Supporting Information

Lack of *in vivo* antibody dependent cellular cytotoxicity with antibody containing gold nanoparticles

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Materials and Methods:

Synthesis of antibody-PEG conjugates:

Cetuximab, rituximab, panitumumab, and trastuzumab were obtained from Dr. Yun Yen at the City of Hope. Antibody-PEG-OPSS conjugates were prepared by reacting amine groups of antibodies with NHS-PEG-OPSS (5kDa, Nanocs) at a 5:1 PEG to antibody ratio in 0.1 M phosphate buffer, pH 7.4. The conjugates were purified by high performance liquid chromatography (HPLC) using 0.1 M phosphate buffered saline (PBS), pH 7.4, as the elution buffer. Collected fractions were analyzed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS).

Synthesis of antibody-functionalized gold nanoparticles:

50 nm gold nanoparticles (AuNPs) (Ted Pella) were surface functionalized with mPEG-SH (5 kDa, Nanocs) and antibody-PEG-OPSS conjugates by mixing 50:10:1 molar ratios of Au:mPEG:antibody-PEG in deionized water. AuNPs were stirred at room temperature for 2 hours. Surface functionalized AuNPs were centrifuged at 14,000 *g* for 10 minutes, the supernatant was removed, and AuNPs were washed with deionized water three times. The purified nanoparticles were re-suspended in deionized water or buffer solution for further characterization. The concentration of antibody-functionalized AuNPs was determined by nanoparticle tracking analysis (NTA) using a Nanosight NS500 system.

Physiochemical Characterization of AuNPs:

mPEG-AuNPs and antibody functionalized AuNPs were pelleted and were re-suspended in saline and deionized water. The hydrodynamic diameter and zeta potential (ζ) of mPEG-AuNPs and antibody functionalized AuNPs were measured using ZetaPALS (Brookhaven Instruments Corporation). Hydrodynamic diameter was also measured by nanoparticle tracking analysis (NTA) using a Nanosight NS500 system.

Quantification of Antibodies per Particle:

Fluorescent Labeling of Antibody-PEG conjugates: Dylight-650 (Pierce) labeled fluorescent antibody-PEG conjugates were prepared according to the manufacturer's protocol. Antibody coated gold nanoparticles were synthesized using the experimental conditions described above. The amount of antibody in the supernatant of AuNPs was estimated by measuring the fluorescence of the labeled antibody using an Infinite M200 microplate reader (Tecan). The fluorescence of the antibody in the supernatant was subtracted from the original fluorescence values measured in the feed amount of antibody per sample.

Ortho-Phthaldehyde Assay (OPA):

Antibody-PEG conjugates bearing a base labile linker, (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone) (BSCOES), were prepared by mixing antibody:NH_2-PEG-SH:BSCOES at molar ratios of 1:10:25, respectively, in 0.1 M PBS, pH 7.4. The conjugates were purified by HPLC. Antibody-functionalized gold nanoparticles were

prepared, as described above. The purified antibody-functionalized AuNPs were dispersed in 8 mM boric acid buffer (pH 10.8) containing 3.5% Birj-35 and were incubated at 37°C overnight. The supernatant was collected and was reacted with 0.06M OPA in the presence of 0.025M 2-mercaptoethanol, and fluorescence was measured using an excitation λ=340 nm and an emission λ=450 nm using an Infinite M200 microplate reader (Tecan). The amount of antibody on the AuNP surface was determined, using a calibration curve of antibody-PEG conjugates.

Cell Line:
H1975 (ATCC) was cultured in RPMI-1640 media (ATCC) containing 10% fetal bovine serum (FBS) in a humidified atmosphere at 37°C with 5% CO_2.

Cell Viability Assay:
H1975 cells were seeded at 5,000 cells per well in 96-well plates and were treated with antibodies and antibody-functionalized gold nanoparticles for 72 hours. H1975 cell viability was evaluated using the MTS assay (Promega).

In vitro Antibody Dependent Cell Cytotoxicity (ADCC) assay:
The H1975 cell line was unable to provide satisfactory cell lysis results even with the cetuximab antibody alone using NKL-cells (DFCI, Boston MA), an immortalized NK cell line, and the lactate dehydrogenase (LDH) assay to measure ADCC *in vitro*. Therefore, another model using the BT474M1 cell line and trastuzumab to see whether ADCC occurs with the antibody, antibody-PEG conjugates, and antibody-functionalized AuNPs was developed. In addition to the Fab fragment of trastuzumab, which does not have the Fc region to which NK cells bind, rituximab was used as a negative control.
Trastuzumab-Fab Synthesis:
Trastuzumab IgG was incubated with a papain-modified agarose gel slurry (Pierce) at 37°C for 8 hours. The enzyme to substrate ratio was 1:160 weight/weight. The digested IgG was neutralized, and free IgG and Fc segments were removed using a protein A column. The purified Fab was analyzed using sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS PAGE).
Cell Lines:
NKL-cells (DFCI, Boston MA) were cultured in the presence of IL-2 (Peprotech) and 10% heat inactivated FBS (Invitrogen). BT474M1 cells (UCSF) were maintained in RPMI-1640 media (Invitrogen) containing 10% ultra-low IgG FBS (Invitrogen) in humidified chambers at 37°C with 5% CO_2. The cells were sub-cultured twice a week and were seeded in 96-well tissue culture plates.
ADCC Assay:
The cells were treated with varying concentrations of antibodies (10-0.001 μM), Fab, antibody-PEG conjugates, and antibody-conjugated gold nanoparticles at a 10:1 ratio of NKL to BT474M1 cells. ADCC was evaluated 3 hours post-treatment using a lactate dehydrogenase (LDH) (Roche) assay according to the manufacturer's protocol.

Because the same chemistry was used for antibody-PEG coupling and AuNP synthesis, the same phenomenon observed with trastuzumab and trastuzumab-AuNPs on the BT474M1 cell line should hold true for cetuximab and cetuximab-AuNPs on the H1975 cell line.

Xenograft Experiments:

All mouse experiments were approved by the California Institute of Technology Institutional Animal Care and Use Committee. $1x10^7$ H1975 cells, suspended in 0.2 mL of serum-free RPMI-1640 media, were injected subcutaneously into the left rear flank of each 8-week old athymic NCr nude mouse (Taconic Biosciences, Inc). Xenograft tumor size was monitored daily or every other day using electronic calipers starting 7 days post-implantation. Tumor volume was calculated using the equation $V = W^2 \times L / 2$, where W is the shortest tumor dimension and L is the longest tumor dimension. When tumors reached 200 mm^3, mice were divided into groups of 7 mice and treated via tail vein IV injection with either saline, cetuximab, or panitumumab at a dose of 9.34 mg/kg, or saline, cetuximab-AuNP, panitumumab-AuNP, rituximab-AuNP, or mPEG-AuNP at a dose of 2.25×10^{12} nanoparticles/25 g mouse, which corresponds to 0.4mg/kg of antibody per mouse. Treatment was repeated 3 times, for a total of 4 doses over 2 weeks. Mice were followed until tumors reached 1500 mm^3 or for 2 months.

Immunohistochemistry (IHC):

Mice were euthanized by CO_2 asphyxiation 24 hours post 2nd injection. Tumors were embedded in Tissue-Tek optimal cutting temperature compound (Sakura) and were frozen at -80^{o}C. Tissue blocks were sectioned (14 μm), and were lightly fixed in 10% buffered formalin for 15 minutes. IHC analysis was performed for immune cell detection in tumor sections. CD45 (BD Biosciences), and CD11b (Biolegend) antibodies were used at 1:100 dilutions. Tissues were imaged using a Zeiss LSM 510 META confocal laser scanning microscope (CLSM). Gold nanoparticles were visualized in tissue sections using a silver enhancement kit (Ted Pella) and imaged with an Olympus IX50 light microscope.

Sample	# of Antibodies by Fluorescent labeling	# of Antibodies by OPA assay
Cetuximab-AuNPs	21±7	16±4
Panitumumab-AuNPs	22±4	18±6
Rituximab-AuNPs	17±3	

Table S1: Quantification of antibodies on AuNPs surface using two different methods.

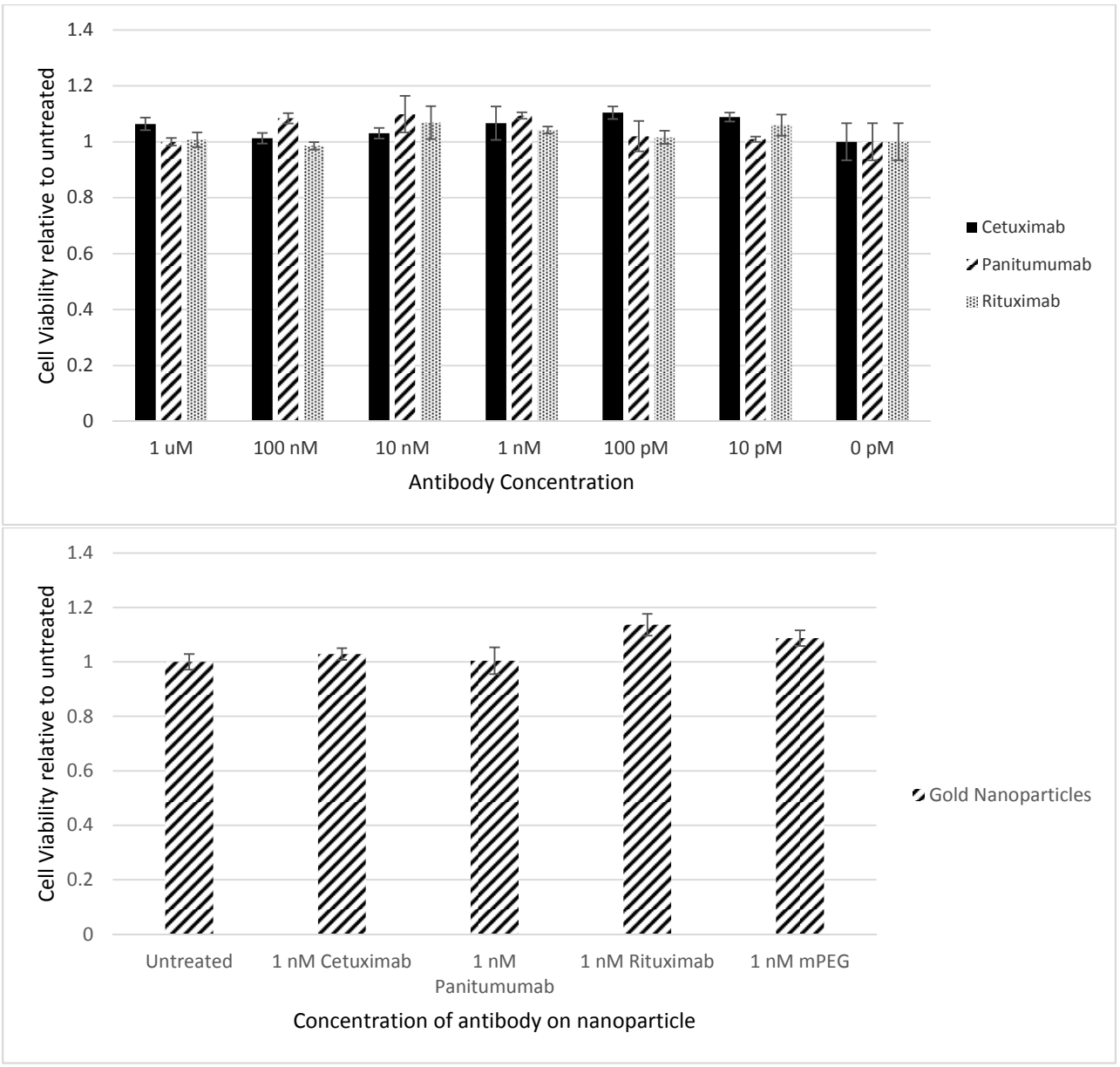


Figure S1: Cell viabilities of H1975 cells 72 hours post-incubation with antibodies (top) and antibody-functionalized AuNPs (bottom), as determined by MTS assay. Results reported in terms of average absorbance compared to untreated cells, with error bars denoting the standard error of 5 replicates.

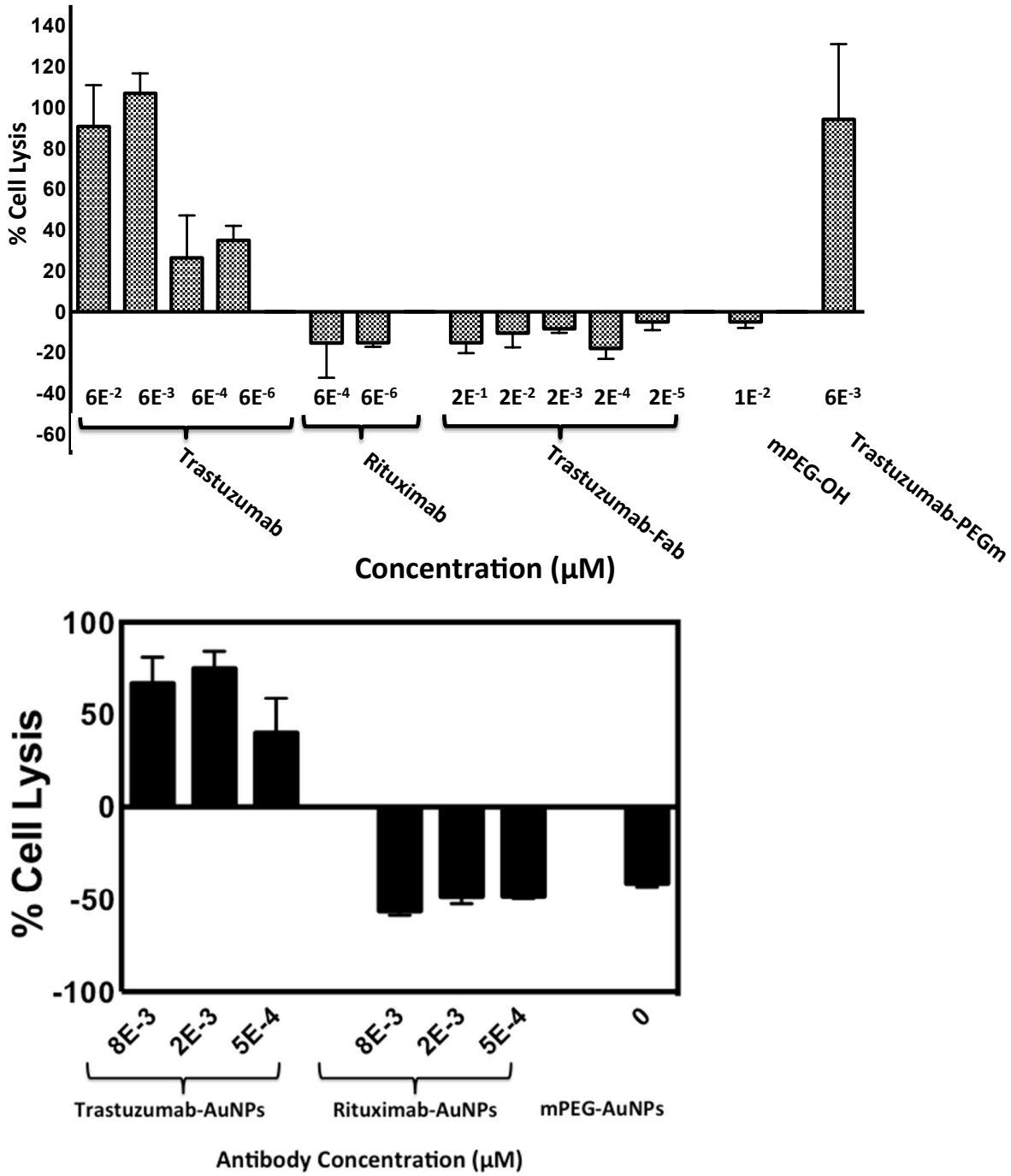
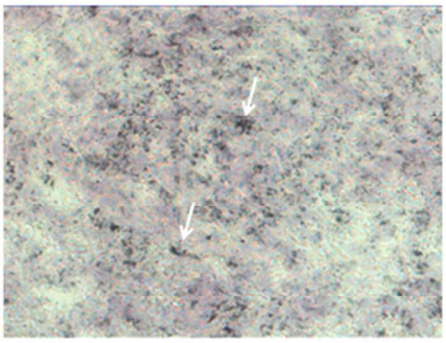
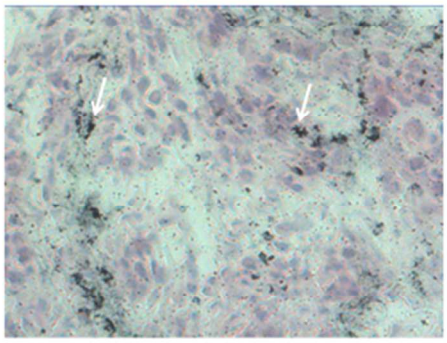


Figure S2: ADCC in the BT474M1 cell line occurs with trastuzumab and trastuzumab-PEG but not trastuzumab-Fab or rituximab (top); and with trastuzumab AuNPs but not rituximab- or mPEG-AuNPs (bottom) as determined by the LDH assay.

Tumor

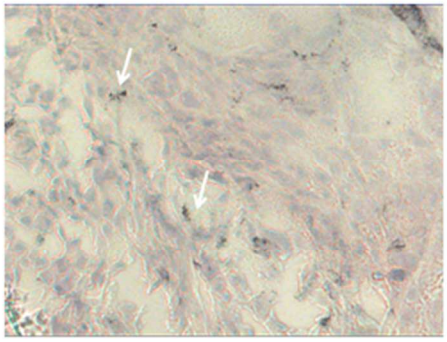


Cetuximab-gold nanoparticle

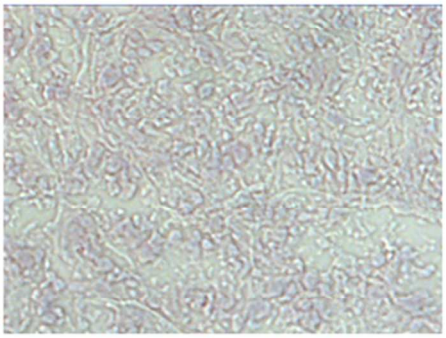


Panitumumab-gold nanoparticle

Rituximab-gold nanoparticle

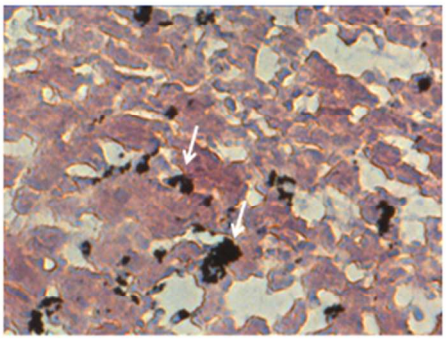


mPEG-gold nanoparticle

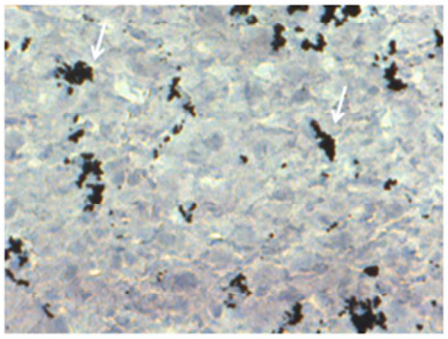


Saline

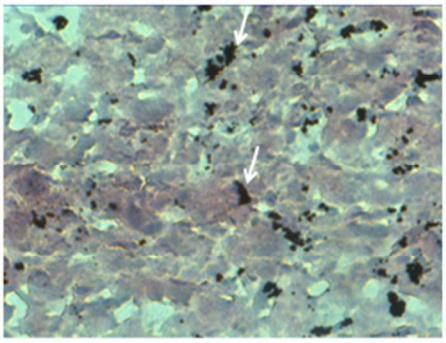
Liver



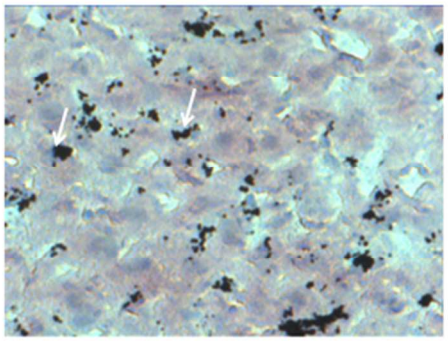
Cetuximab-gold nanoparticle



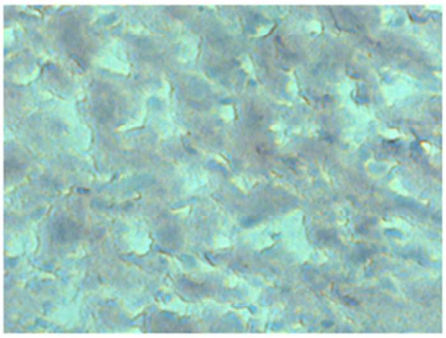
Panitumumab-gold nanoparticle



Rituximab-gold nanoparticle

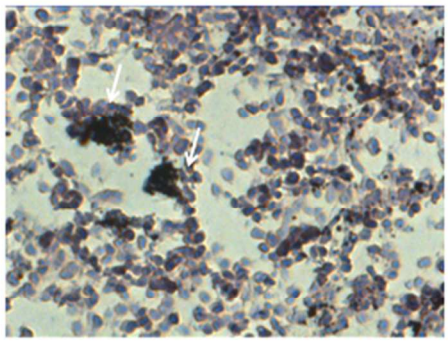


mPEG-gold nanoparticle

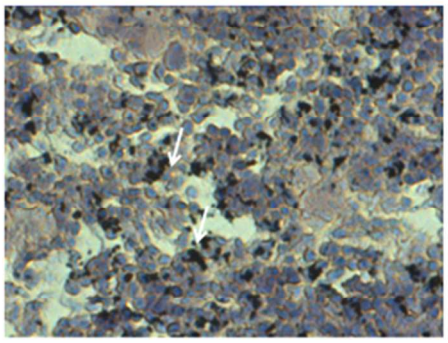


Saline

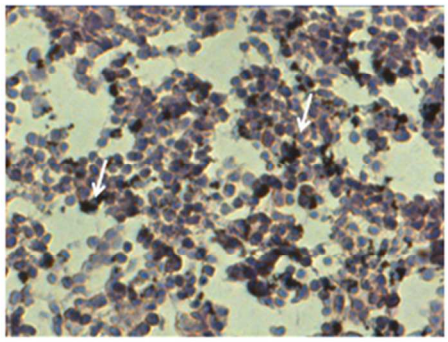
Spleen



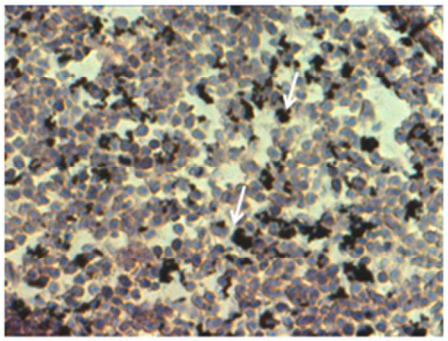
Cetuximab-gold nanoparticle



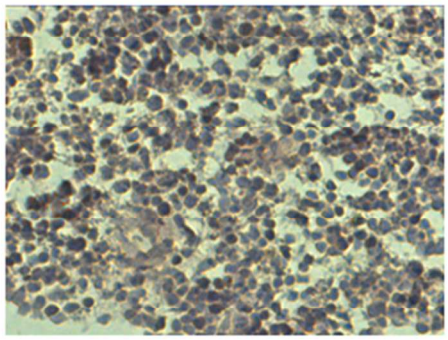
Panitumumab-gold nanoparticle



Rituximab-gold nanoparticle



mPEG-gold nanoparticle



Saline

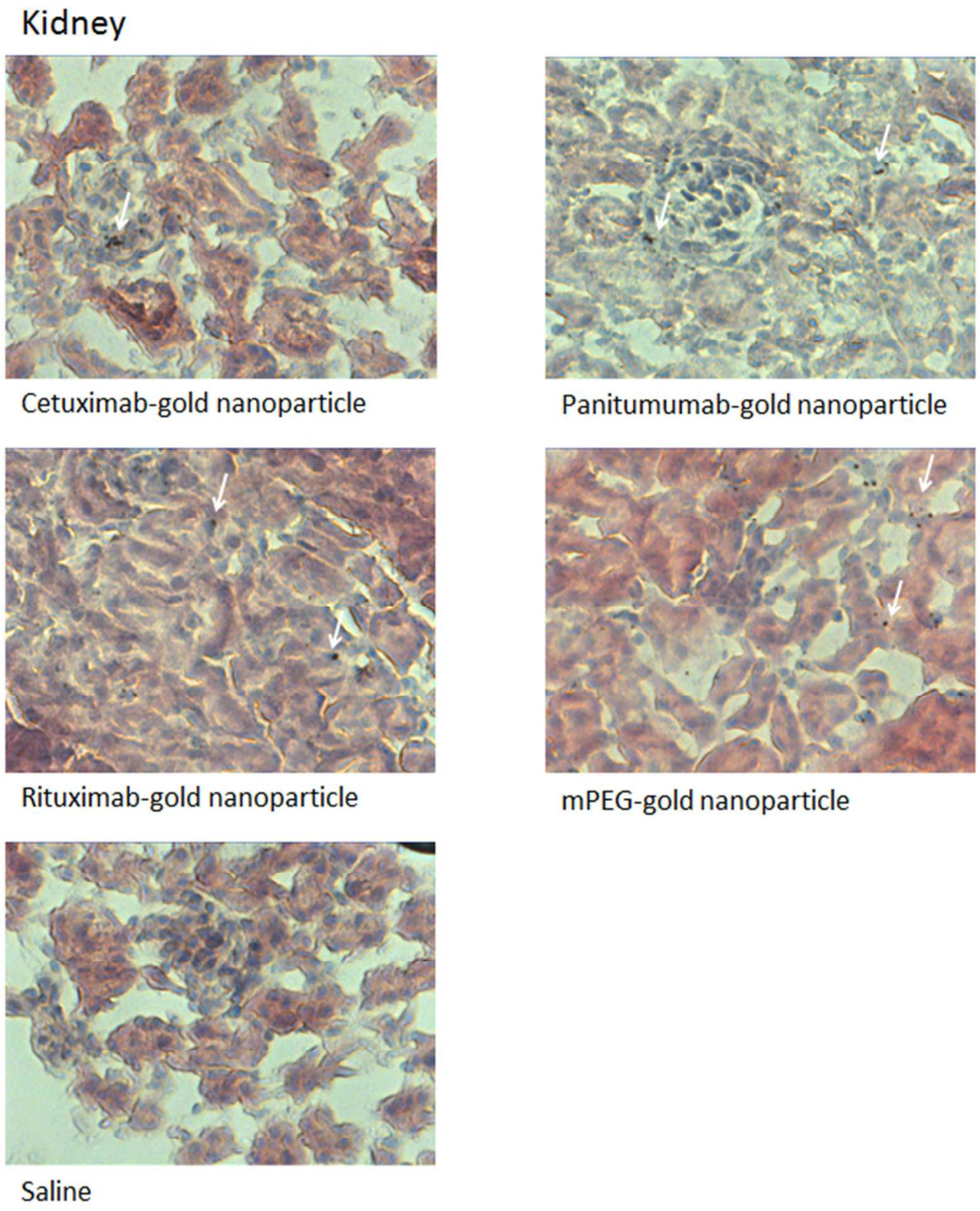


Figure S3: Silver enhancement of gold nanoparticles in frozen tissue sections of tumor (pg. S7), liver (pg. S8), spleen (pg. S9), and kidney (pg. S10). The black patches denoted by the white arrows indicate the presence of gold nanoparticles in the tissue sections, which are absent from tissues of saline-treated mice.

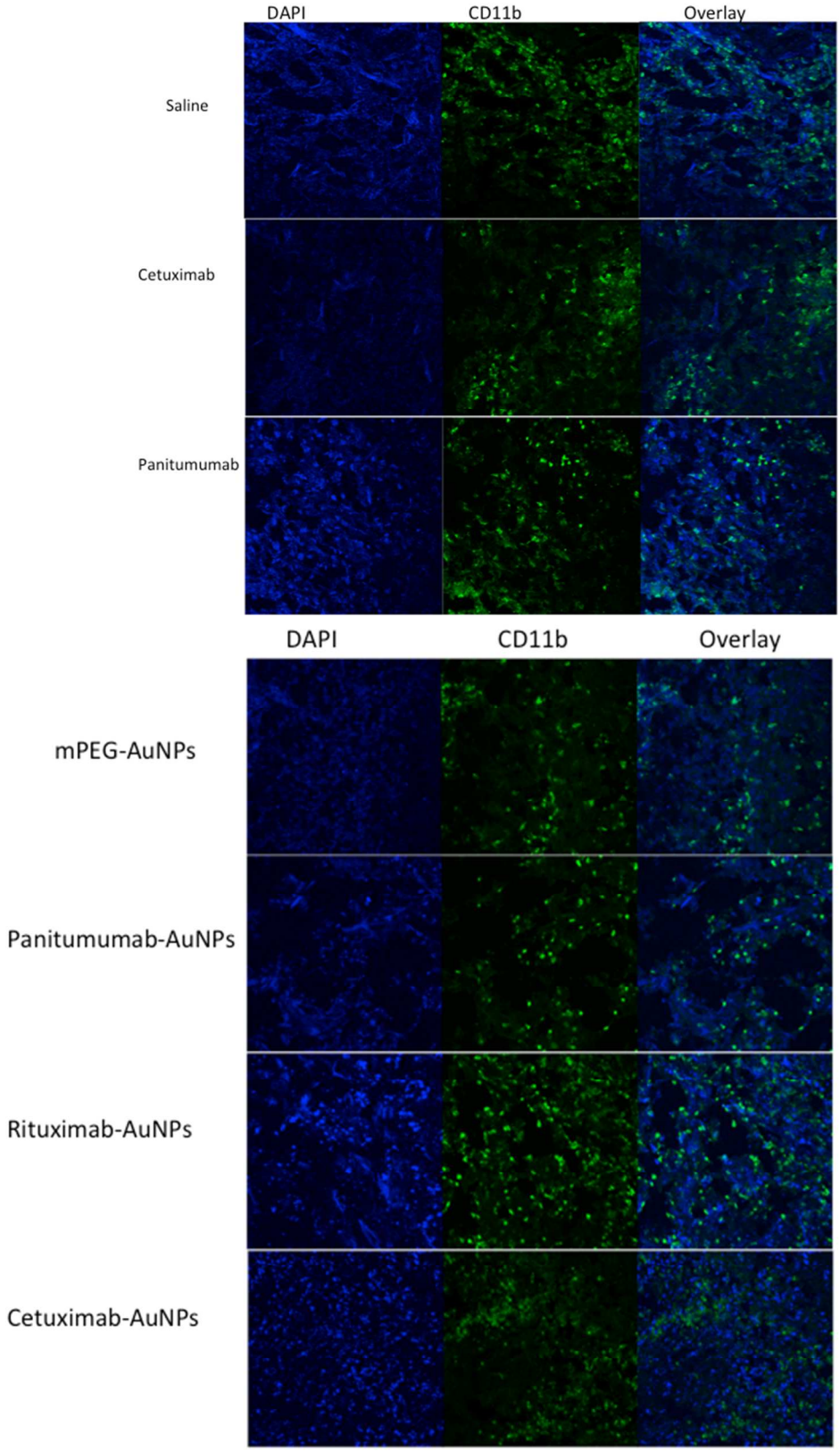
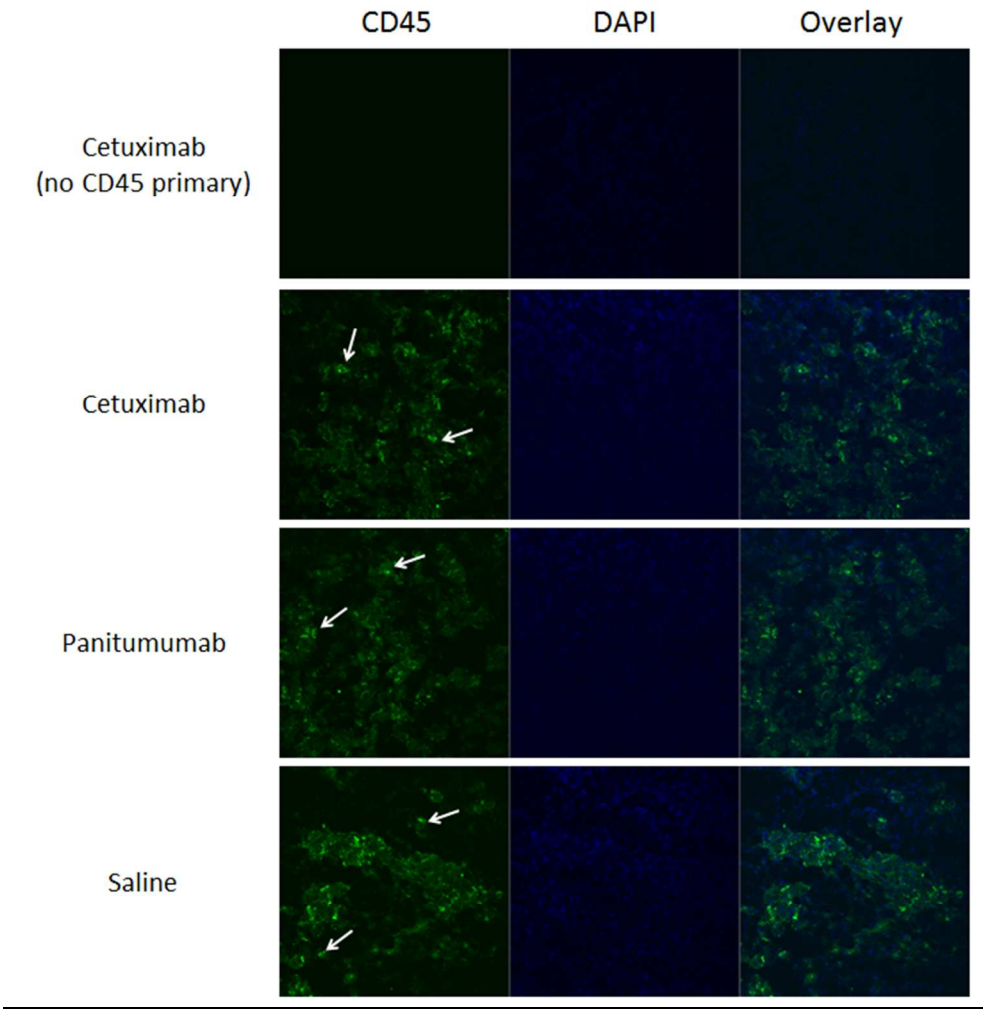


Figure S4: CD11b staining of NK cells in tumor tissues. No difference in NK cells present in the tumors can be seen between the various treatment groups.


CD45
DAPI
Overlay
Cetuximab
(no CD45 primary)
Cetuximab
Panitumumab
Saline

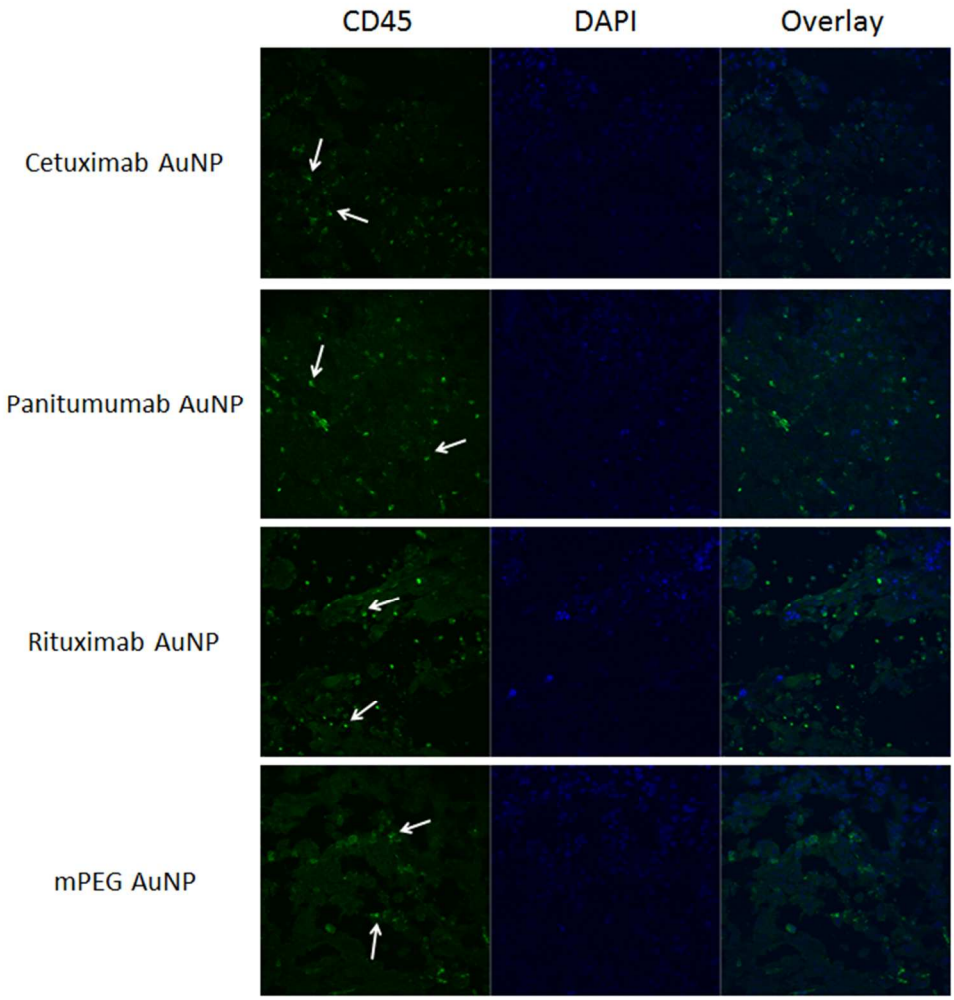


Figure S5: CD45 staining of frozen tumor sections as an indication of total immune cell infiltration present in tumors. Antibody (pg. S12) and antibody-functionalized gold nanoparticle (pg. S13) treated mice are shown. No difference can be seen between the various treatment groups.