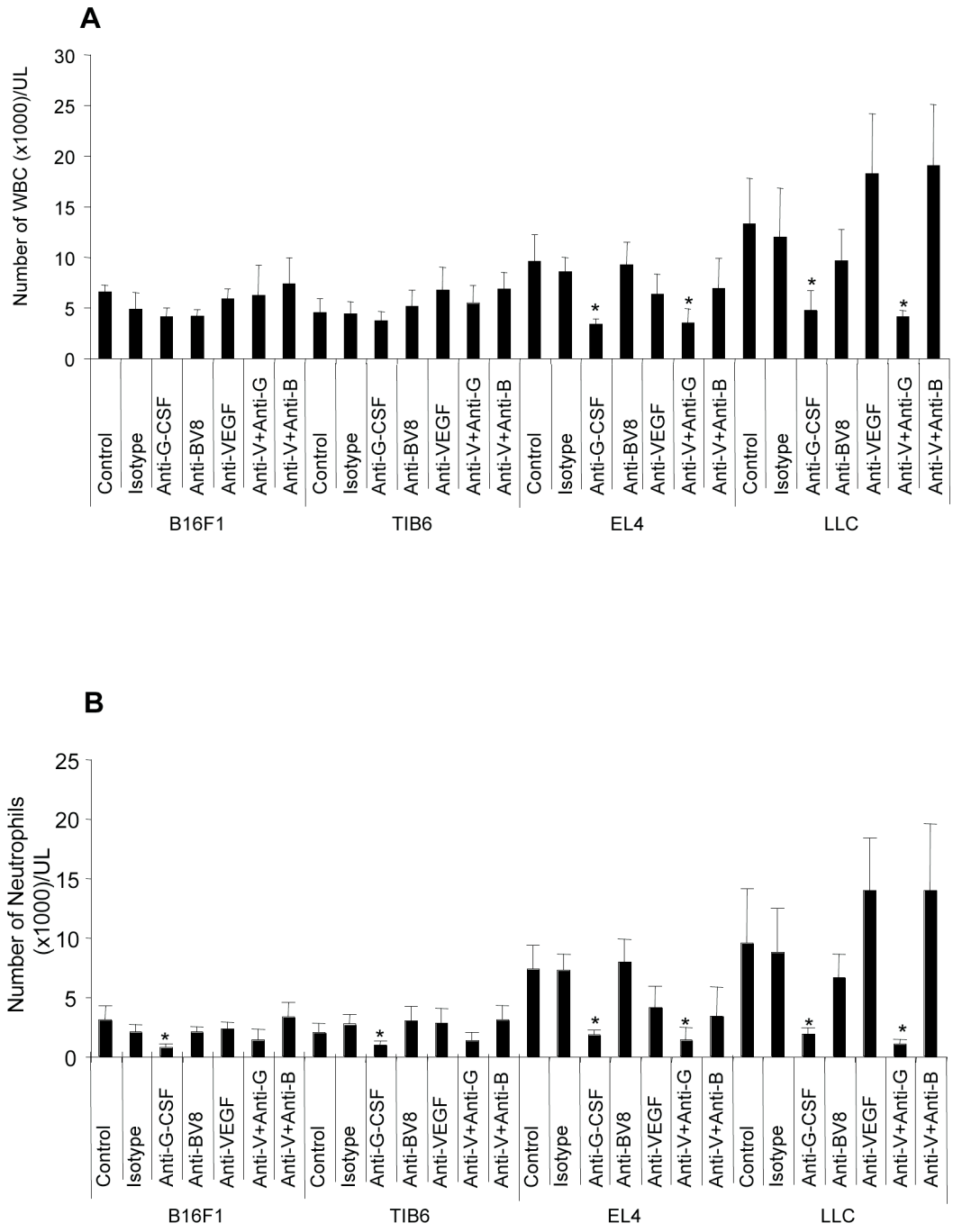

Fig. S3. Anti-G-CSF reduces the number of circulating leukocytes and neutrophils in mice bearing refractory tumors. Balb-c nude mice were implanted with sensitive or refractory tumors ($n = 5$), and were treated with Mabs as described. At terminal analysis, when tumors reached ≈2,000 mm^3, the total number of blood leukocytes (*A*) and neutrophils (*B*) were measured as described in *SI Methods*. The number of circulating leukocytes and neutrophils was significantly greater in animals bearing refractory tumors compared with sensitive ones. Anti-G-CSF monotherapy or combination treatment resulted in a significant reduction ($P < 0.05$) in the number of circulating leukocytes and neutrophils. *, significant difference in the number of cells in each treatment in refractory tumors vs. the corresponding treatment in sensitive tumors.

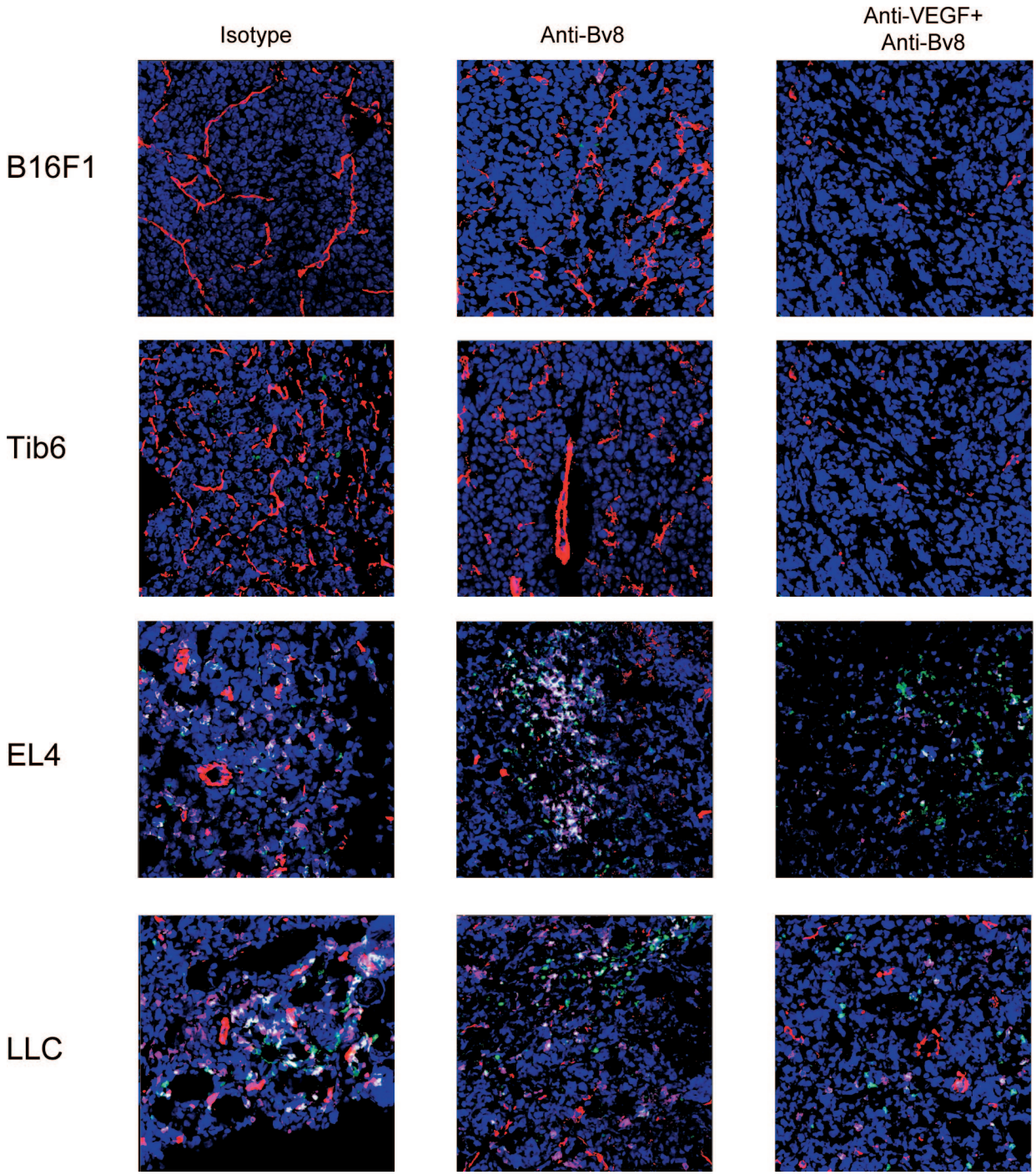

Fig. S4. Anti-Bv8 treatment reduces angiogenesis in refractory tumors. Section of tumors (Tib6, B16F1, EL4, and LLC) from Isotype control, anti-Bv8, and combination (Anti-Bv8 plus Anti-VEGF) treatments were stained with anti-CD31 (red), anti-CD11b (green), and anti-Gr1 (pink) antibodies as described, and images (20×) were captured in a confocal microscope (Zeiss). Image analyses indicated that anti-Bv8 treatment may not inhibit infiltration of myeloid cells in refractory tumors, but rather suppresses tumor growth through inhibition of tumor angiogenesis in both monotherapy and combination treatment with anti-VEGF Mab. Data shown are part of the study illustrated in Fig. 3*A*.

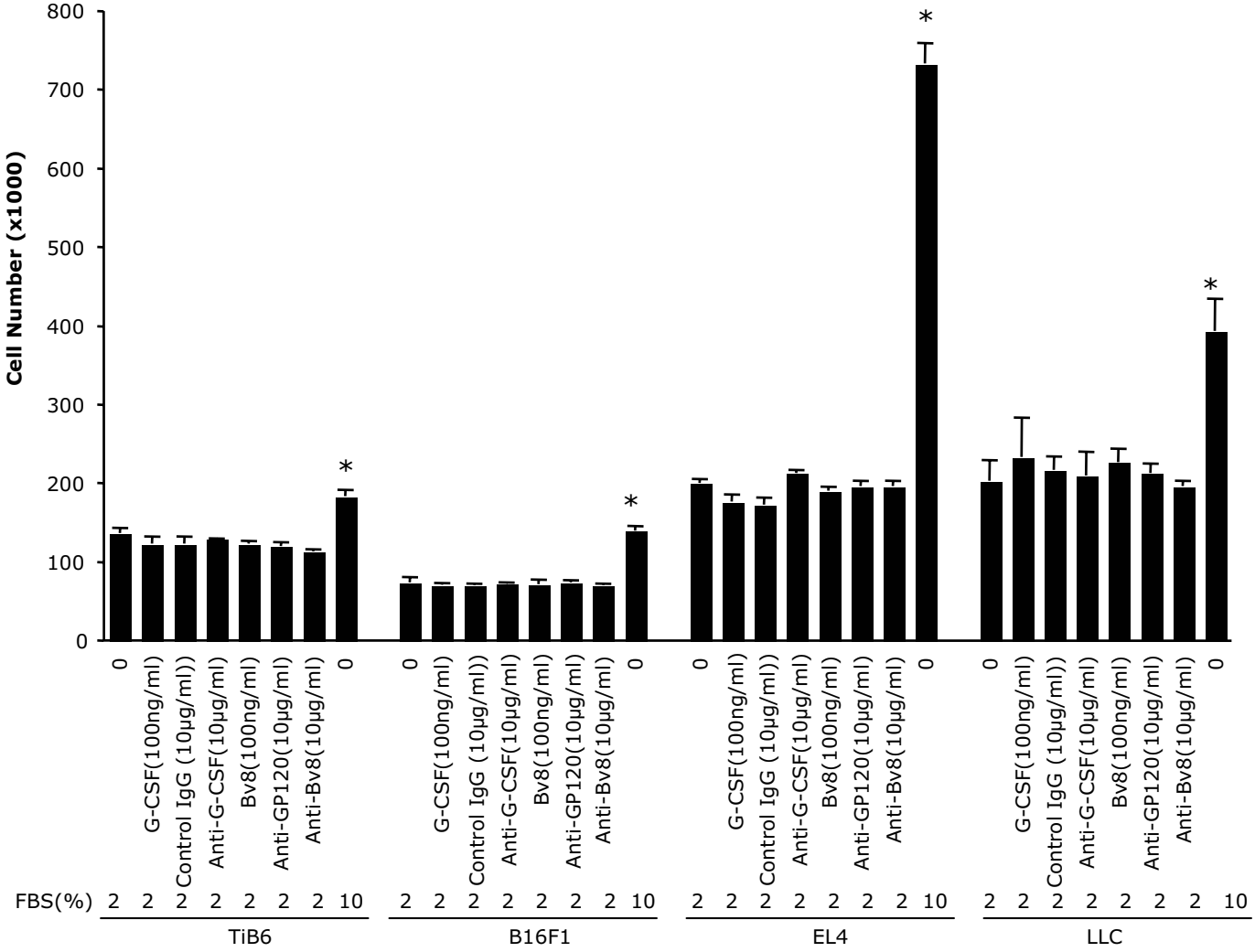

Fig. S5. G-CSF, anti-G-CSF, Bv8, or anti-Bv8 has no effect on tumor cell proliferation in vitro. LLC, TiB6, EL4, and B16F1 cells ($n = 3$) were treated with human G-CSF, or anti-G-CSF antibody or the matching isotype IgG control, or recombinant human Bv8, or anti-Bv8 antibody or the matching control antibody in media containing 2% FBS for 3 days, and cell numbers were determined by a Coulter Counter. Error Bars represent SEM. * indicates significant difference ($P < 0.5$) compared with control groups (2% FBS).

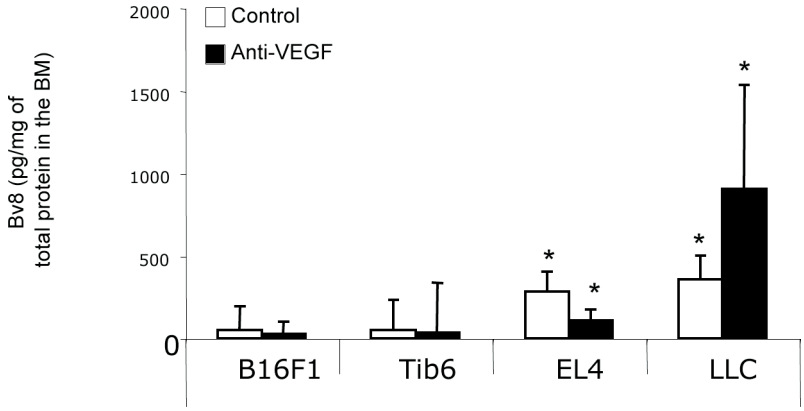

Fig. S6. Increased Bv8 levels in the BM of mice harboring refractory tumors. Bv8 concentrations were measured by ELISA in BMMNCs isolated from mice bearing sensitive or refractory tumors. Similar to tumors and plasma, refractory tumors are associated with increased levels of Bv8 in the BM relative to the sensitive tumors. Asterisks indicate significant difference when comparing levels of Bv8 in refractory tumors vs. sensitive ones ($P < 0.05$).