Supplementary Materials for

Noninvasive imaging of CD206-positive M2 macrophages as an early biomarker for post-chemotherapy tumor relapse and lymph node metastasis

Chenran Zhang[1], Xinhe Yu[1], Liquan Gao[1], Yang Zhao[1], Jianhao Lai[1], Dehua Lu[1], Rui Bao[1], Bing Jia[1,2], Lijun Zhong[2], Fan Wang[1,3], Zhaofei Liu[1]

[1] Medical Isotopes Research Center and Department of Radiation Medicine, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China

[2] Medical and Healthy Analytical Center, Peking University, Beijing 100191, China

[3] Key Laboratory of Protein and Peptide Pharmaceuticals, CAS Center for Excellence in Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Corresponding author: Zhaofei Liu, Ph.D., Medical Isotopes Research Center and Department of Radiation Medicine, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China. Phone: +86-1082802871; E-mail: liuzf@bjmu.edu.cn.

Supplementary Materials and Methods

Pilot study of cyclophosphamide treatment

Cyclophosphamide (CTX) treatment started when tumors in 4T1 tumor-bearing mice reached a tumor volume of 100–150 mm^3. Mice were intraperitoneally administered CTX (75 mg/kg, on days 0, 3, 6, and 9). On days 0, 8, and 16, CD206-targeted NIRF imaging (n = 5 per group) and flow cytometry analysis of CD206 and F4/80 (n = 5 per group) were performed using the protocols described in the main text.

Characterization of CD206-targeting probes

The purity of Dye-αCD206 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by near-infrared fluorescence (NIRF) imaging using a Maestro In-Vivo Imaging System (CRI, Woburn, MA). The purity of ^{125}I-αCD206 was determined by the instant thin-layer chromatography (ITLC) using 85% methanol as the mobile phage.

In vitro targeting specificity of ^{125}I-αCD206

$CD206^+$ RAW264.7 cells grown in 24-well plates were incubated with 0.1 μCi ^{125}I-αCD206 (with or without a blocking dose of unlabeled αCD206) or ^{125}I-IgG (isotype-matched control) for 2 h at 4°C. After washing with pre-chilled PBS, cells were collected by trypsinization using a 0.25% trypsin solution, and cell-associated radioactivity was measured using a γ-counter (Packard, Meriden, CT). Results are expressed as the percentage of the total added dose per 10^5 cells (%AD/10^5 cells).

Zoledronic acid and CTX combination treatment

4T1 tumor-bearing mice were separated into 4 groups (n = 8 per group) and treated with (1) PBS, (2) zoledronic acid (ZA, 150 μg/kg daily in PBS, from day 0 to day 13), (3) a single dose of CTX (150 mg/kg in PBS, once on day 0) or (4) ZA plus CTX. Tumor size was monitored every other day. On day 8, 5 mice from each group were i.v. administered 0.5 nmol Dye-αCD206, and NIRF imaging was performed at 24 h p.i.; mice were NIRF-imaged using a Meastro In-Vivo Imaging System (CRI, Woburn, MA).

NIRF imaging in a dual inflammation and 4T1 tumor-bearing mouse model

For the dual inflammation and 4T1 tumor-bearing mouse model, 50 μL of turpentine was injected in the left thigh muscle of the 4T1 tumor-bearing BALB/c mice at 24 h before the NIRF imaging experiments. For NIRF imaging, mice were intravenously administered 0.5 nmol of Dye-αCD206 or Dye-IgG (n = 5 per group). NIRF imaging was performed at 24 h postinjection using a Maestro In-vivo Imaging System (CRI, Woburn, MA).

For *ex vivo* validation of CD206 and F4/80 expression, 3 mice bearing inflammation and 4T1 tumors were euthanized. The tumors, inflamed tissues, and muscles were harvested and frozen cut into 8-μm thick sections. The sections were stained for F4/80 and CD206 using the protocol described in the maintext.

Supplementary Figures and Figure Legends

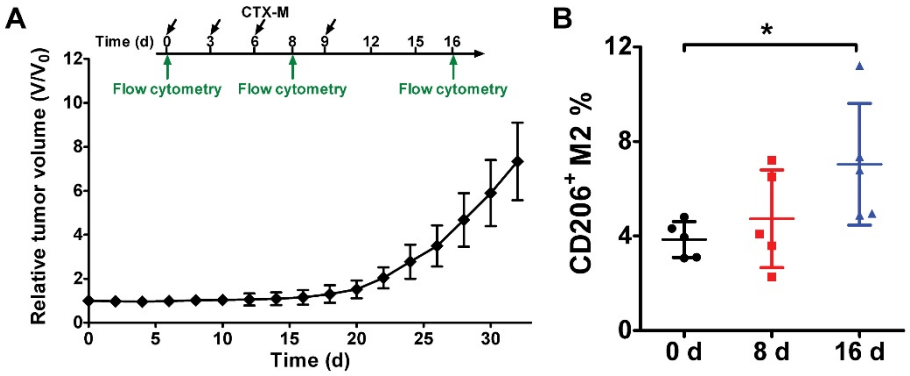

Figure S1. Pilot study of CTX treatment and flow cytometry. (A) Tumor growth curves for 4T1 tumor-bearing mice treated with CTX (75 mg/kg, every 3 days, for 4 times, n = 12). Inset, Schedule of CTX treatment and flow cytometry. (B) Quantified $CD206^{+}F4/80^{+}$ macrophage percentage from flow cytometry analysis of CD206 and F4/80 in 4T1 tumors treated with CTX on days 0, 8, and 16 (n = 5 per group). *, P <0.05

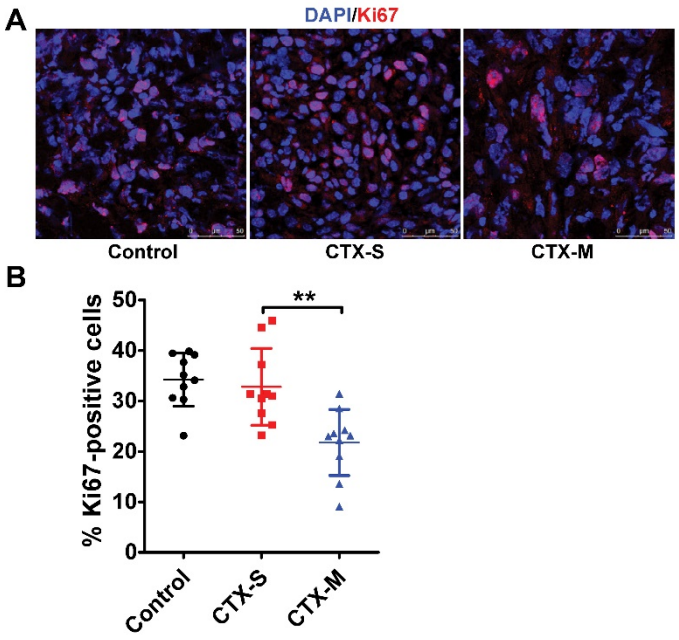

Figure S2. Immunofluorescence staining of Ki67 after CTX treatment. (A) Representative images of Ki67 staining in 4T1 tumors after 8 days of PBS (control), single dose of CTX (CTX-S, 150 mg/kg, i.p. injection) and multiple doses of CTX (CTX-M, 75 mg/kg, i.p. injection every 3 days, six times) treatment. (B) Quantitative analysis of Ki67-positive cells shown in panel A. **, P <0.01.

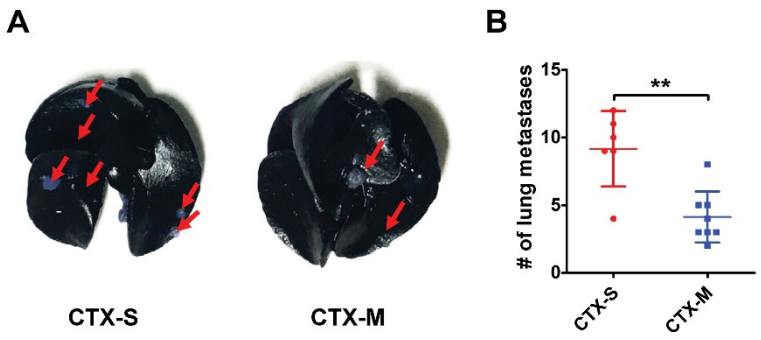

Figure S3. Examination of lung metastatic lesions in 4T1 tumor-bearing mice after CTX treatment. Photographs of India ink-filled lungs (A) and average number of tumor metastatic lesions in the lungs (B) from 4T1 tumor-bearing mice (on day 32) treated with a single dose or multiple doses of CTX. Tumor metastases appear as white nodules on the black lung surfaces and are indicated by red arrows (CTX-S, n = 6; CTX-M, n = 8). **, P <0.01.

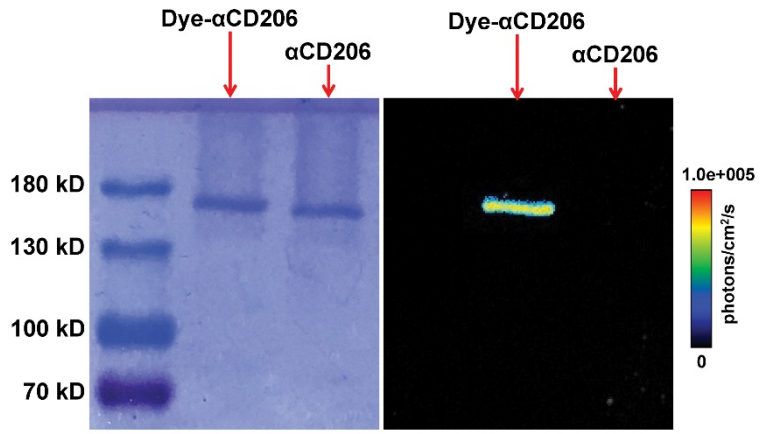

Figure S4. High purity (>98%) of Dye-αCD206 determined by SDS-PAGE and subsequent NIRF imaging.

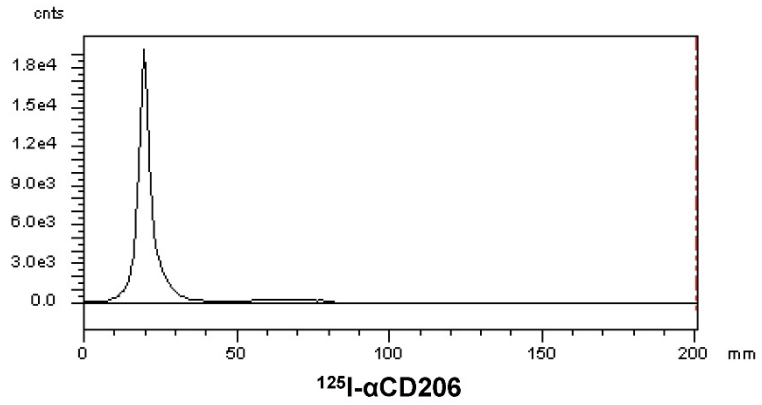

Figure S5. High radiochemical purity (>98%) of ^{125}I-αCD206 determined by ITLC.

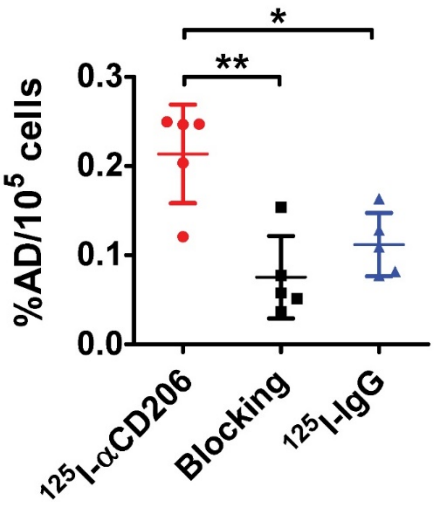

Figure S6. Binding of ^{125}I-αCD206 (with or without an excess dose of cold αCD206) or ^{125}I-IgG to CD206^{+} RAW264.7 cells (n = 5 per group). *, P <0.05; **, P <0.01.

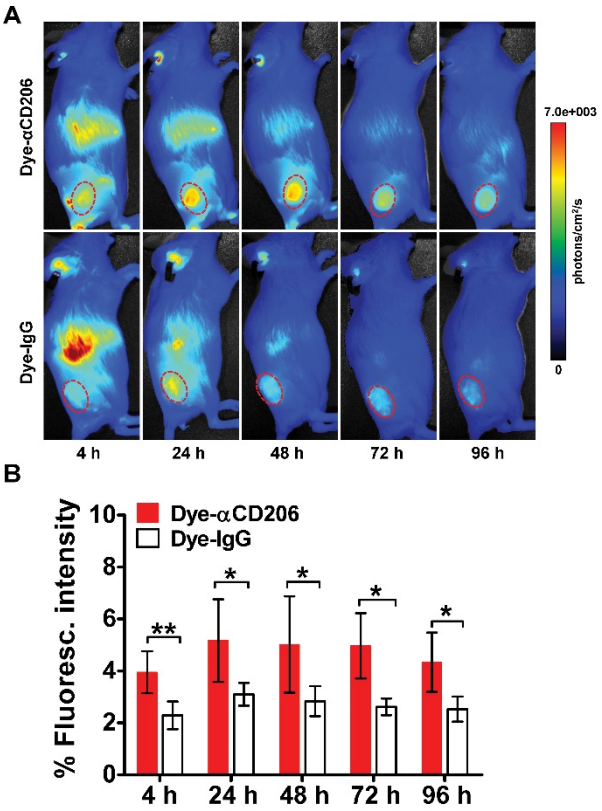

Figure S7. *In vivo* tumor-specific targeting of Dye-αCD206 in 4T1 tumor-bearing mice. Representative NIRF images (A) and quantified tumor uptake (B) 4, 24, 48, 72 and 96 h after Dye-αCD206 or Dye-IgG injection (n = 5 per group). Tumors are indicated with circles. *, P <0.05; **, P <0.01.

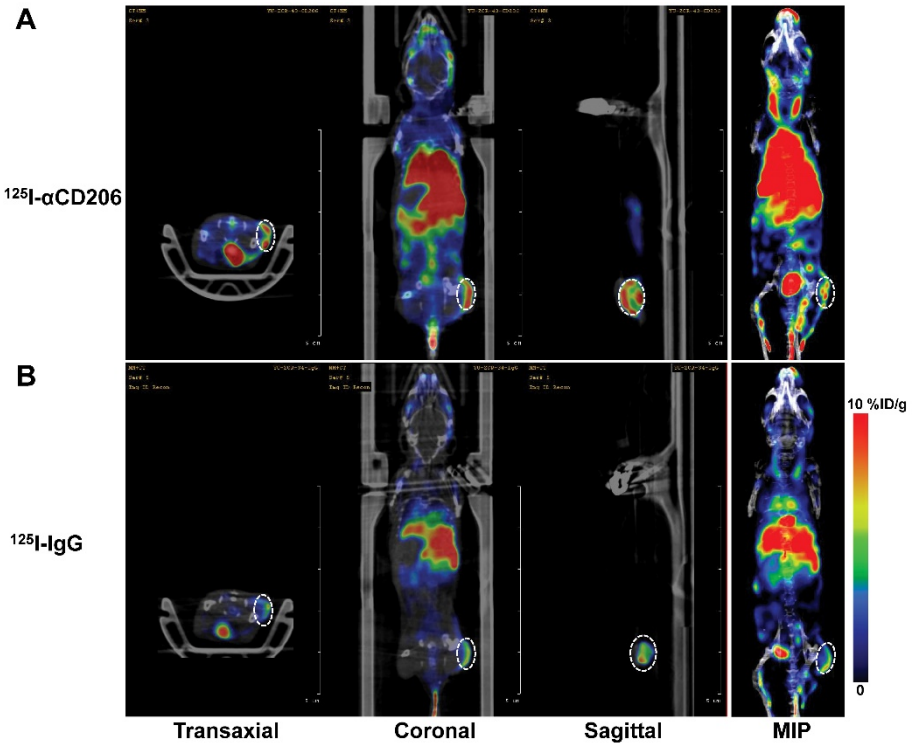

Figure S8. Representative transaxial, coronal, sagittal, and MIP of SPECT/CT images 24 h after ^{125}I-αCD206 (A) and ^{125}I-IgG (B) injection. Tumors are indicated with circles.

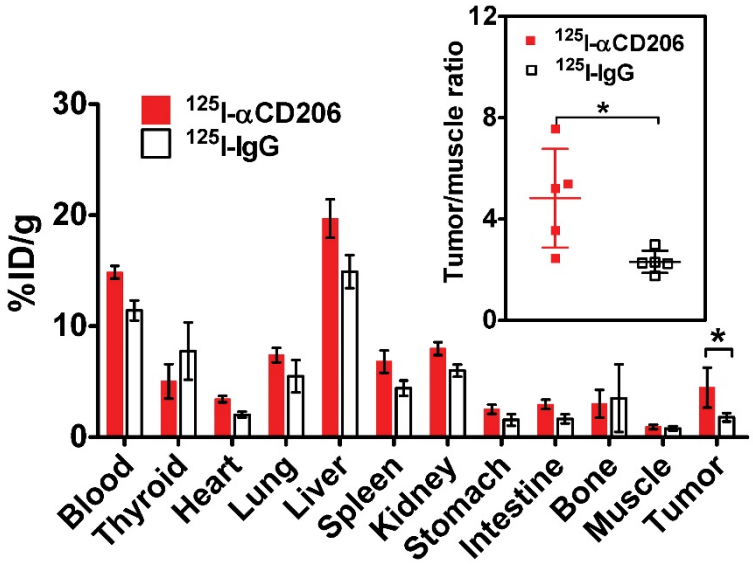

Figure S9. Biodistribution of ^{125}I-αCD206 and ^{125}I-IgG in major organs and tumors (n = 5 per group). Inset, tumor-to-muscle ratios for ^{125}I-αCD206 and ^{125}I-IgG. *, P <0.05.

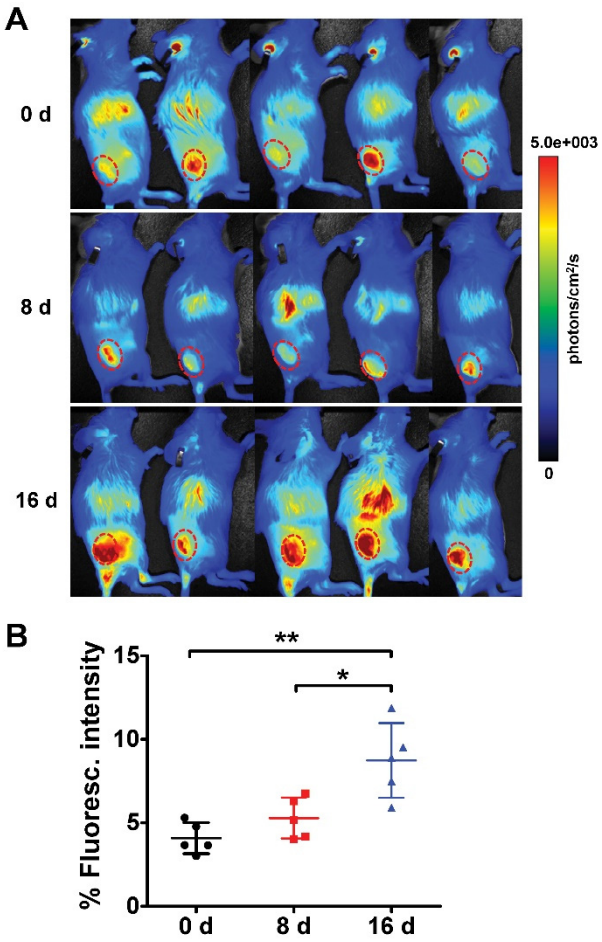

Figure S10. NIRF images (A) and quantified tumor uptake (B) of 4T1 tumor-bearing mice treated with CTX (75 mg/kg on days 0, 3, 6, and 9) 24 h after Dye-αCD206 injection on days 0, 8, and 16 (n = 5 per group). Tumors are indicated with circles. *, P <0.05; **, P <0.01.

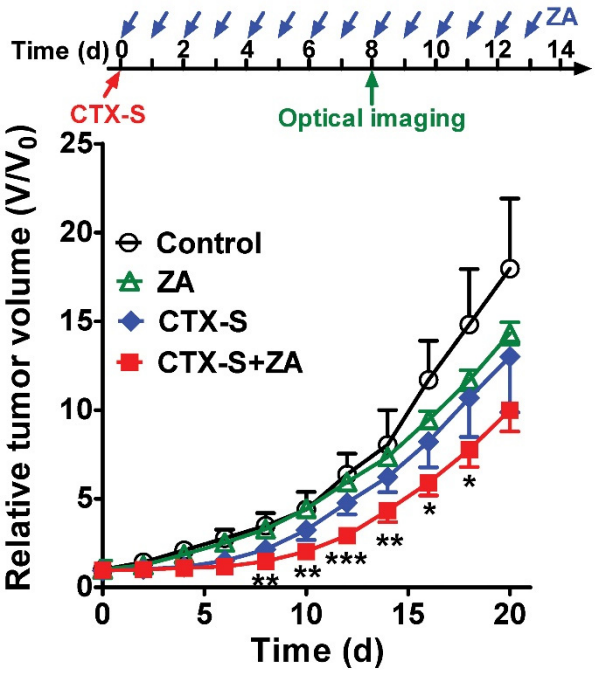

Figure S11. Improved antitumor efficacy using combination therapy of ZA and CTX in 4T1 tumor model. (A) 4T1 tumor growth curve after treatment with vehicle (PBS), ZA (150 μg/kg, every day for 14 days), CTX (single dose, 150 mg/kg) and ZA (150 μg/kg, every day for 14 days) plus CTX (single dose, 150 mg/kg) (n = 8 per group). Inset, ZA and CTX treatment schedule. *, P <0.05; **, P <0.01; ***, P <0.001.

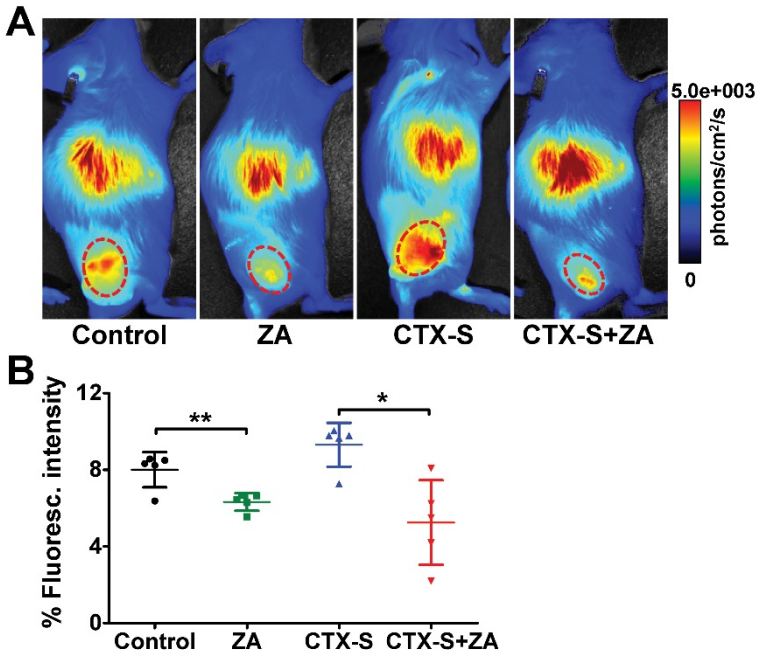

Figure S12. ZA treatment depletes CD206^{+} M2 macrophages in 4T1 tumor model. Representative NIRF images (A) and quantitative analysis of tumor uptake (B) after indicated treatment of 4T1 tumor-bearing mice 24 h after Dye-αCD206 injection (n = 5 per group). Tumors are indicated with circles. *, P <0.05; **, P <0.01.

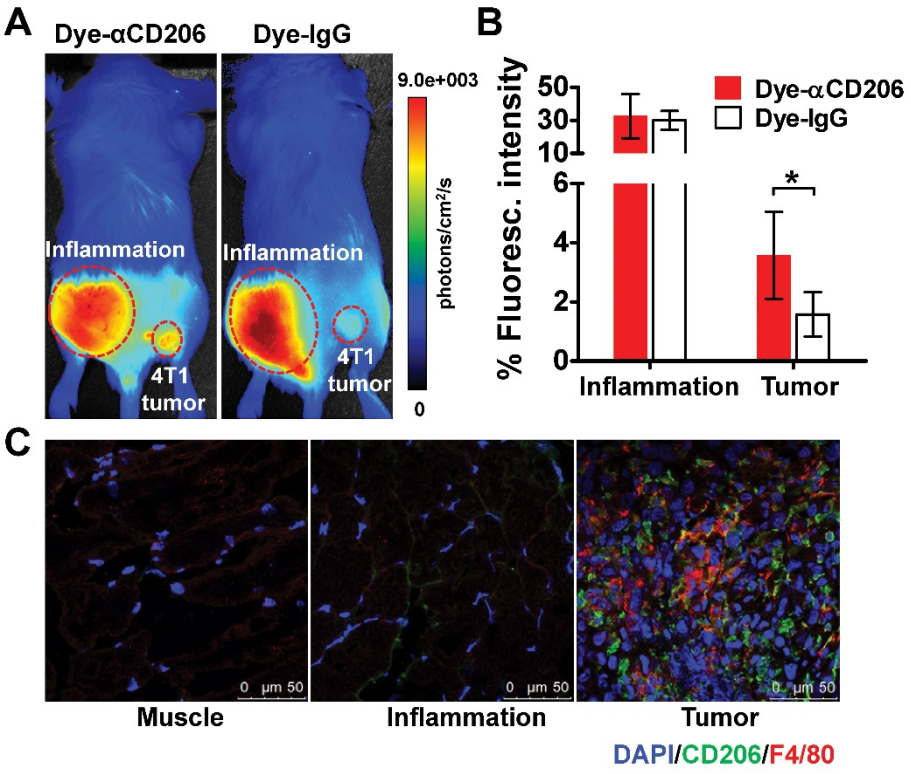

Figure S13. CD206-targeted NIRF imaging of Dye-αCD206 or Dye-IgG in a dual inflammation and 4T1 tumor-bearing mouse model. Representative NIRF images (A) and quantified inflammation and tumor uptake (B) of 4T1 tumor-bearing mice 24 h after Dye-αCD206 or Dye-IgG injection (n = 5 per group). Inflammatory areas and tumors are indicated with circles. *, P <0.05. (C) Immunofluorescence staining of CD206 and F4/80 in muscle as well as in inflamed and 4T1 tumor tissues.