Supporting Information

A Molecularly Targeted Near-Infrared Fluorescence Intraoperative Imaging Agent for High Grade Serous Ovarian Cancer

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Materials and Methods

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were used without further purification. All water used was ultrapure (>18.2 $M\Omega cm^{-1}$ at 25 °C), and dimethylsulfoxide was of molecular biology grade (>99.9%). IRDye® 800CW-NHS ester and IRDye® 800CW-DBCO were purchased from LI-COR Biosciences (Lincoln, Nebraska), and Alexa Fluor™ 488-DBCO was purchased from ThermoFisher Scientific (Eugene, Oregon). The B43.13 antibody was obtained from Oncoquest, Inc. (Edmonton, Alberta), and mouse immunoglobulin G (mIgG) was purchased from Thermo Fisher Scientific (Waltham, MA). All *in vivo* experiments were performed in accordance with published protocols approved by the Institutional Animal Care and Use Committees of Hunter College, Weill Cornell Medical College, and Memorial Sloan Kettering Cancer Center.

Syntheses

Synthesis of mIgG-IR800

mIgG (1.5 mg, 10.03 nmol) was dissolved in 100 µL of phosphate-buffered saline (PBS, pH 7.4), and the pH of the solution was adjusted to 8.8-9.0 with Na_2CO_3 (0.1 M). 6 equivalents of IRDye® 800CW-NHS Ester (11.9 µL, 5.9 mg/mL in DMSO) were added to the solution in small aliquots. The resulting solution was incubated at 37 °C for 1 hour with shaking at 500 rpm. The IR800CW-modified antibody was then purified using size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume: 2.5 mL, eluted with 2 mL of Chelex PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut-off (Amicon™ Ultra 2 mL Centrifugal Filtration Units, MilliporeSigma Corp., Burlington, MA).

Synthesis of ^{ss}B43.13-AF488 and ^{ss}B43.13-IR800

Glycans Modification: For the deglycosylation of B43.13, B43.13 (4.5 mg) was combined with 1.5 units of recombinant EndoS enzyme (New England BioLabs, Ipswich, MA) per 1 µg of antibody, 50 µL 500 mM sodium phosphate, pH 7.5 (10X Glycobuffer 1 from New England Biolabs), and H_2O to make up a total reaction volume of 500 µL. The reaction was incubated at 37 °C for 24 h and then purified using magnetic chitin beads (New England Biolabs). The deglycosylated B43.13 antibody was buffer exchanged into pre-treatment buffer (50 mM Bis-Tris, 100 mM NaCl, pH 6.0) using a centrifugal filter (Amicon™ Ultra 2 mL Centrifugal Filtration

Units, MilliporeSigma Corp., Burlington, MA). To perform the buffer exchange, the filter was first equilibrated with 50 mM Bis-Tris, pH 6.0 and then centrifuged for 6 minutes at 5,000 g. After the final spin, the deglycosylated antibody was isolated in 100 µL (22.58 mg/mL).

GalNAz Labeling: After the EndoS treatment and buffer exchange, 75 µL H_2O, 15 µL 1M Tris buffer pH 7.6, 2.5 µL 1M $MnCl_2$, 5.3 µL UDP-GalNAz (from a stock solution at 40 mM in H_2O) and 13.2 µL Gal-T1(Y289L) (stock solution at 2 mg/mL) were added to the reaction solution for a final volume of 265 µL. This resultant solution contained final concentrations of 13.12 mg/mL antibody, 9.5 mM $MnCl_2$, 0.8 mM UDP-GalNAz, and 0.1 mg/mL Gal-T1(Y289L) and was incubated overnight at 30 °C.

Fluorophore Ligation: After the GalNAz labeling step, the resulting solution was purified via centrifugal filtration using 2 mL Amicon filtration units and tris-buffered saline (TBS, pH 7.4). After centrifugation, the modified B43.13 (2 sets of 1,613 µg in 88 µL of TBS) was combined with 86.0 µL DBCO-IR800 or DBCO-AF488 (from a 2 mM stock solution) and TBS (pH 7.4) to yield solutions with a final volume of 1.5 mL containing 1.08 mg/mL B43.13 and 0.1 mM DBCO. The solutions were incubated overnight at 25 °C.

Purification: After labeling the antibody with DBCO-IR800 or DBCO-AF488, the site-specifically modified antibodies were purified via size exclusion chromatography (PD-10 column, GE Healthcare) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 2 mL Centrifugal Filtration Units, MilliporeSigma Corp., Burlington, MA) and PBS (pH 7.4).

Characterization

SDS-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to characterize the constructs – B43.13, EndoS-B43.13, and ^{ss}B43.13-IR800. 2 µg antibody were combined with 5 µL 500 mM dithiothreitol (NuPAGE® 10X Sample Reducing Agent, Thermo Fisher Scientific, Waltham, MA), 12.5 µL 4X electrophoresis buffer (NuPAGE® LDS Sample Buffer, Thermo Fisher Scientific, Waltham, MA), and H_2O to make up a total volume of 30 µL. The mixture was then denatured by heating to 90 °C for 15 min using an Eppendorf ThermoMixer® F1.5 (Hauppauge, NY). Subsequently, 20 µL of each sample was loaded alongside an appropriate molecular weight marker (Mark12™ Unstained Standard, Thermo Fisher Scientific) onto a 1 mm,

10 well 4-12% Bis-Tris protein gel (NuPAGE™ 4-12% Bis-Tris Protein Gels, Thermo Fisher Scientific) and run for ~ 4 h at 10 V/cm in MOPS buffer. The resulting gel was washed 3 times with H_2O, stained with SimplyBlue™ SafeStain (Thermo Fisher Scientific) for 1 h, and destained overnight in H_2O. Last, the gel was analyzed using the Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

Degree of Labeling Determination

To determine the degree of labeling (DOL) of the immunoconjugates, UV-Vis measurements were obtained using a Shimadzu BioSpec-Nano Micro-Volume UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). Absorbance measurements were taken at 280 nm (for both immunoconjugates), 494 nm (for ^{ss}B43.13-AF488), and 774 nm (for ^{ss}B43.13-IR800), and the DOL was calculated using the following formulas:

$$A_{mAb} = A_{280} - A_{max}(CF)$$

$$DOL = [A_{max} * MW_{mAb}]/[[mAb] * \varepsilon_{fluorophore}]]$$

For both immunoconjugates, MW_{mAb} = 150,000 and $\varepsilon_{280,\,mAb}$ = 210,000 $cm^{-1}M^{-1}$.
For ^{ss}B43.13-IR800, the correction factor (CF) is 0.03 and ε_{IR800} = 240,000 $cm^{-1}M^{-1}$.
For ^{ss}B43.13-AF488, the correction factor (CF) is 0.11 and ε_{AF488} = 71,000 $cm^{-1}M^{-1}$.

***In Vitro* Studies**

Cell Culture

Human ovarian cancer cell line SKOV3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in McCoy's 5A Medium, supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 units/mL streptomycin in an incubator (Heracell™ 150i, ThermoFisher Scientific) set to 37 °C and 5% CO_2. Human ovarian cancer cell OVCAR3 was purchased from the ATCC and maintained in RPMI 1640 Medium, supplemented with heat inactivated fetal bovine serum (20% v/v), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 0.01 mg/mL bovine insulin (Gemini Bio-Products, 700-112P), 100 units/mL penicillin, and 100 units/mL streptomycin. The cell lines were harvested and passaged upon reaching 80% confluency using

0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium. All media was purchased from the Media Preparation Facility at Memorial Sloan Kettering Cancer Center.

Flow Cytometry

Flow cytometry experiments were performed with two cell lines, CA125-positive OVCAR3 and CA125-negative SKOV3 human ovarian cancer cells. Either 10^6 OVCAR3 or SKOV3 cells were incubated with 1 μg/mL, 4 μg/mL, 8 μg/mL, and 16 μg/mL ^{ss}B43.13-AF488 immunoconjugate for 30 min on ice. A blocking condition was also created by incubating the OVCAR3 or SKOV3 cells with 2 μg/mL ^{ss}B43.13-AF488 and a 75-fold excess of unmodified B43.13 (150 μg/mL). Cells were washed by pelleting, resuspension, and analyzed on a BD LSR II (BD Biosciences, San Jose, CA). Binding data was collected in triplicate, averaged, and plotted.

Immunostaining and Fluorescence Microscopy

Upon splitting from 80% confluent T150 flasks, OVCAR3 and SKOV3 cells were grown separately on sterile glass coverslips placed in 6-well tissue culture plates. Cells were seeded at a density of 250,000 cells in 3 ml growth medium per well. The cells were incubated for 48 h in a sterile incubator set to 37 °C and supplied with 5% CO_2. Thereafter, the cells were rinsed with PBS and fixed in 100% methanol for 30 min at -20°C. The fixed cells were incubated in 5% non-fat dry milk (Carnation) in PBS and stained separately for 1 h with 50 μL of a 1:100 dilution of ^{ss}B43.13-AF488 (0.67 mg/mL); 50 μL of 1:250 dilution of unmodified B43.13 (3.0 mg/mL) spotted on parafilm. 50 μL of 1:500 dilution of AF488-conjugated goat anti-mouse antibody (1 mg/mL) (ThermoFisher Scientific, A-11001) suspended in PBS containing 5% non-fat dry milk was used as a secondary antibody. All antibody incubations were followed by three rinses using 0.1% PBST (0.1% Tween-20-supplemented phosphate-buffered saline) for 10 min each. Upon completion of incubations and washes, the coverslips were mounted on microscopy slides (Fisherbrand) using Mowiol® mounting medium (Calbiochem, 475904) supplemented with DAPI (50 μg/ml). Immunofluorescence was observed through a Zeiss Plan Apochromat 10× objective lens on a digital axioscope fluorescence microscope.

***In Vivo* Studies**

Xenograft Models

Ten to twelve-week-old female athymic nude and NSG mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimatize for approximately 1 week prior to inoculation. Animals were housed in ventilated cages and given water and food *ad libitum*.

Subcutaneous Xenografts: Mice were anaesthetized by inhalation of 2% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture and xenografted subcutaneously on the right shoulder with 5×10^6 OVCAR3 cells in a 150 µL cell suspension of a 1:1 mixture of fresh media: Matrigel (Corning Life Sciences, Corning, NY). The OVCAR3 tumors reached the ideal size for imaging and biodistribution studies (~200-300 cm^3) after approximately 4 weeks.

Orthotopic Xenografts: Mice were anaesthetized by inhalation of 1.5% isoflurane/oxygen gas mixture, and surgery was performed on a heated surface to maintain body temperature. One dose of meloxicam (2 mg/kg) and buprenorphine (0.5 mg/kg) was given preemptively via subcutaneous injection. Bupivacaine, a local anesthetic agent, was injected into the tissue adjacent to the incision line. The skin was then prepped for surgery by alternating scrubs of povidone-iodine and 70% ethanol. A dorsolateral incision (1-2 cm in length) was made on the skin on the top right of the spleen, and the retroperitoneum was dissected to expose the fat pad surrounding the ovary. 3×10^6 cells OVCAR3-Luc in 15 µL of PBS were injected into the left ovary. The retroperitoneum was closed using Vicryl sutures. The skin was closed with sterile wound clips and the wound edges were sealed with a few drops of tissue adhesive. The tumors reached the ideal size for experiments after approximately 8 weeks.

Patient-Derived Xenografts (PDX): The PDX seed was revived from frozen stocks and xenografted subcutaneously on the right shoulder. The tumors reached the ideal size for experiments after approximately 4 weeks.

NIRF Imaging

^{ss}B43.13-IR800 was administered 72 h prior to the NIRF imaging. Approximately 5 min prior to imaging, mice (n = 5 per cohort) were anesthetized by inhalation of 2% isoflurane/oxygen gas mixture and placed on the scanner bed. Anesthesia was maintained by inhalation of 1% isoflurane/oxygen gas mixture. Fluorescent images were acquired with an IVIS Spectrum Preclinical Imaging System (PerkinElmer) using an excitation wavelength of 740 nm and emission

wavelength of 820 nm. For *ex vivo* imaging, the mice were sacrificed via CO_2 asphyxiation. Select tissues (including tumor) were collected, rinsed in water, dried, and placed on either a black sheet of paper or petri dish. Fluorescent images were acquired with the same wavelength settings on the IVIS Spectrum. The resulting images were processing using the Living Image (v4.4) software.

***Ex Vivo* Analyses**

Histopathology

The tumors and metastases harvested for the biodistribution were fixed in 10% neutral buffered formalin. The formalin-fixed tissue samples were processed in ethanol and xylene, embedded in paraffin, sectioned into 5 μm thick sections, and stained with hematoxylin and eosin (H&E). Sections from the tumors and metastases were also stained by immunohistochemistry for MUC16/CA125 on a Leica Bond RX automated staining platform (Leica Biosystems). Subsequently, heat-induced epitope retrieval was conducted in a pH 9.0 buffer, and the primary antibody (Novus Biologicals, NBP1-96619) was applied using a 1:250 dilution. This was followed by applying a polymer detection system (Novocastra Bond Polymer Refine Detection, Leica Biosystems, DS9800). The resulting slides were interpreted by a board-certified veterinary pathologist from the Laboratory of Comparative Pathology at Memorial Sloan Kettering Cancer Center. The slides were digitized using Pannoramic Flash scanners (3DHistech). The scanned images were processed using Pannoramic Viewer software (3DHistech).

Supplemental Figures

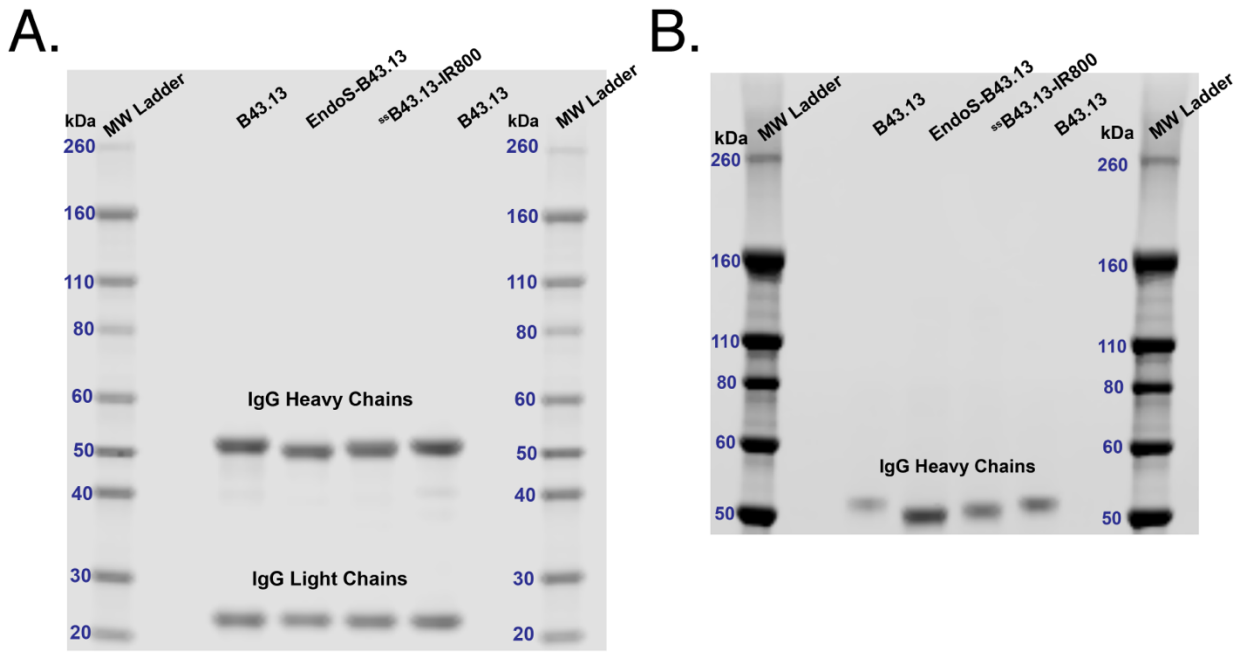

Figure S1. SDS-PAGE of ^{ss}B43.13-IR800. (A) A reducing SDS-PAGE assay showing the heavy and light chains of (from left to right) unmodified B43.13, B43.13 following EndoS treatment (EndoS-B43.13), ^{ss}B43.13-IR800, and unmodified B43,13. Note that the mass of the heavy chain decreases upon treatment with EndoS and subsequently increases slightly upon the conjugation of DBCO-IR800. In contrast, the mass of the light chain does not change upon the modification of the parent antibody. (B) A reducing SDS-PAGE assay that has been run for a longer period of time, thereby resolving the heavy chains more fully. Again, the mass of the heavy chain decreases upon treatment with EndoS and subsequently increases slightly upon the conjugation of DBCO-IR800

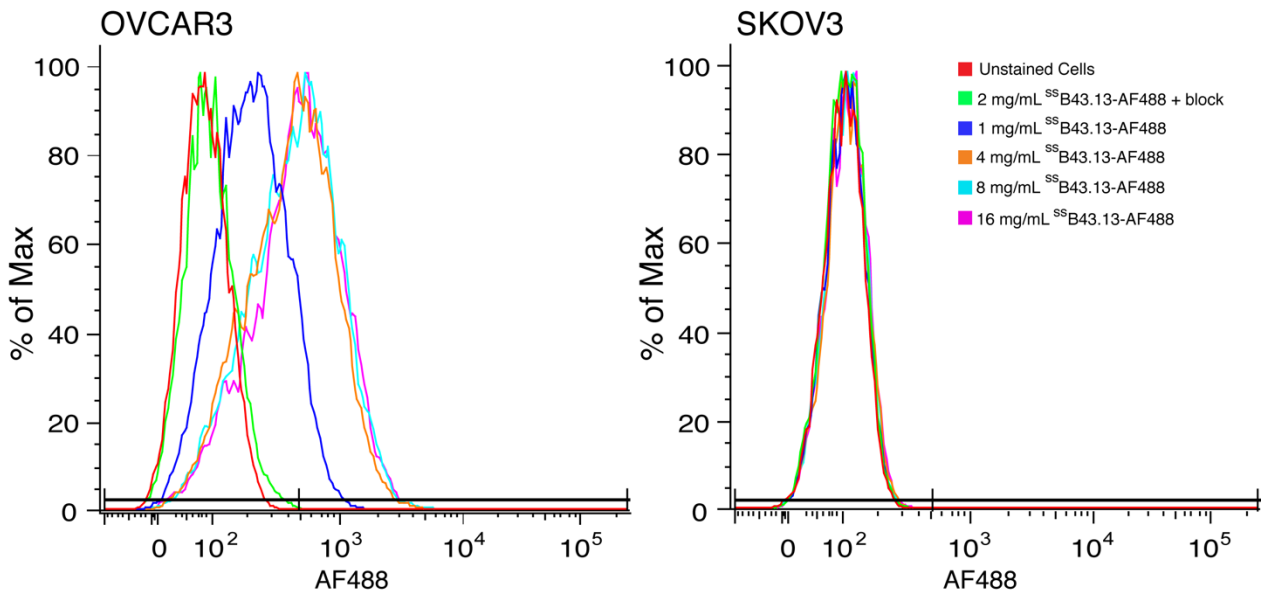

Figure S2. ***In vitro*** **selectivity.** FACS histograms illustrating the selectivity of a ^{ss}B43.13-AF488 using CA125-positive OVCAR3 (left) and CA125-negative SKOV3 (right) ovarian cancer cells. ^{ss}B43.13-AF488 staining of CA125-positive OVCAR3 cells showed a rightward shift of fluorescence from the baseline (unstained OVCAR3 cells). In contrast, the blockade of ^{ss}B43.13-AF488 using a vast excess of unlabeled B43.13 *did not* show a rightward shift in fluorescence (similar to unstained cells). On the other hand, the histograms for CA125-negative SKOV3 cells treated with similar concentrations of the ^{ss}B43.13-AF488 showed no shift in the fluorescence signal, indicative of a lack of binding.

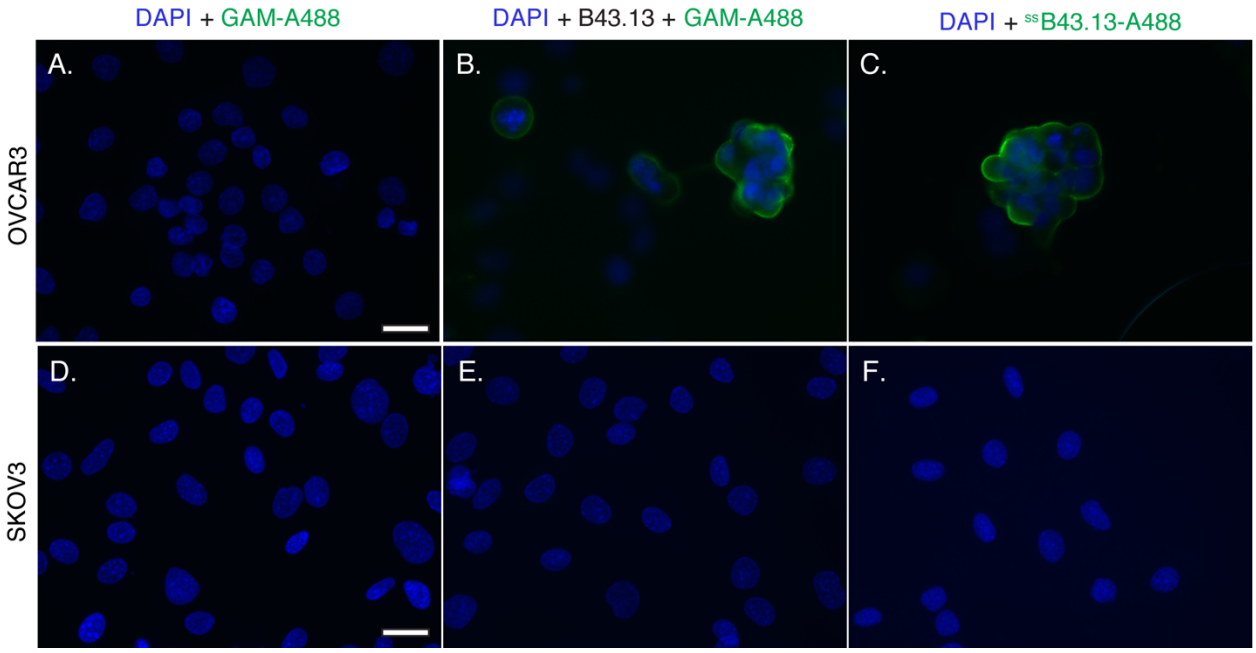

Figure S3. ssB43.13-AF488 retains the ability to bind to CA125-expressing OVCAR3 cells. Fluorescence microscopy images (representative of n = 3 experiments): (A) OVCAR3 cells stained with AF488-modified goat anti-mouse antibody (GAM-A488) and counterstained with DAPI for the delineation of cell nuclei; (B) OVCAR3 cells stained with unmodified B43.13 followed by GAM-A488 and counterstained with DAPI; (C) OVCAR3 cells stained with ssB43.13-AF488 and counterstained with DAPI; (D-F) CA125-negative SKOV3 cells stained using conditions identical to those used in panels A-C, respectively.

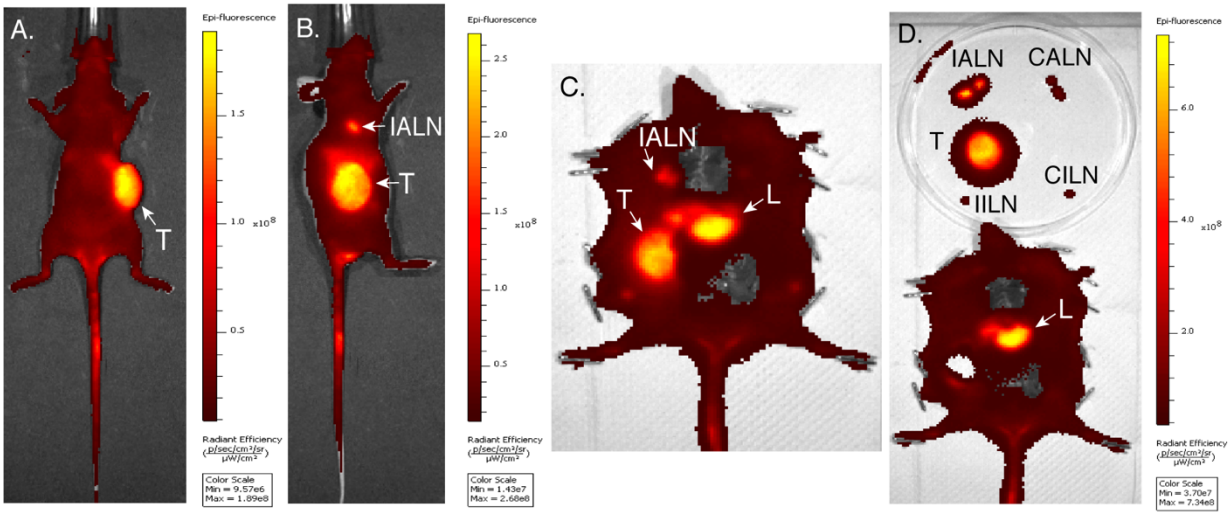

Figure S4. ***In vivo*** **NIRF imaging with ^{ss}B43.13-IR800.** NIRF images of a nude mouse bearing a CA125-positive OVCAR3 xenograft (right flank) in a (A) prone and (B) side position collected 72 h after the administration of ^{ss}B43.13-IR800 (100 µg; 0.66 nmol). Note the clear visualization of the primary tumor as well as the ipsilateral axillary lymph node (IALN). (C) NIRF image of the same mouse with the internal organs exposed, further illustrating the NIRF signal in the tumor, IALN, and liver. (D) *Ex vivo* NIRF image showing the degree of uptake of ^{ss}B43.13-IR800 in the tumor (T), ipsilateral axillary lymph nodes (IALN), contralateral axillary lymph nodes (CALN), ipsilateral inguinal lymph nodes (IILN), contralateral inguinal lymph nodes (CILN), and liver (L). NIRF image shown is representative of n = 9 mice. Only 3/9 mice contained NIRF-positive ipsilateral lymph nodes.

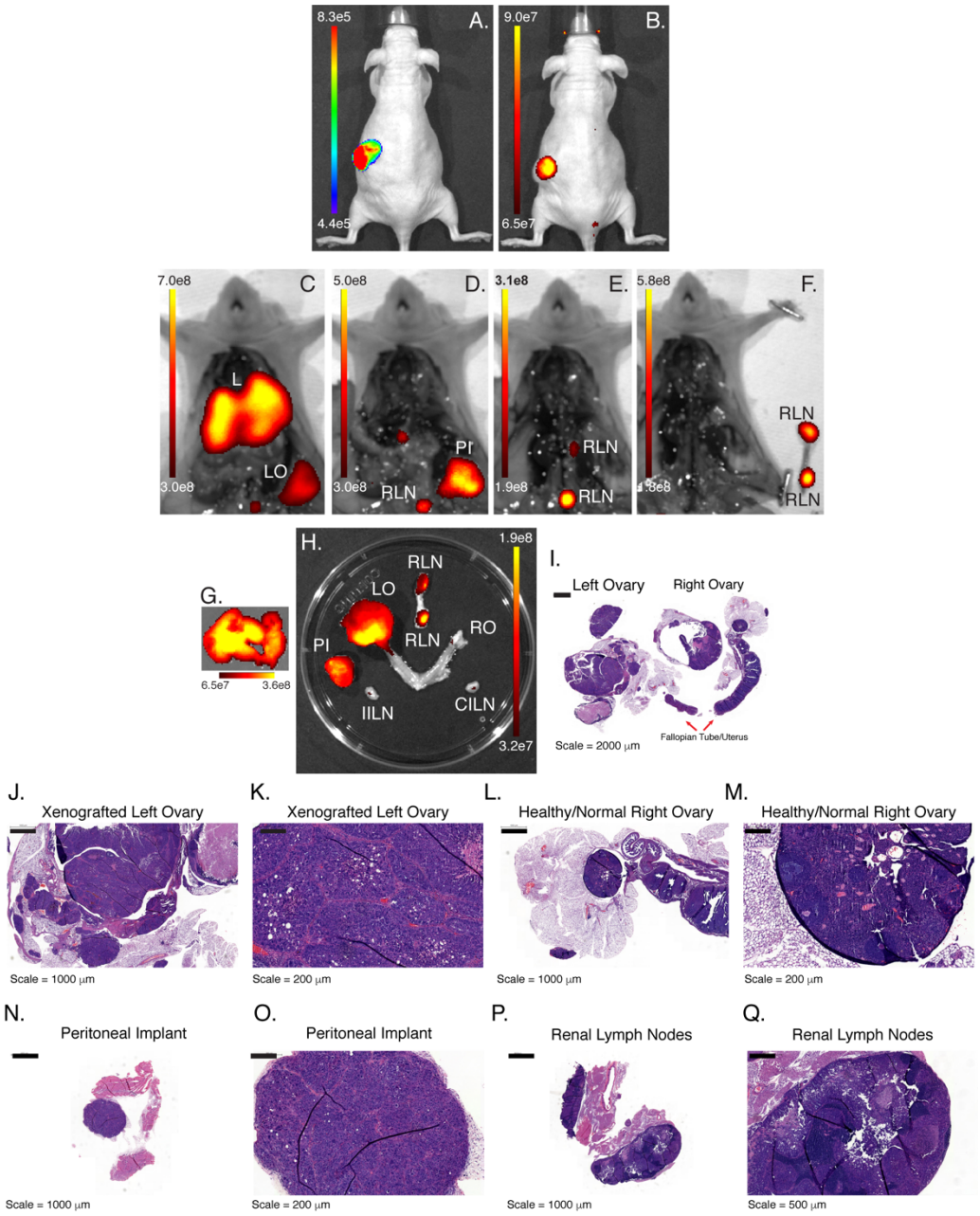

Figure S5. *In vivo* and *ex vivo* NIRF imaging using ^{ss}B43.13-IR800. (A) Bioluminescence image of a mouse bearing an orthotopic, OVCAR3-Luc xenograft implanted in the left ovary; (B) NIRF image of a mouse bearing an orthotopic, CA125-positive OVCAR3 xenograft implanted in the left ovary obtained 120 h after the administration of ^{ss}B43.13-IR800 (100 μg; 0.66 nmol); NIRF images obtained after (C) the exposure of the peritoneal cavity, (D) the removal of the liver [L] and left ovary [LO], (E) the removal of the peritoneal implant [PI], and (F) the removal of the renal lymph nodes [RLN]; *ex vivo* NIRF images obtained of (G) the liver as well as (H) the left ovary, right ovary [RO], renal lymph nodes [RLN], ipsilateral inguinal lymph nodes [IILN] and contralateral inguinal lymph nodes [CILN]; histopathologic analysis of the resected (I) reproductive tract, (J, K) xenografted left ovary, (L, M) healthy right ovary, (N, O) peritoneal implant, and (P, Q) renal lymph nodes

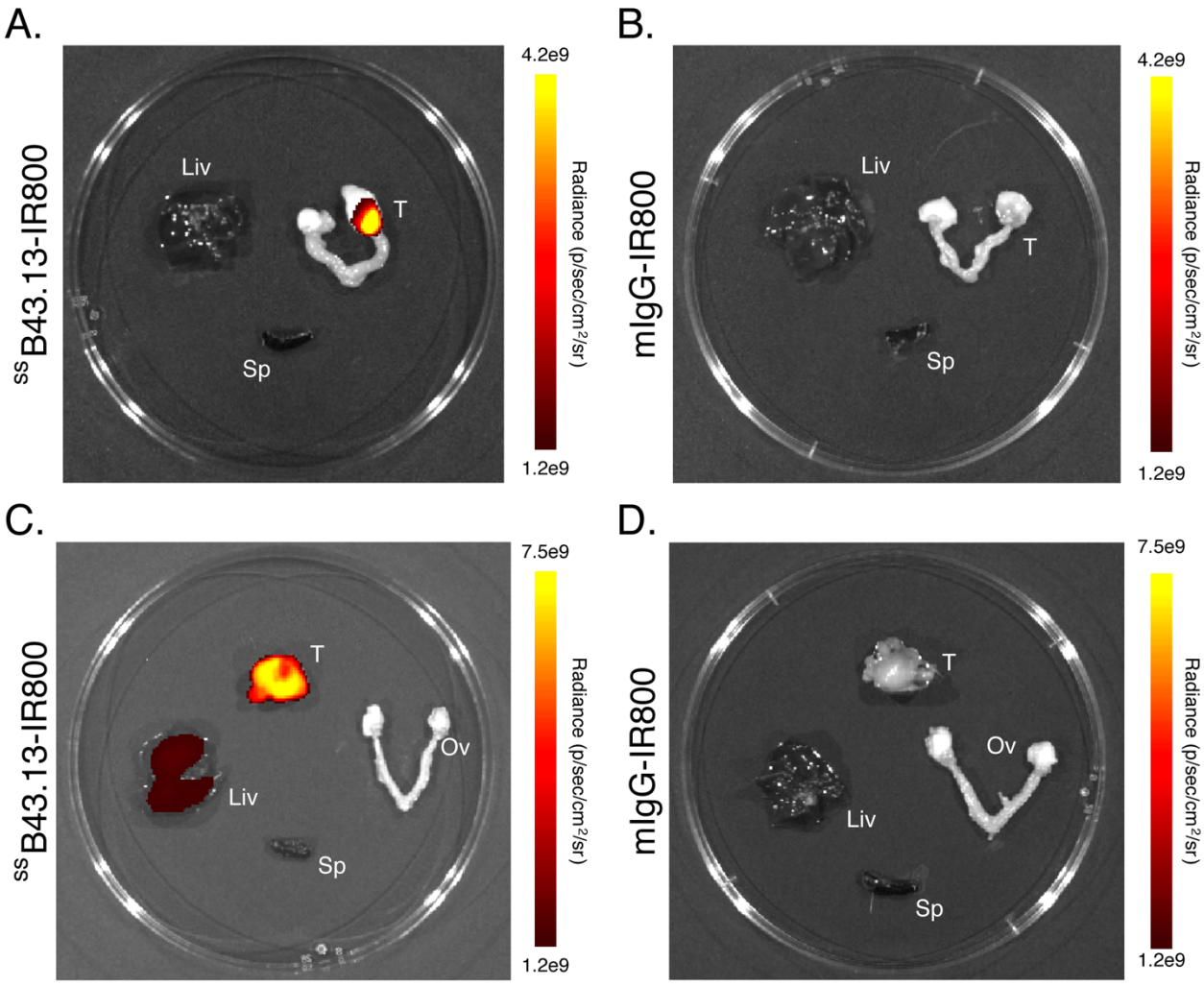

Figure S6. *Ex vivo* NIRF imaging using ^{ss}B43.13-IR800. *Ex vivo* NIRF imaging of selected organs from (A) a mouse bearing an orthotopic CA125 OVCAR3-Luc xenograft in the left ovary collected 72 h after the intravenous administration of ^{ss}B43.13-IR800 (50 µg; 0.33 nmol); (A) a mouse bearing an orthotopic CA125 OVCAR3-Luc xenograft in the left ovary collected 72 h after the intravenous administration of mIgG-IR800 (50 µg; 0.33 nmol); (C) a mouse bearing a subcutaneous patient-derived xenograft collected 72 h after the intravenous administration of ^{ss}B43.13-IR800 (50 µg; 0.33 nmol); (A) a mouse bearing a subcutaneous patient-derived xenograft collected 72 h after the intravenous administration of mIgG-IR800 (50 µg; 0.33 nmol). Liv = liver; T = tumor; Sp = spleen; Ov = ovary.

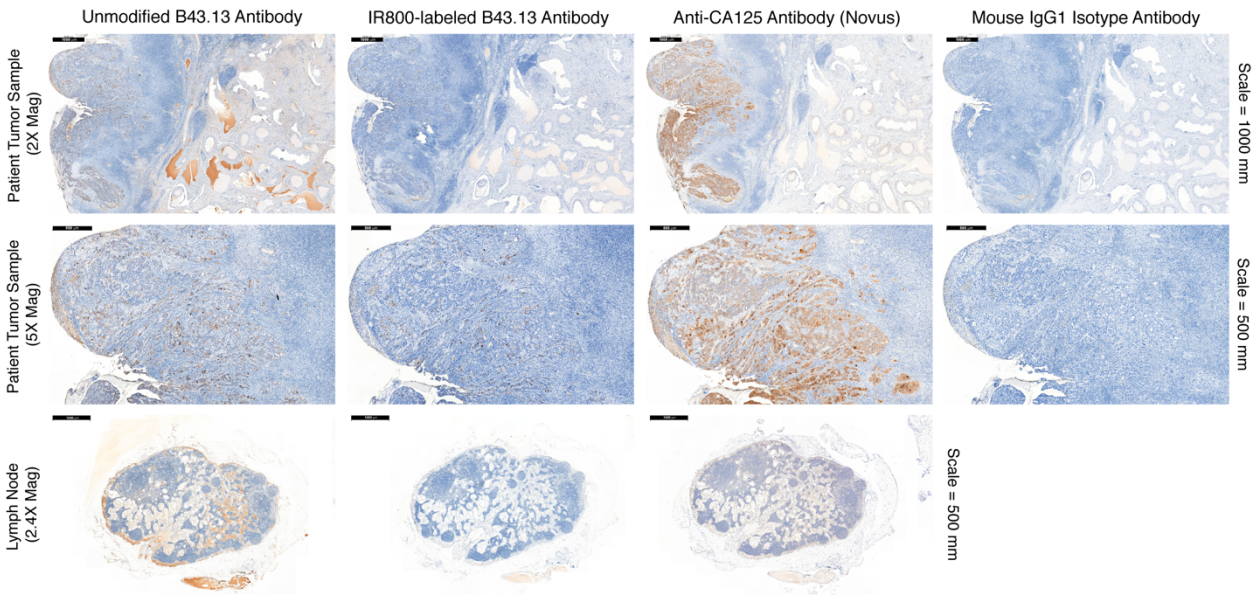

Figure S7. Histopathologic analysis of tumor and lymph node samples from a clear cell ovarian cancer patient. Immunohistochemical staining of CA125 in tumor and lymph node samples obtained from a patient with clear cell ovarian cancer who underwent surgical debulking to remove peritoneal tumor masses and associated lymph nodes at Memorial Sloan Kettering Cancer Center. The extreme right-hand side panel shows staining of the tumor sections with mouse isotype IgG1 antibody. Though the anti-CA125 antibody from Novus Biologicals (NBP1-96619) showed slight background staining (brown), a high concordance was observed in the staining patterns when equivalent dilutions of unmodified B43.13 and ^{ss}B43.13-IR800 were employed for manual immunohistochemistry on the tumor sections. These data demonstrate the lack of B43.13 binding to human tumors that do not express CA125 on the tumors or in the serum. IHC images are representative of samples from 1 clear cell carcinoma patient.