SUPPLEMENTARY METHODS

Generation, transduction, and utilization of pUltra-Chili-Luc lentiviral vector for tracking metastasis progression in mice xenograft models

We infected LM8 cells with a lentiviral expression vector, pUltra-Chili-Luc. This simultaneously expresses a fluorescent protein, Chili (dTomato-absorption max and emission max at 554 nm and 581 nm, respectively) and a bioluminescence generating protein, firefly luciferase (Luc) that catalyzes the oxidation of luciferin substrate into an end product with emission max–550–570 nm).

We designed the plasmid pUltra-Chili-Luc in the backbone of a third generation lentiviral plasmid (1). First, we engineered the pUltra-Chili (dimer [d]Tomato, aka dTomato) plasmid (deposited as Addgene plasmid #48687) by excising the eGFP sequence in pUltra plasmid (Addgene plasmid #24129) with *AgeI* and *BsRGI* and replacing it with the dTomato cDNA (Takara Bio, USA) without a stop codon from pRSET-B-dTomato (Roger Y. Tsien Lab) 5' in-frame with the P2A sequence (2). Next, the firefly luciferase cDNA from an unpublished plasmid (Malcolm Moore laboratory) with its stop codon was inserted into the *XbaI* and *BamHI* site 3' in-frame with the P2A sequence in the above generated pUltra-Chili plasmid; this was named as pUltra-Chili-Luc (now we deposited as Addgene plasmid #48688; **Supplementary Fig. S1A**). Sanger sequencing was used to verify each step of the PCR-mediated cloning by standard procedures. Complete DNA sequence information of the above plasmid #48688, including restriction sites, can be found at www.addgene.org.

Upon transfection of HEK 293T cells with the pUltra-Chili-Luc in a split packaging-system, viral particles pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV.G) were generated and released into the media; these were subsequently harvested, and viral particles were concentrated by ultracentrifugation according to Dull et al. (1). Transduction of the above concentrated pUltra-Chili-Luc lentiviral particles resulted in the synthesis of the luciferase and the fluorescent proteins in LM8 cells. Here, independent syntheses of the dTomato and luciferase are facilitated through ribosome skipping during the peptide bond formation in between the Glycine and Proline residues of the P2A sequence. Transduced LM8 cells were purified from the untransduced cells in the bulk population by the fluorescence activated cell sorting (FACS), named *LM8-Luc*. The course of metastasis development *in vivo* is assayed by the substrate luciferin injection and the resulting bioluminescence measurement with the IVIS imager.

Protein extraction and Western Blotting

To isolate protein from bone marrow-derived macrophage (BMDM), lysis was performed in NP-40 buffer containing protease and phosphatase inhibitors (Thermo Scientific, Waltham, MA). Before cell lysis, cells were stimulated with macrophage colony-stimulating factor (M-CSF) (PeproTech, Inc, USA) at 50 ng/ml or 50% tumor-conditioned media (TCM) for one, five, 10, and 15 minutes, respectively, in order to measure the stimulatory effects of M-CSF and TCM on BMDMs. The inhibitory effects of PLX3397 on the CSF-1 signaling cascade was measured by starving BMDMs of M-CSF or TCM for four hours. PLX3397 (dose range, 50 to 250 nM), pazopanib (dose range, 0.1 to 10 μM), neutralizing monoclonal antibody for CSF-1 (dose range, 0.1 to 10 μg/ml) and CSF-1R (dose range, 0.1 to 10 μg/ml), rat IgG1 isotype control (10 μg/ml), and rat IgG2a isotype control (10 μg/ml) were added to each sample for one hour. Then, cells were stimulated with CSF-1 at 50 ng/ml or 50% TCM for 10 minutes before cell lysis. Extracted lysates were run on gel electrophoresis (Criterion™ TGX™ Precast Gel; Bio-Rad Laboratories, USA), transferred to PVDF membrane (Millipore, Billerica, MA, USA), and blocked with 5% non-fat milk. Membranes were incubated overnight at 4° C with primary antibodies against pERK1/2 (Cell Signaling Technology, Danvers, MA, USA; cat#4370s) and GAPDH (Cell Signaling Technology; cat#2118s) at a dilution of 1:2000. Following incubation with anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technologies; cat #70745) at a dilution of 1:10,000 for one hour at room temperature, target protein was detected using SuperSignalWest Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL.)

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted after 48 hours of treatment with PLX3397 using the RNeasy Mini Kit (Qiagen, Austin, TX) according to manufacturer's instructions. cDNA was synthesized using a cDNA Synthesis Kit (Thermo Scientific, Rockford, IL). For each qPCR, equal amounts of cDNA were mixed with PerdeCTa SYBR Green qPCR FastMix (Quanta Biosciences, USA) and the following, specific primers: IL-1β, iNOS, CD80, CD206, and CCL2. Relative gene expression was calculated to the established comparative threshold cycle delta Ct equation and normalized to the housekeeping gene HPRT.

Primers:

IL-1β:	Forward (5'-Sequence-3'):	FAGTTGACGGACCCCAAAAG
	Reverse (5'-Sequence-3'):	TACTGCCTGCCTGAAGCTCT
iNOS:	Forward (5'-Sequence-3'):	CAGCTGGGCTGTACAAACCTT

	Reverse (5'-Sequence-3'):	CATTGGAAGTGAAGCGTTTCG
CD80:	Forward (5'-Sequence-3'):	ACCCCCAACATAACTGAGTCT
	Reverse (5'-Sequence-3'):	TTCCAACCAAGAGAAGCGAGG
CD206:	Forward (5'-Sequence-3'):	CTCTGTTCAGCTATTGGACGC
	Reverse (5'-Sequence-3'):	TGGCACTCCCAAACATAATTTGA
CCL2:	Forward (5'-Sequence-3'):	AGGTCCCTGTCATGCTTCTG
	Reverse (5'-Sequence-3'):	TCATTGGGATCATCTTGCTG
HPRT:	Forward (5'-Sequence-3'):	AGCTACTGTAATGATCAGTCAACG
	Reverse (5'-Sequence-3'):	AGAGGTCCTTTTCACCAGCA

Cell viability assay

Survival of BMDMs in response to PLX3397 was measured by MTT assay. BMDMs were seeded at 2×10^4 cells/well on a 96-well plate and incubated α-MEM with either M-CSF (50ng/ml) or 50% TCM. After 24 hours, PLX3397 (dose range, 50 to 500 nM), pazopanib (dose range, 0.1 to 10 μM), and neutralizing monoclonal antibody for CSF-1 (dose range, 0.1 to 10 μg/ml) and CSF-1R (dose range, 0.1 to 10 μg/ml), were added at the indicated doses. After 24 hours, 20μl of MTT aqueous solution® (Promega Madison, WI) was added to each well, and cells were incubated for four hours. Absorbance was measured at a wavelength of 565 nm using spectrophotometer (Infinite M22 PRO, Tecan).

Transwell chemotaxis assay

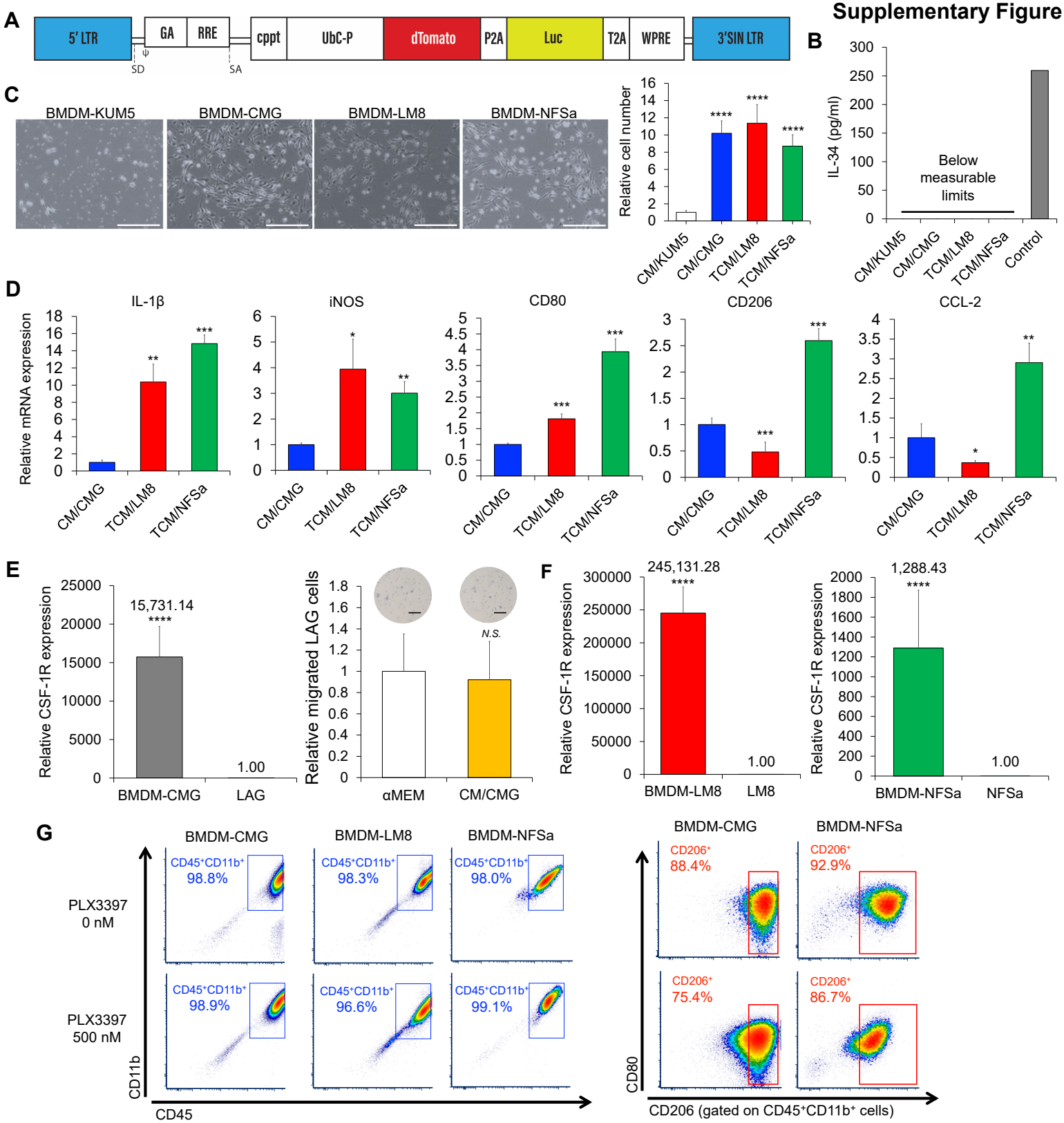
The migration of BMDMs was measured by using 24-well trans-well plates (Cell Culture Insert, Falcon) and 8 μm pore size transparent PET membrane transwell inserts were used to separate the upper and lower chambers. A total of 2.5×10^5 cells were suspended in 100 μm α-MEM and added to the upper chamber. Prior to the cell seeding, BMDMs were treated with PLX3397 (dose range, 50 to 1000 nM), pazopanib (dose range, 0.1 to 10 μM), and neutralizing monoclonal antibody for CSF-1 (dose range, 0.1 to 10 μg/ml) and CSF-1R (dose range, 0.1 to 10 μg/ml) for one hour and then trypsinized and detached; this ensured the cell viability using trypan blue staining. Chemoattractant media was added to the lower chamber at 500 μl/well; TCM, α-MEM containing 200 ng/ml M-CSF, and α-MEM (negative control) were used. After incubation for six hours, the cells on the upper surface of the filter were completely removed by wiping with cotton swabs. The filters were fixed 70% EtOH and stained with crystal violet. The filters were mounted onto slides, and the cells on the lower surfaces were counted.

Flow cytometry

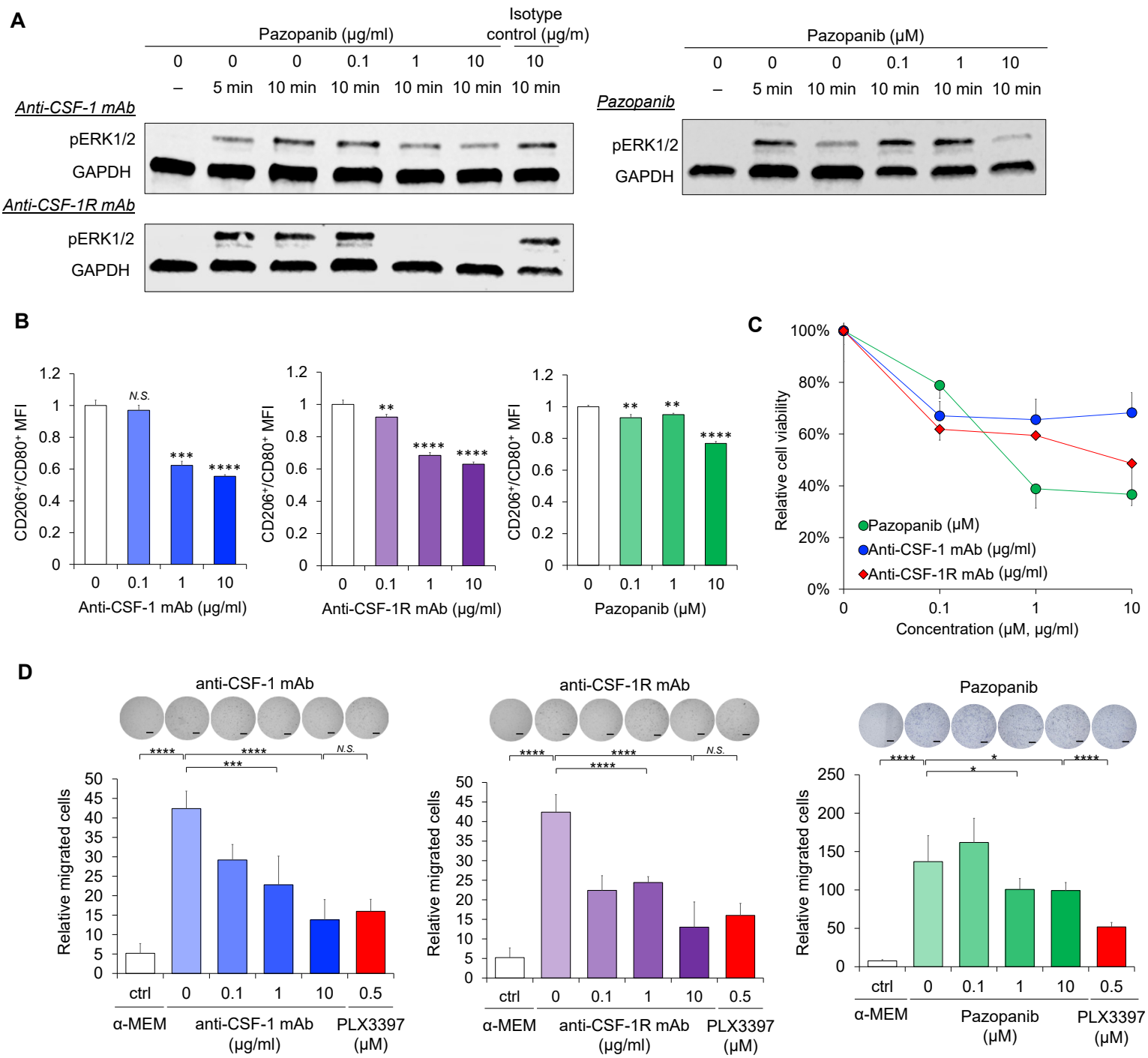
Single cell suspensions were prepared from BMDMs treated with PLX3397 (dose range, 50 to 500 nM), pazopanib (dose range, 0.1 to 10 μM), and neutralizing monoclonal antibody for CSF-1 (dose range, 0.1 to 10 μg/ml) and CSF-1R (dose range, 0.1 to 10 μg/ml), and dissected osteosarcomas. Tumors were harvested, minced, and enzymatically digested using 2.0 mg/ml type I collagenase (Worthington Biochemical Corp., Freehold, NJ) for 60 minutes at 37° C. Digestion mixtures were filtered through 40-μm nylon strainers (Fischer Scientific). Red blood cells were lysed by incubating cells in ACK lysis buffer for 10 minutes. Mononuclear cells were washed with phosphate-buffered saline. Cell suspensions were treated with a rat anti-mouse CD16/CD32 mAb (1:200, Clone 2.4G2, Fc-block; BD Biosciences, Franklin Lakes, NJ) for 10 minutes at 4° C and stained using fluorophore-conjugated anti-mouse antibodies at the manufactures' recommended concentrations: CD45 (30-F11; eBioscience Thermo Fisher, Rockford, IL), CD11b (M1-70; eBioscience), CD206 (C068C2; BioLegend, San Diego, CA), CD80 (16-10A1; BioLegend), CD3 (17A2; BioLegend), CD4 (GK1.5; BioLegend), CD8a (53-6.7; BioLegend), CD19 (6D5; BioLegend), and the matching isotype controls. The samples were analyzed on a BD FACS LSR Fortessa (BD Biosciences). Viability was determined using 7-AAD (Biolegend) to exclude dead cells. The results were analyzed using the FCS Express version 7 program (DeNovo, Los Angeles, CA).

Supplementary References

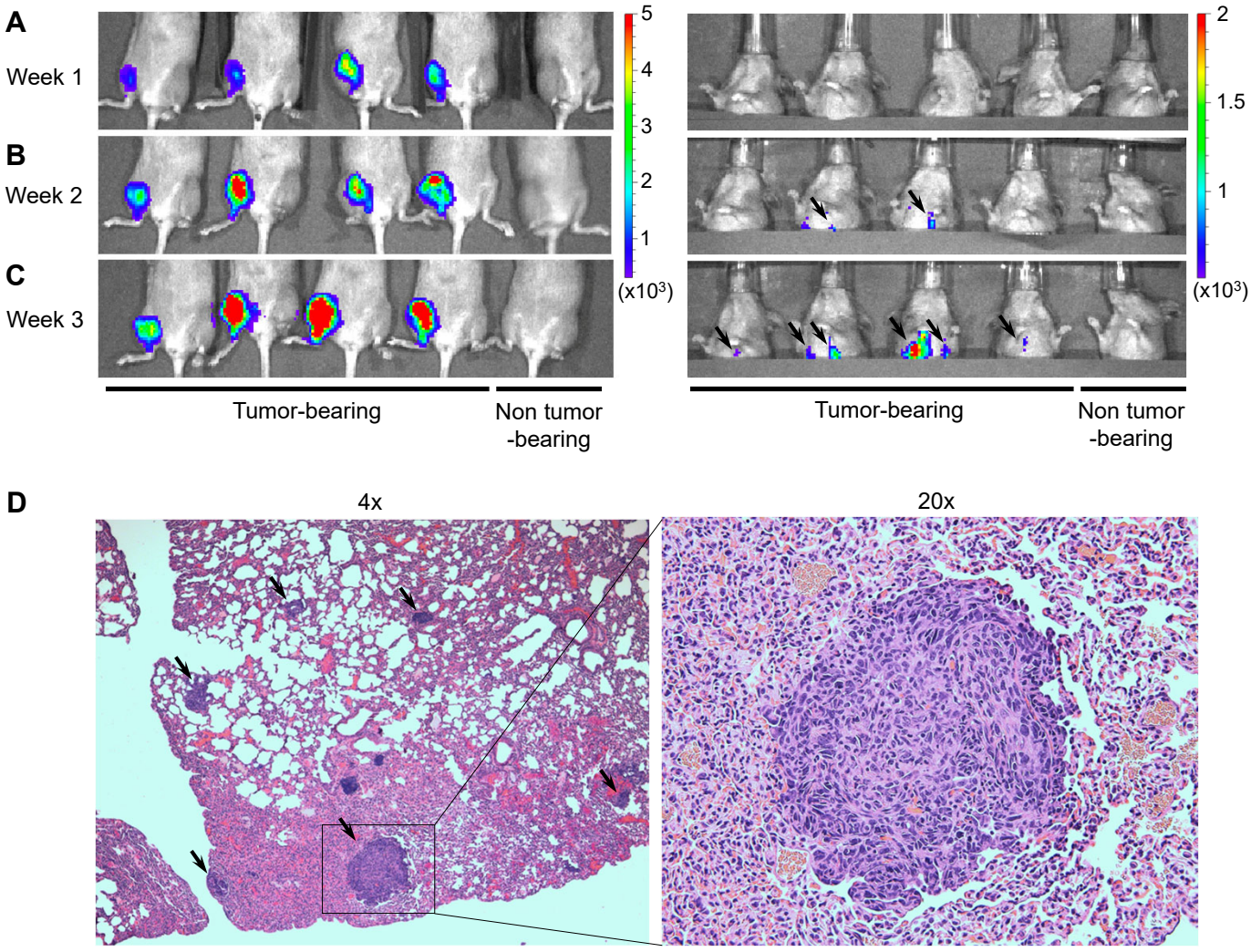
1. Dull T, Zufferey R, Kelly M, Mandel R, Nguyen M, Trono D*, et al.* A third-generation lentivirus vector with a conditional packaging system. Journal of virology **1998**;72:8463-71
2. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF*, et al.* Correction of multi-gene deficiency in vivo using a single'self-cleaving'2A peptide–based retroviral vector. Nature biotechnology **2004**;22:589-94



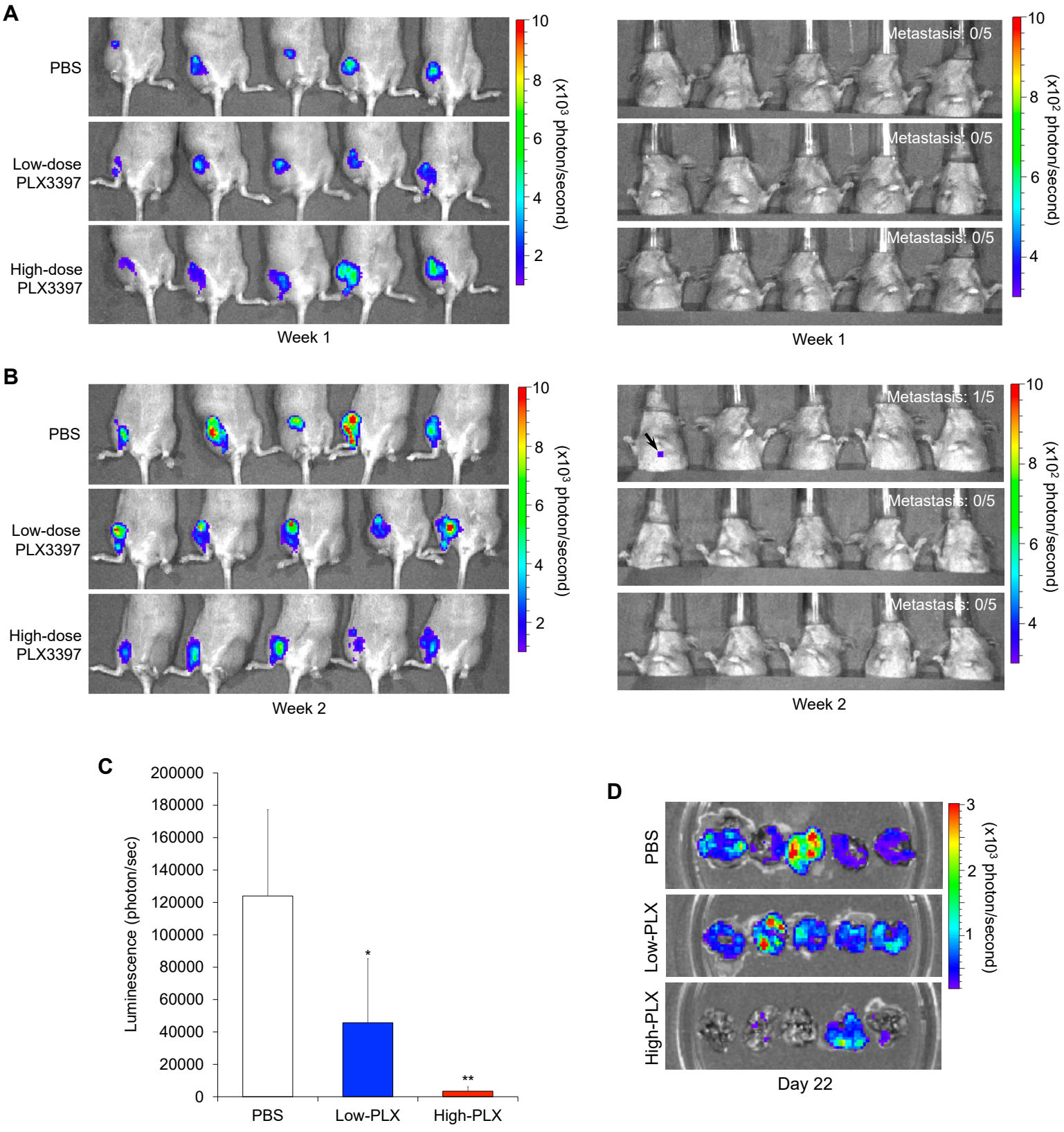
Supplementary Figure 1. Establishment of the *in vitro* sarcoma TAM model. **A,** Plasmid #48688 contains the following: 5' LTR, Chimeric Rous sarcoma virus (RSV)-HIV 5' long terminal repeat; SD, splice donor; ψ=packaging signal; GA, truncated gag sequence; RRE, Rev-Responsive Element; SA, splice acceptor; cppt, central polypurine tract; UbC-P, Ubiquitin C promoter; dTomato, red fluorescent dimer tomato (with 5' Kozak sequence, GCCACC[ATGG]); P2A, porcine teschovirus 2A element; adapted from Szymczak et al (2004); luc, firefly luciferase; T2A, Thosea asigna virus 2A element adapted from Szymczak et al (2004); WPRE, woodchuck hepatitis virus posttranscriptional regulatory element and 3'SIN-LTR, U3-deleted 3' LTR to generate self-inactivating (SIN) vector. **B,** IL-34 secretion from KUM5, CMG, LM8, and NFSa. IL-34 expressions in culture media from CMG, LM8, and NFSa were below measurable limits compared to control solution containing recombinant mouse IL-34. **C,** Morphology of BMDM-KUM5, CSF-1, LM8, and -NFSa after expansion of BMDMs with culture supernatant of KUM5, CMG, LM8, and NFSa cell lines (left). Significantly increased number of BMDMs were observed with CM from CMG, LM8, and NFSa compared to KUM5 (right). ****, $p < 0.0001$ Bars, 100 μm. **D,** Quantification of mRNA expression of M1- and M2-related genes in BMDM-CSF-1, BMDM-LM8, and BMDM-NFSa. HPRT was used as an internal control. Data are presented as mean ± SD (n = 3 per group). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$; Student *t* test. **E,** Left, relative mRNA expression of CSF-1R in BMDM-CSF-1 versus LAG. ****, $p < 0.0001$; Student *t* test. Right, transwell chemotaxis. The migration of LAG was not promoted by CSF-1. Number of migrated cells were photographed (upper) and counted (lower). Scale bar, 200 μm. **F,** Relative mRNA expression of CSF-1R in BMDM-LM8 versus LM8, and BMDM-NFSa versus NFSa. ****, $p < 0.0001$; Student *t* test. **G,** Left; surface marker profile of BMDM-TAMs produced by CSF-1/CMG, TCM/LM8, and TCM/NFSa, assessed by flow cytometry. Upper: Cellular distribution of $CD45^+/CD11b^+$ cells. PLX3397 treatment did not affect cellular distribution of $CD45^+/CD11b^+$ cells in BMDMs-CSF-1, BMDMs-LM8, and BMDMs-NFSa. Lower: Cellular distribution of $CD45^+CD11b^+CD206^+/CD45^+CD11b^+CD80^+$ cells. The number of $CD45^+CD11b^+CD206^+$ decreased by PLX3397 at a dose of 500 nM in BMDMs-CSF-1 and BMDMs-NFSa.



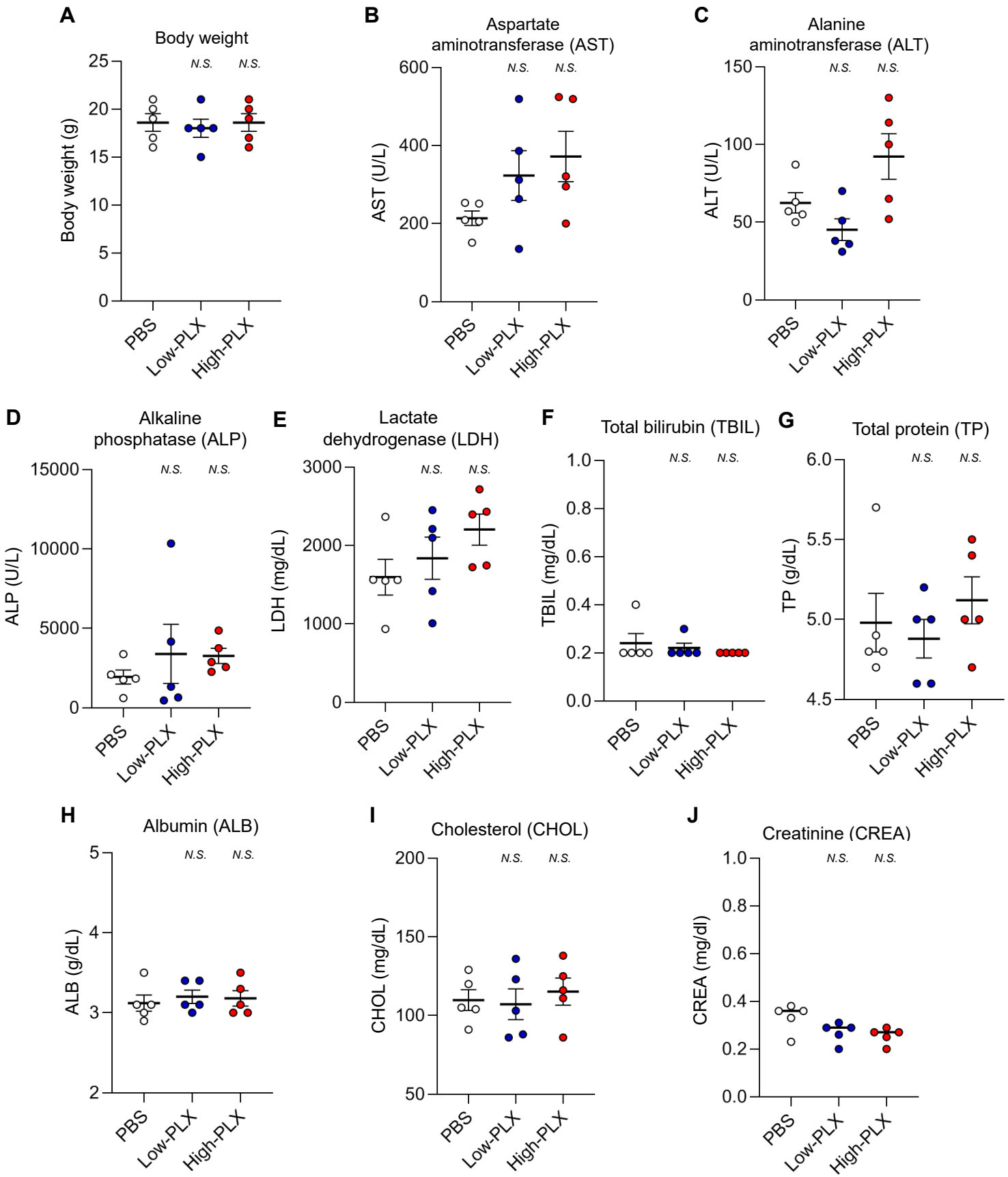
Supplementary Figure 2. Effect of anti-CSF-1 and anti-CSF-1R monoclonal antibody (mAb), and pazopanib on *in vitro* sarcoma TAMs. **A,** Eradication of pERK1/2, stimulated with TCM/LM8, at the indicated doses of anti-CSF-1 mAb, anti-CSF-1R mAb, and pazopanib. **B,** Effect of anti-CSF-1 mAb, anti-CSF-1R mAb, and pazopanib on polarization of BMDM-LM8. Data of $CD206^{+}/CD80^{+}$ MFI are shown. **C,** Survival of TAMs in response to anti-CSF-1 mAb, anti-CSF-1R mAb, and pazopanib. **D,** Trans-well chemotaxis. Macrophage migration was reduced in a dose-dependent manner by anti-CSF-1 mAb, anti-CSF-1R mAb, and pazopanib. The number of migrated cells were photographed (upper) and counted (lower). Scale bar, 200 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; Student's *t*-test.



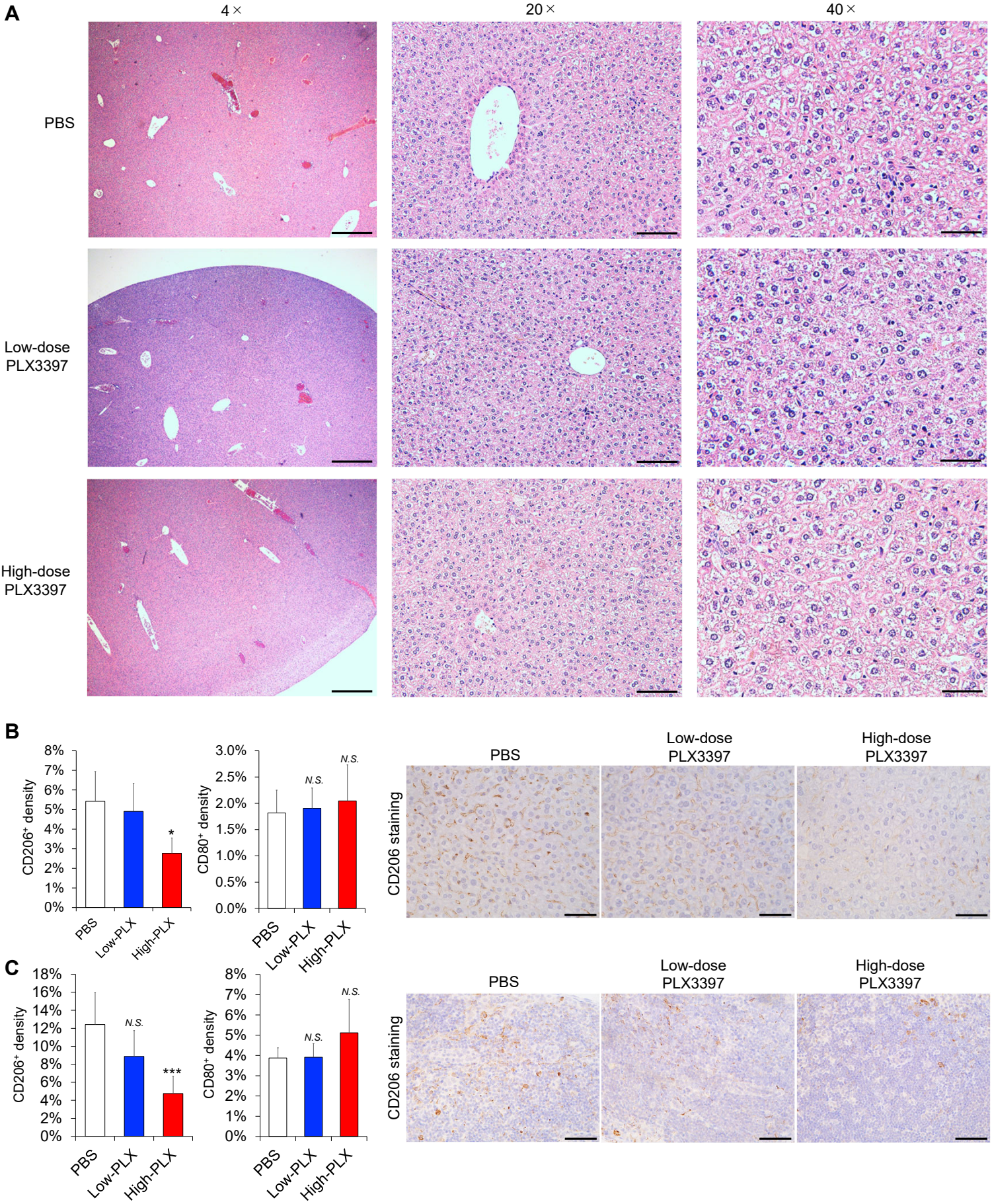
Supplementary Figure 3. Spontaneous lung metastasis in LM8 orthotopic osteosarcoma mouse model as seen in bioluminescent images of mouse inoculated with LM8-luc cells and histological evaluation of the primary tumors and lung metastasis. **A,** Five days after inoculation of 1.0×10^6 LM8-luc cells, the signal from labeled cells was localized in the inoculation site of the right proximal tibia of the mouse. **B,** At 2 weeks after inoculation, a first luminous signal was evident at the pulmonary area (arrows). **C,** At 3 weeks after inoculation, all mice exhibited luminous signal at the pulmonary area. **D,** Many nodules (arrows) were seen at the surface of resected lungs (left), and they were confirmed microscopically as an osteosarcoma metastatic lesion (right).



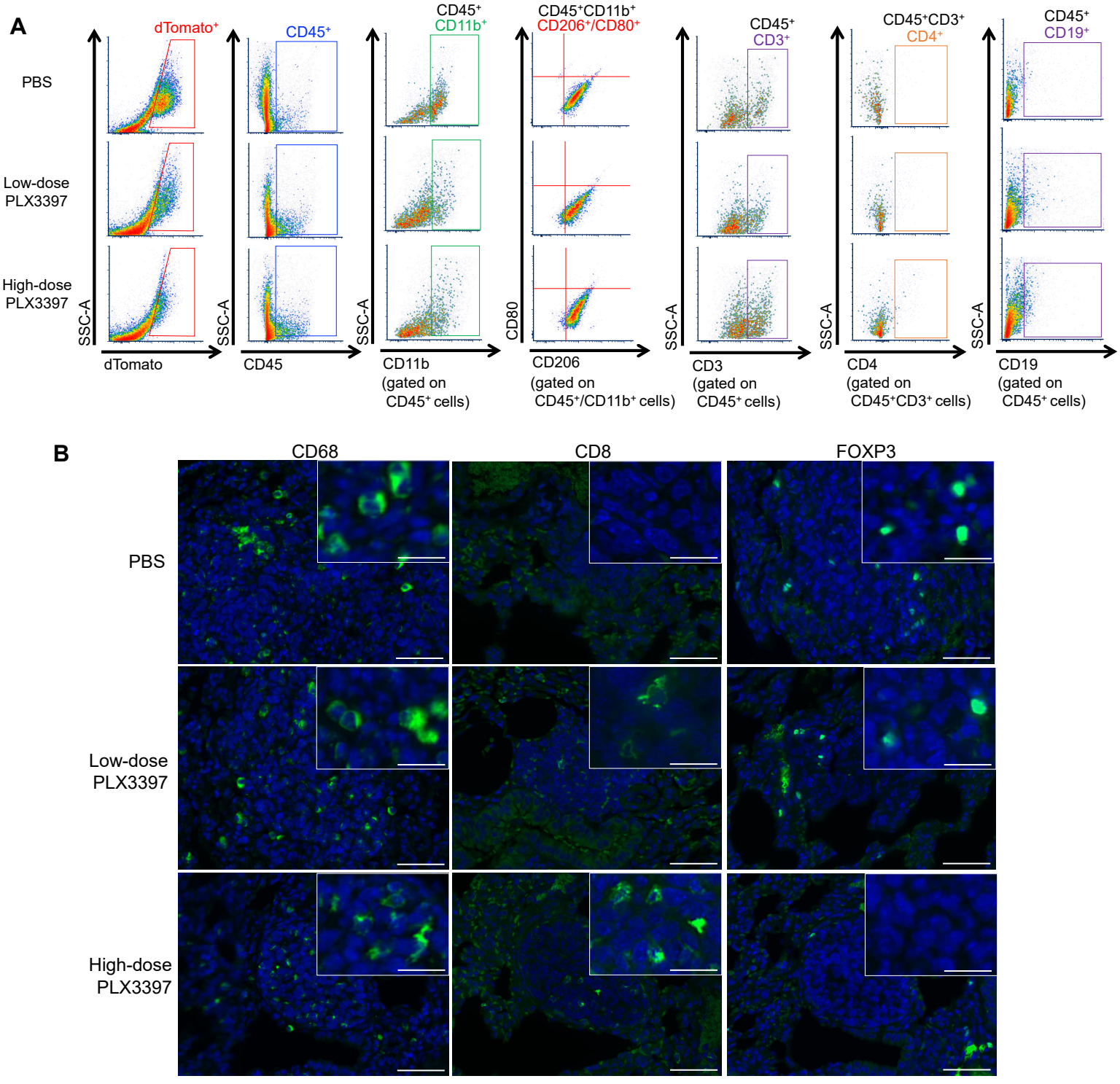
Supplementary Figure 4. CSF-1R inhibition by PLX3397 blocks LM8-luc tumor growth and lung metastasis in orthotopic osteosarcoma mouse model. **A,** Luminescence intensity from the primary tumors (left) and chest lesions (right) of each treatment group measured on week 1 (day 5) using an IVIS. **B,** Luminescence intensity from the primary tumors (left) and chest lesions (right) of each treatment group measured on week 2 (day 12) using an IVIS. **C,** Signal intensity from the chest lesions of mice on week 3 (day 19). *, $p < 0.05$, **, $p < 0.01$; Student *t* test, in comparison with control PBS group. **D,** Luminescence intensity from the resected lungs of each treatment group.



Supplementary Figure 5. Toxicity assessment by body weight and blood chemistry per treatment group at the end of therapeutic experiments (day 22). **A,** Body weight. **B,** Aspartate aminotransferase. **C**, Alanine aminotransferase. **D**, Alkaline phosphatase. **E**, Lactate dehydrogenase. **F**, Total bilirubin. **G**, Total protein. **H,** Albumin. **I**, Cholesterol. **J**, Creatinine. These data showed no significant difference between treatment groups. Data are presented as mean ± s.e.m. (n = 5 per group). *N.S.*, not significant; Student *t* test in comparison with control PBS group.



Supplementary Figure 6. Histopathological examination of orthotopic osteosarcoma mouse models. **A,** Histopathological examination on H&E-stained sections from the livers of the LM8-luc-bearing mice. These samples revealed no evidence of necrosis, fibrosis, steatosis, inflammation, or biliary changes in any of the three groups. Scale bars, 200 μm (left), 40 μm (middle), and 20 μm (right). **B** and **C,** Representative images of immunohistochemical staining for CD206 and CD80 in the liver (B) and lymph node (C) of of the LM8-luc-bearing mice. Scale bars, 50 μm.



Supplementary Figure 7. Alteration of the TME composition in PLX3397-treated mice after CSF-1R inhibition. **A,** Flow cytometry analysis of the dissociated LM8 tumor cells in each treatment group. Representative flow data showing dTomato$^+$, CD45$^+$, CD45$^+$11b$^+$, CD206$^+$/CD80$^+$, CD45$^+$CD3$^+$, CD45$^+$CD3$^+$CD4$^+$, and CD45$^+$CD19$^+$ are shown. All analyzed flow data are shown in **Figure 5**. **B,** Fluorescent IHC showing the distribution of infiltrating CD68$^+$ macrophages (green), CD8$^+$ T cells (green), and FOXP3$^+$ regulatory T cells (green) in metastatic tumors of PLX3397- or PBS-treated mice. Nuclei were stained with DAPI (blue). Scale bars, 50 μm (lower) and 20 μm (upper).