Supplementary Information

Universal Endogenous Antibody Recruiting Nanobodies capable of Triggering Immune Effector for Targeted Cancer Immunotherapy

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General Information

Materials. Gene and primers were synthesized in General Biosystems (Chuzhou, China). The host strain *E. coli* BL21 (DE3) and vector pET22b were purchased from Novagen (Madison, USA). Restriction enzymes, HRP-conjugated goat anti-mouse IgE antibodies and Dylight488-conjugated goat anti-mouse IgG antibodies were purchased from Thermo Fisher Scientific (Waltham, USA). Mouse anti-His6 tag antibodies was from Proteintech (Chicago, USA). Dulbecco's modified eagle medium (DMEM), roswell park memorial institute medium (1640), fetal bovine serum (FBS), penicillin-streptomycin solution and HiPrep desalting column were from GE Healthcare (Chicago, USA). The recombinant human EGFR was from Genscript (Nanjing, China). Rabbit total complement was from Sigma-Aldrich (Santa Clara, USA). The pooled human serum was from CellChip (Beijing, China). The human total IgGs, rabbit total IgGs, mouse total IgGs, HRP-conjugated goat anti-human IgG antibodies, HRP-conjugated goat anti-rabbit IgG antibodies, HRP-conjugated goat anti-mouse IgG antibodies, DiO dye, DiI dye, BCA kit, TMB kit, LDH cytotoxicity assay kit and CCK8 assay kit were all purchased from Beyotime (Shanghai, China). Ni-NTA agarose was from Yeasen (Shanghai, China). All other materials and reagents were purchased commercially.

Cell Lines. The human TNBC cell line MDA-MB-468 (EGFR high expression), human squamous carcinoma cell line A431 (EGFR high expression), and human breast cancer cell line MCF7 (EGFR low expression) were kindly provided by Professor Feng Guan (Northwest University, China). All the above lines were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37^{o}C in a 5% CO_2 humidified atmosphere. The human monocyte cell line THP-1 was maintained in 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 0.05 mmol/L 2-mercaptoethanol at 37^{o}C in a 5% CO_2 humidified atmosphere.

Mice. Balb/c mice (female, 6-8 weeks-old), Balb/c nude mice (female, 4-6 weeks-old) and C57BL/6J mice (female, 6-8 weeks-old) were purchased from SLAC (Shanghai, China). All animal experiments were performed according to the guidelines and

protocols approved by the Institutional Animal Care and Use Committee of the Jiangnan University (JN. Nos. 20190315b0650625 and 20201130c0650220).

Plasmids Construction. The commercially constructed plasmids pET28a-7D12 and pET28a-ZZ were applied as the PCR templates. To generate 7D12-ZZ, 7D12 gene was amplified by primers 7D12-ZZ-F1 (5'-3': GGA ATT CCA TAT GCA GGT GAA ACT GGA AGA AAG TGG T) and 7D12-ZZ-R1 (5'-3': GCT GCC GCC GCC GCC GCT ACC ACC ACC ACC GGC TGC GCC GTT CAG ATC), ZZ gene was amplified by primers 7D12-ZZ-F2 (5'-3': GGT GGT GGT GGT AGC GGC GGC GGC GGC AGC GCC CAG CAT GAT GAA GCC) and 7D12-ZZ-R2 (5'-3': CCG CTC GAG TCA GTG GTG GTG ATG ATG ATG AC). After a subsequent fusion PCR step, the gel-purified 7D12-ZZ fragment was cloned into vector pET22b. The resultant plasmid pET22b-7D12-ZZ was transformed into *E. coli* BL21(DE3) and confirmed by sequencing. To generate ZZ-7D2, ZZ gene was amplified with primers ZZ-7D12-F1 (5'-3': GGA ATT CCA TAT GGC CCA GCA TGA TGA AGC C) and ZZ-7D12-R1 (5'-3': GCT GCC GCC GCC GCC GCT ACC ACC ACC ACC GCT GCT GCT ATT GGC ATC C), 7D12 gene was amplified with primers ZZ-7D12-F2 (5'-3': GGT GGT GGT GGT AGC GGC GGC GGC GGC AGC CAG GTG AAA CTG GAA GAA AGT GG) and ZZ-7D12-R2 (5'-3': CCG CTC GAG TCA GTG GTG GTG ATG ATG ATG AC). After a subsequent fusion PCR step, the gel-purified ZZ-7D12 fragment was cloned into vector pET22b. The resultant plasmid pET22b-ZZ-7D12 was transformed into *E. coli* BL21(DE3) and confirmed by sequencing.

Protein Expression and Purification. A positive clone was propagated in Luria–Bertani (LB) medium containing 100 μg/mL kanamycin (for 7D12 and ZZ domain expression) or ampicillin (for 7D12-ZZ and ZZ-7D12 expression) at 37°C overnight with constant shaking at 200 rpm. The overnight seed culture was diluted (1:50) in 100 mL of Terrific-Broth (TB) medium and incubated at 37°C until the OD_{600} reached 0.6-0.8, following which recombinant proteins were induced with 0.5 mmol/L isopropyl-b-D-thiogalactopyranoside. After another incubation period at 16°C for 24 h, cell pellets were harvested by centrifugation and disrupted on ice by sonication. The collected lysate supernatant was loaded onto a gravity-flow column and

incubated with Ni-NTA agarose for 2 h at 4°C. After a washing step using imidazole (10–40 mmol/L), the His6-tagged proteins were eluted from the agarose with 250 mmol/L imidazole and desalted on ÄKTA avant system. The concentration of proteins was determined by a BCA kit.

Enzyme-Linked Immunosorbent Assay (ELISA). The high binding 96-well plates were coated with 100 μL of protein samples (0.025 μmol/L) at 4°C for 12 h and followed by 37°C for 1 h. After washing with PBST and blocking with 3% BSA, wells were successively treated with 100 μL of diluted IgGs and 100 μL of HRP-conjugated secondary antibodies at 37°C for 1 h. The results were determined by using a TMB kit.

Competitive ELISA for Binding Affinity (IC50) Assay. A431 (EGFR positive) were seeded in 96-well plates at a final concentration of 3×10^3 cells/well. After a washing step to remove old medium, the cells were fixed with 4% paraformaldehyde for 10 min. Next, the wells were blocked with 3% BSA at 25°C for 2 h. With a subsequent washing step, 10 μL of 10% BSA was added, followed by 90 μL of pre-mixed (at 25°C for 1 h) solution containing 5 nmol/L nanobody samples and increasing concentrations (ranging from 0.016 to 500 nmol/L) of recombinant human EGFR. After around 10 min incubation, the wells were rinsed with PBST and then successively treated with anti-His6 tag antibodies and HRP-modified goat anti-mouse IgG antibodies at 37°C for 1 h. The results were finally determined using a TMB kit. The binding rate was calculated using the following equation:

$$\%\ \text{Binding rate} = \left(\frac{\text{A (experimental)} - \text{A}(negative)}{\text{A (maximum)} - A\ (negative)}\right) \times 100$$

Where A(experimental) is the OD_{450} value of wells treated with nanobody samples in the presence of recombinant human EGFR; A(maximun) is the OD_{450} value of wells treated with 5 nmol/L nanobody samples in the absence of recombinant human EGFR; A(negative) is the OD_{450} value of wells treated with 0 nmol/L of nanobody samples.

Immunofluorescence: Cells seeded on sterile coverslips in 24-well plates were fixed with 4% paraformaldehyde for 10 min, and blocked using 1% BSA (in PBS) for 1 h.

Then, cells were treated with 50 nmol/L protein samples (in 1% BSA) in the presence of mouse total IgGs at 37°C for 1 h, and labeled with 4 μg/mL Dylight488-conjugated anti-mouse IgG antibodies (in 1% BSA) for another 1 h. After permeabilization with 0.1% TritonX-100 (in 1% BSA), cells were treated with a drop of antifade mounting medium (containing 4',6-diamidino-2-phenylindole (DAPI)) and finally imaged using a confocal microscope.

Flow Cytometry. To a tube containing 100 μL of protein samples (or PBS as the negative control), 4×10^4 of cells in flow cytometry buffer (2% BSA in PBS) were added, immediately followed by mouse total IgGs. After the incubation period on ice for 30 min and a washing step, cells were treated with 10 μg/mL Dylight488-conjugated anti-mouse IgG antibodies for 30 min on ice. After another washing step, cells were resuspended in flow cytometry buffer and detected using Accuri C6 Flow Cytometer. The results were analyzed on FlowJo software.

ADCC Assay. Target cells were seeded in 96-well plates at a concentration of 4×10^3 cells/well. Then, 50 μL of antibody samples (or PBS as the negative control) and 50 μL of 2% mouse serum were added, and incubated on 37°C for 1 h. After a washing step, cells were treated with 8×10^4 freshly isolated PBMCs at 37°C for 4 h. The cell maximum killing was achieved by adding Triton X-100 to a final concentration of 1%. Cytotoxicity was then detected using an LDH cytotoxicity kit. Spontaneous LDH release by PBMC were also detected. Cell lysis was calculated using the following equation:

$$\%\ \text{ADCC} = \left(\frac{A\ (\text{experimental}) - A\ (\text{spontaneous})}{A\ (\text{maximum})}\right) \times 100$$

Where A(experimental) is the OD_{490} value of LDH released by cells treated with protein samples in the presence of mouse serum and PBMC; A(spontaneous) is the OD_{490} value of LDH spontaneously released by PBMC; A(maximum) is the OD_{490} value of cells completely lysed with 1% Triton X-100.

CDC Assay. Target cells were seeded in 96-well plates at a concentration of 4×10^3 cells/well. Then, 50 μL of 100 nmol/L antibody samples (or PBS as the negative

control) and 50 μL of 2% mouse serum were added, and incubated on 37°C for 1 h. After a washing step, cells were treated with 100 μL of 1% RC for another 4 h (or 1% HIRC as the complement control). The cell maximum killing was achieved by adding Triton X-100 to a final concentration of 1%. The cell viability was measured using a CCK8 assay kit, and cell lysis was calculated using the following equation:

$$\% \text{ CDC} = \left(1 - \frac{\text{A (experimental)} - \text{A (maximum)}}{\text{A (negative)} - \text{A (maximum)}}\right) \times 100$$

Where A(experimental) is the OD_{450} value of cells treated with protein samples in the presence of MS and complement; A(maximum) is the OD_{450} value of cells completely lysed with 1% Triton X-100. A(negative) is the OD_{450} value of cells treated with PBS.

ADCP Assay. DiO-prestained target cells (green) were seeded in 24-well plates at a concentration of 3×10^4 cells/well, and then treated with 100 μL of 50 nmol/L antibody samples (or PBS as the negative control) in the presence of 10% pooled human serum. After 1 h of incubation at 37°C, target cells were washed with PBS to remove excess antibodies and co-cultured with 1.5×10^5 DiI-prestained THP-1 cells (orange red) for another 6 h. Subsequently, the medium containing THP-1 cells were collected in the tubes, and the remaining target cells were detached with trypsin and added to the same tubes. After another washing step to remove trypsin, cells were collected for further confocal fluorescence and flow cytometry assays.

For confocal fluorescence assay, the collected cells were seeded on sterile coverslips in 24-well plates by centrifugation. After fixing with 4% paraformaldehyde, cells were blocked with 1% BSA. Thereafter, cells were treated with a drop of antifade mounting medium (containing DAPI) and imaged by confocal microscope.

For flow cytometry assay, the collected cells were resuspended in flow cytometry buffer and detected using Accuri C6 Flow Cytometer. The cell phagocytosis was calculated using the following equation:

$$\% \text{ ADCP} = \left(\frac{R1}{R1 + R2}\right) \times 100$$

Where R1 is the number of double positive cells; R2 is the number of remaining target cells.

***In vivo* Pharmacokinetics Assay.** UEAR Nbs 7D12-ZZ and ZZ-7D12 (5 μmol/L, 100 μL) were i.v. injected into the tail vein of a group of 3 Balb/c mice, respectively. Blood samples collected at different time points were analyzed using ELISA. As for the standard curves, high binding 96-well plates were coated with recombinant human EGFR (5 μg/mL) at 4^{o}C overnight and then at 37^{o}C for 1 h. After blocking with 3% BSA, the plates were successively treated with different concentrations (0.2-10 nmol/L) of UEAR Nbs and HRP-conjugated anti-mouse IgG antibodies. The results were analyzed using a TMB kit. As for pharmacokinetics assay, after blocking step, the plates were successively incubated with diluted blood samples (1:100 in PBS) and HRP-conjugated anti-mouse IgG antibodies. The plasma concentration of UEAR Nbs were finally calculated as the standard curves.

***In vivo* Antitumor Evaluation.** Balb/c nude mice retain functional NK cells, macrophages and complement system, which make them capable of evoking some anti-tumor mechanisms including ADCC, CDC, etc.[2-5] Thus, Balb/c nude mice is a suitable model for studying cancer immunotherapy in this study. To evaluate the *in vivo* antitumor efficacy of UEAR Nbs, Balb/c nude mice were injected subcutaneously on the right flanks with 2×10^6 MDA-MB-468 cells. Then the mice with 40-80 mm^3 of tumor were randomly divided into four groups. PBS group treated by i.p. injection with 50 μL of PBS and 50 μL of pooled normal mouse serum, 7D12 group treated by i.p. injection with 50 μL of 7D12 (30 μmol/L in PBS) and 50 μL of pooled normal mouse serum, 7D12-ZZ group treated by i.p. injection with 50 μL of 7D12-ZZ (30 μmol/L in PBS) and 50 μL of pooled normal mouse serum, ZZ-7D12 group treated by i.p. injection with 50 μL of ZZ-7D12 (30 μmol/L in PBS) and 50 μL of pooled normal mouse serum. Treatments were given every two days in 10 days. Tumors were detected every two days by caliper using the formula: $1/2\times length\times width^2$.

Histological Analysis. The kidney, liver, spleen and lung tissues from sacrificed mice were immediately fixed with 10% formalin. After a washing step using 70% ethanol,

the samples were embedded in paraffin and cut into 4-6 μm thick sections. Then, hematoxylin and eosin (H&E) staining was performed and samples were imaged under an Olympus upright microscope.

Anaphylactic Shock Analysis. To evaluate the anaphylactic shock risk of UEAR Nbs, C57BL/6J mice were i.p. injected with 100 μL of 7D12-ZZ or ZZ-7D12 (1 mg/mL in PBS). Treatments were given at day 0, followed by day 7 to 15 with a frequency of every two days. In addtion, serum samples at day 0 and 15 were collected for the eveluation of nanobody-specific IgE level by using ELISA. The high binding 96-well plates were coated with 100 μL of nanobody 7D12 (5 μg/mL) at 4^{o}C for 12 h, followed by 37^{o}C for 1 h. After washing with PBST and blocking with 3% BSA, wells were treated with 100 μL of diluted mouse serum samples (1: 300 to 24300 in PBS) at 37^{o}C for 2 h. Thereafter, wells were incubated with 100 μL of HRP-conjugated goat anti-mouse IgE antibodies at 37^{o}C for another 1 h. The results were finally determined using a TMB kit.

Statistical Analysis. Statistical analysis was conducted on GraphPad Prism 6.0 software. A *P* value of <0.05 was regarded as statistically significant. Number of asterisks in the figures indicates the level of statistical significance as follows: * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$.

Supplementary Figures

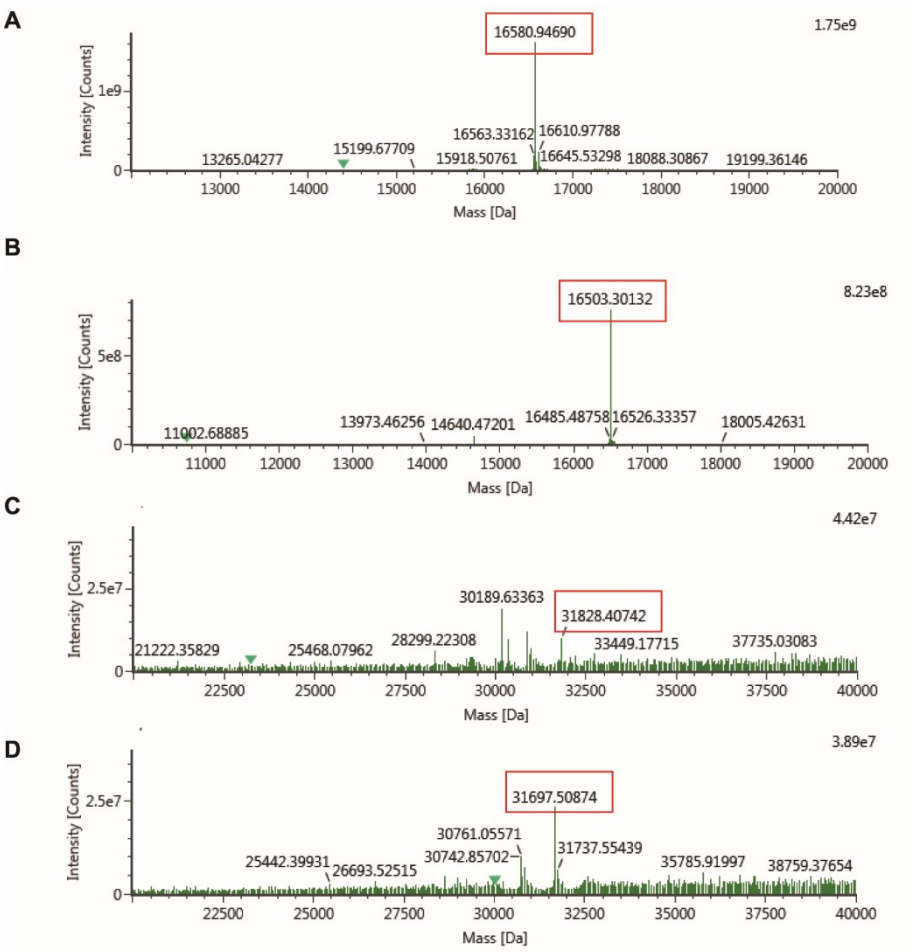

Fig. S1. Mass spectrums (MS) of 7D12, ZZ domain, 7D12-ZZ and ZZ-7D12. (**A**) MS of 7D12[1], observed: 16580.9 Da; calculated MW: 16581.1 Da. (**B**) MS of ZZ domain, observed: 16503.3 Da; calculated MW: 16503.9 Da. (**C**) MS of 7D12-ZZ (with the N-terminus Methionine residue), observed: 31828.4 Da; calculated MW: 31828.7 Da. (**D**) MS of ZZ-7D12, observed: 31697.5 Da; calculated MW: 31697.5 Da.

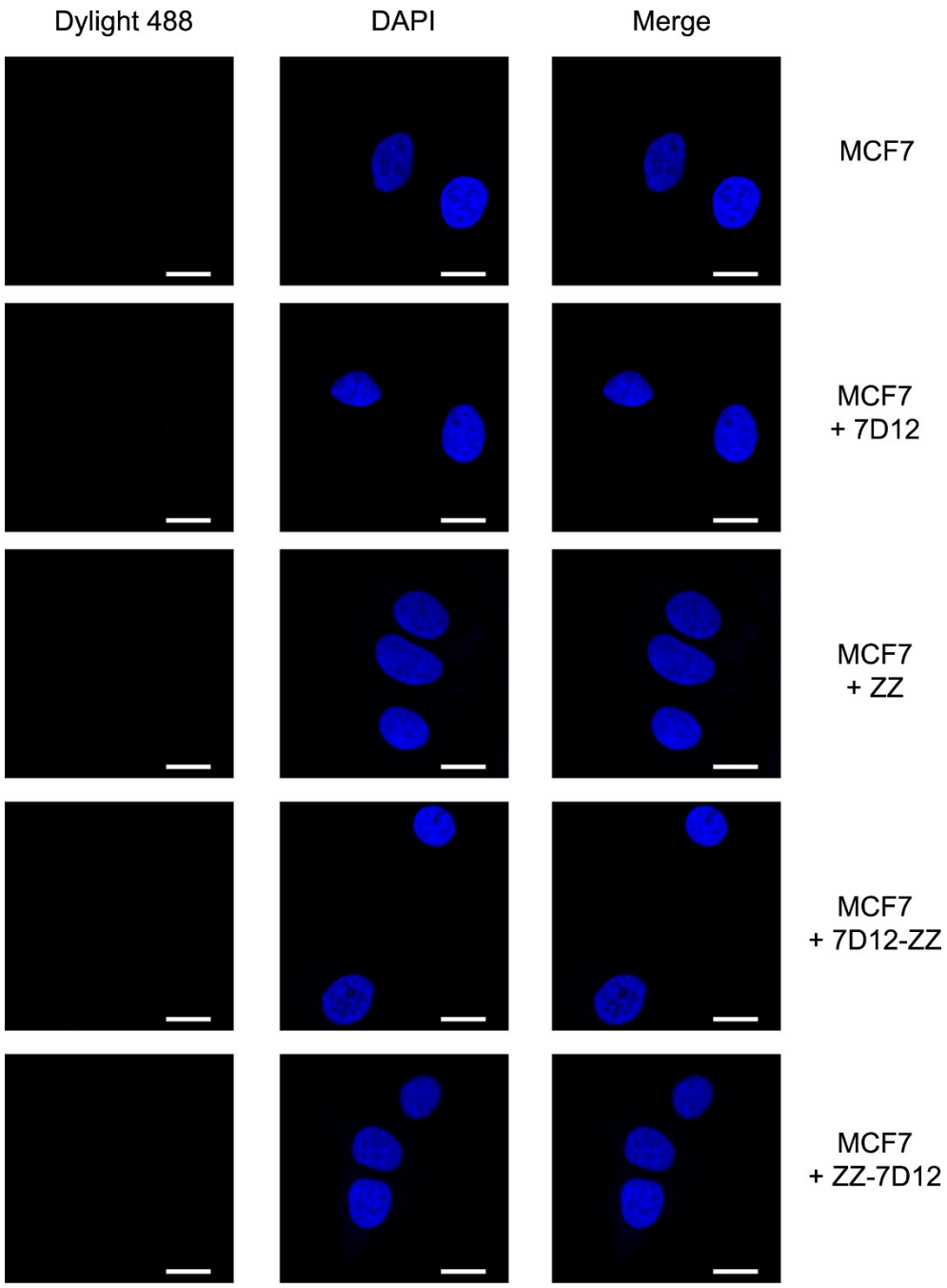

Fig. S2. Immunofluorescence of MCF7 cells treated with nanobody 7D12, ZZ domain, 7D12-ZZ or ZZ-7D12. Scale Bar: 20 μm.

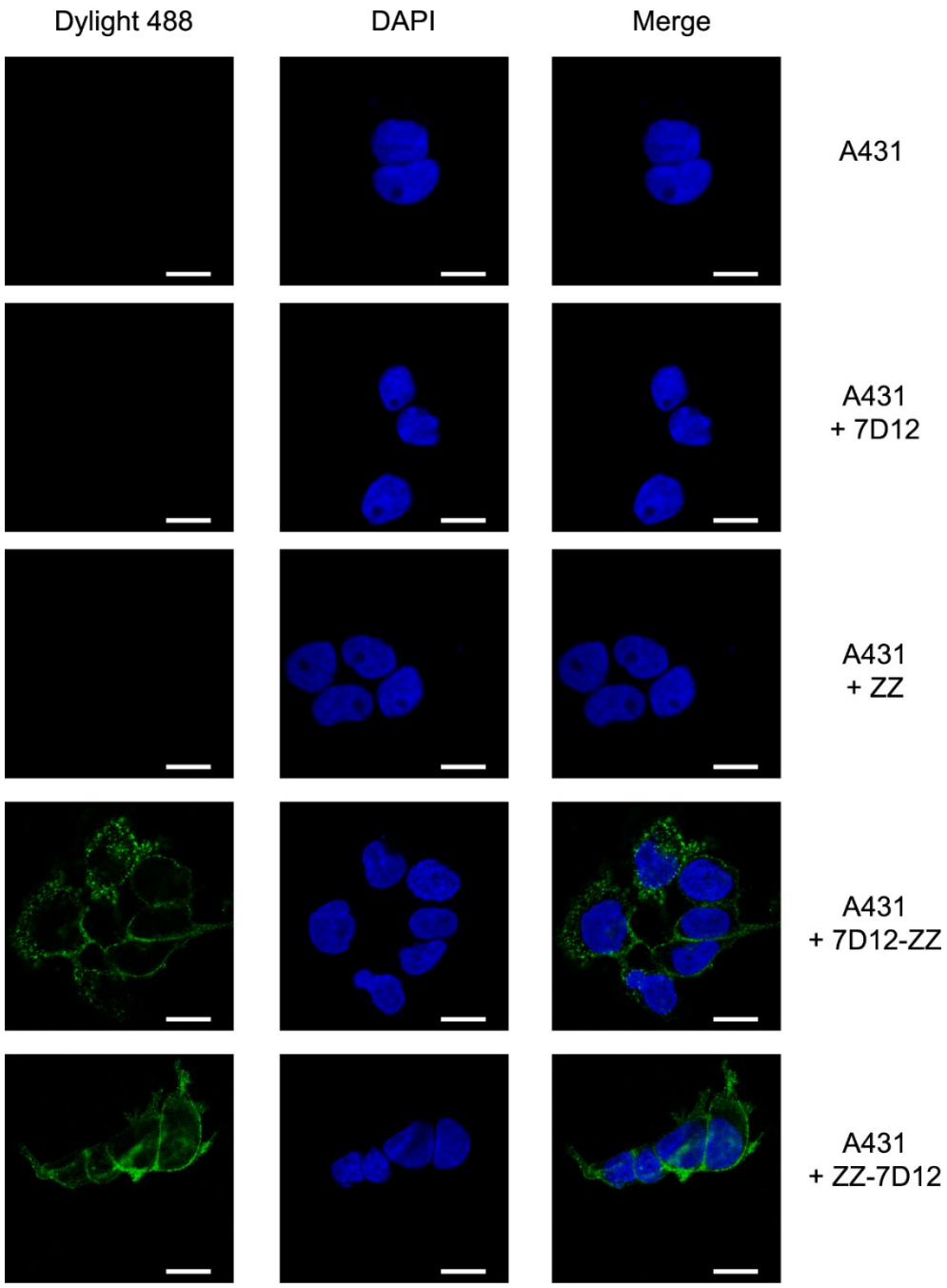

Fig. S3. Immunofluorescence of A431 cells treated with nanobody 7D12, ZZ domain, 7D12-ZZ or ZZ-7D12. Scale Bar: 20 μm.

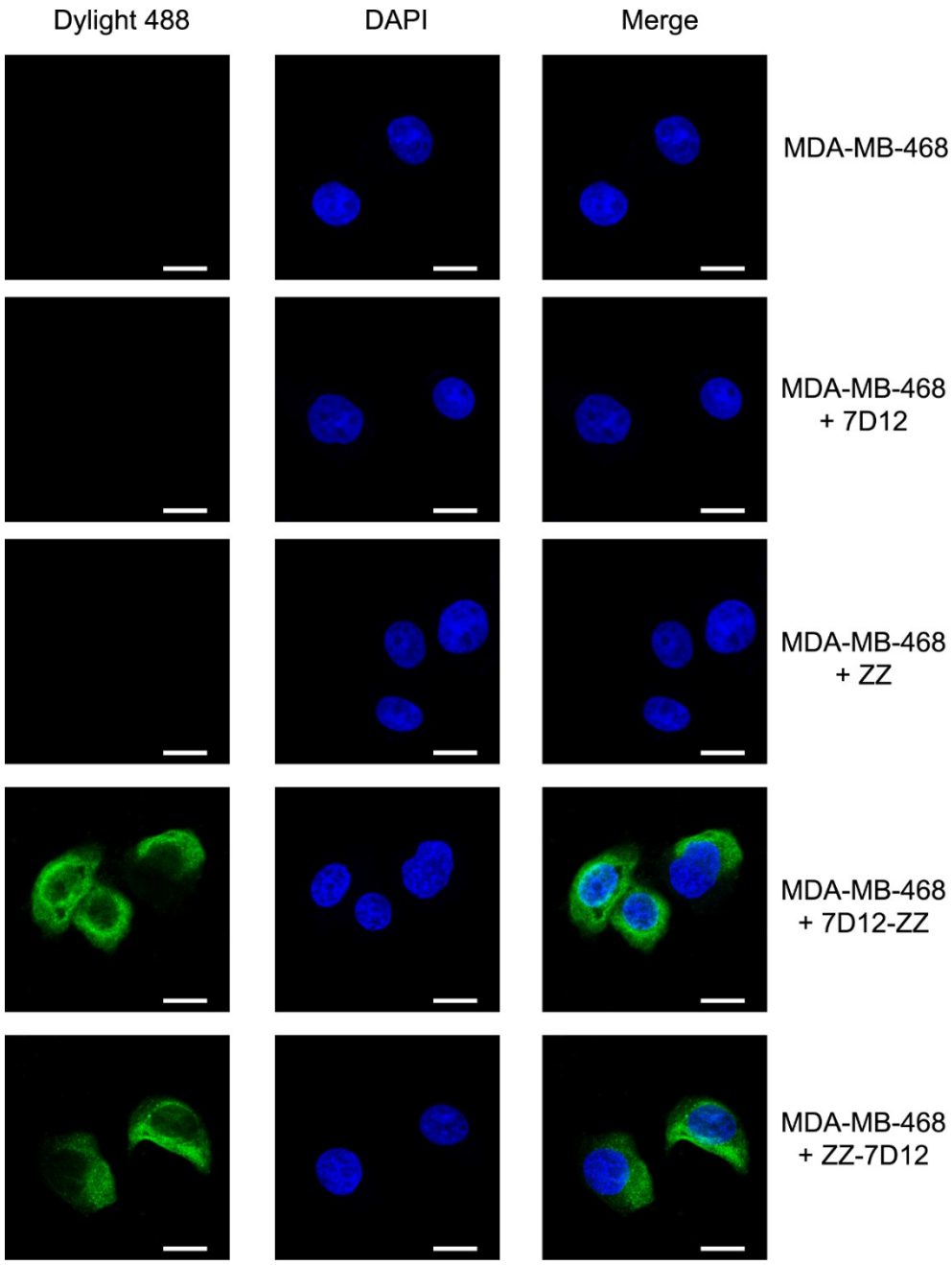

Fig. S4. Immunofluorescence of MDA-MB-468 cells treated with nanobody 7D12, ZZ domain, 7D12-ZZ or ZZ-7D12. Scale Bar: 20 μm.

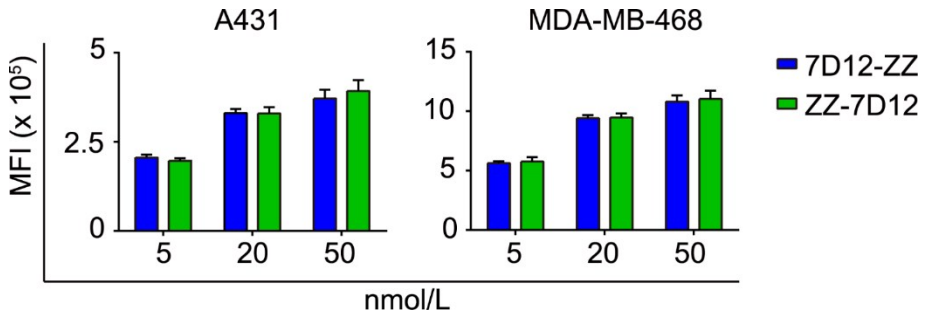

Fig. S5. Flow cytometry analysis of A431 or MDA-MB-468 cells treated with different concentrations of UEAR Nbs (7D12-ZZ or ZZ-7D12). Error bars represent the SD of three parallel experiments.

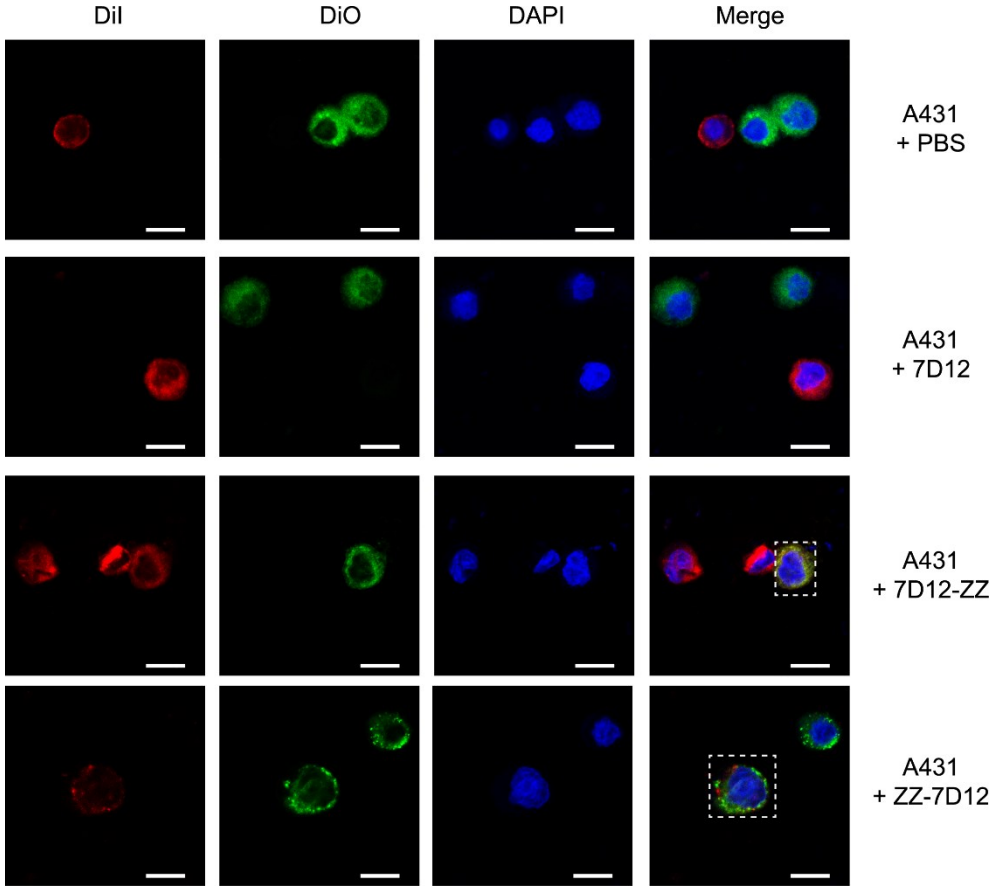

Fig. S6. Confocal fluorescence analysis of A431 cells treated with PBS, 7D12, 7D12-ZZ or ZZ-7D12 in the presence of THP-1 cells. The A431 cells phagocytosed by THP-1 cells were pointed out with white dotted box in merge channel. Scale Bar: 20 μm.

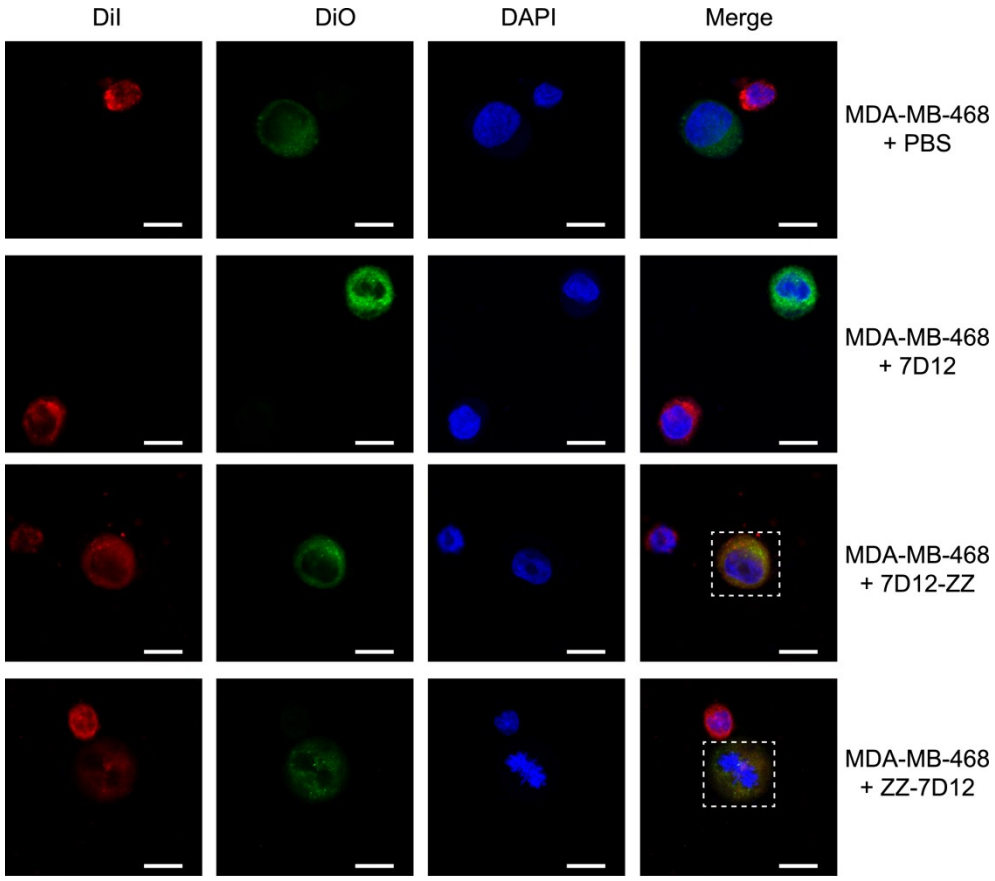

Fig. S7. Confocal fluorescence analysis of MDA-MB-468 cells treated with PBS, 7D12, 7D12-ZZ or ZZ-7D12 in the presence of THP-1 cells. The MDA-MB-468 cells phagocytosed by THP-1 cells were pointed out with white dotted box in merge channel. Scale Bar: 20 μm.

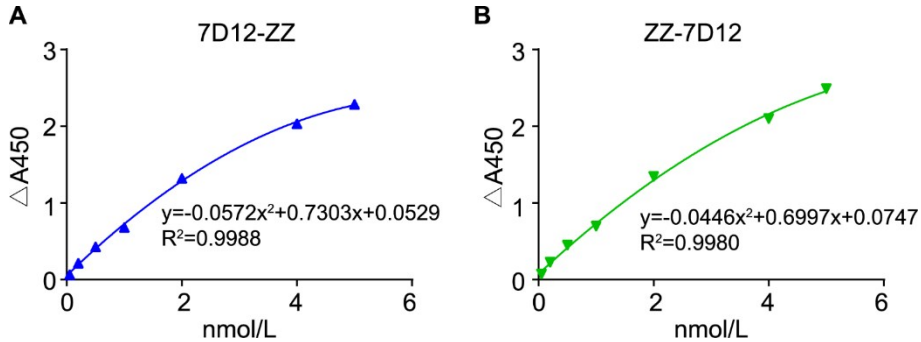

Fig. S8. The standard curves for pharmacokinetics analysis. The standard curve of (**A**) 7D12-ZZ and (**B**) ZZ-7D12 were generated by ELISA using human recombinant EGFR as the antigen. △A450= Ax-A0, where Ax is the OD450 value of samples containing x nmol/L of 7D12-ZZ or ZZ-7D12, A0 is the OD450 value of sample containing 0 nmol/L of 7D12-ZZ or ZZ-7D12.

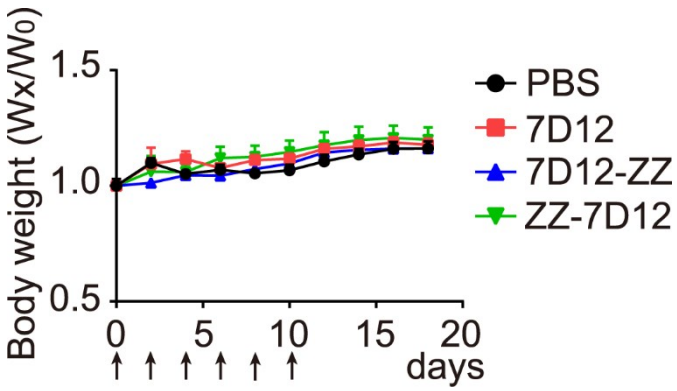

Fig. S9. Body weights of the treated mice during the course of experiment. Wx represents the body weight at day x, W_0 represents the body weight at day 0. Each arrow represents a treatment. Error bars represent the SEM (n=5).

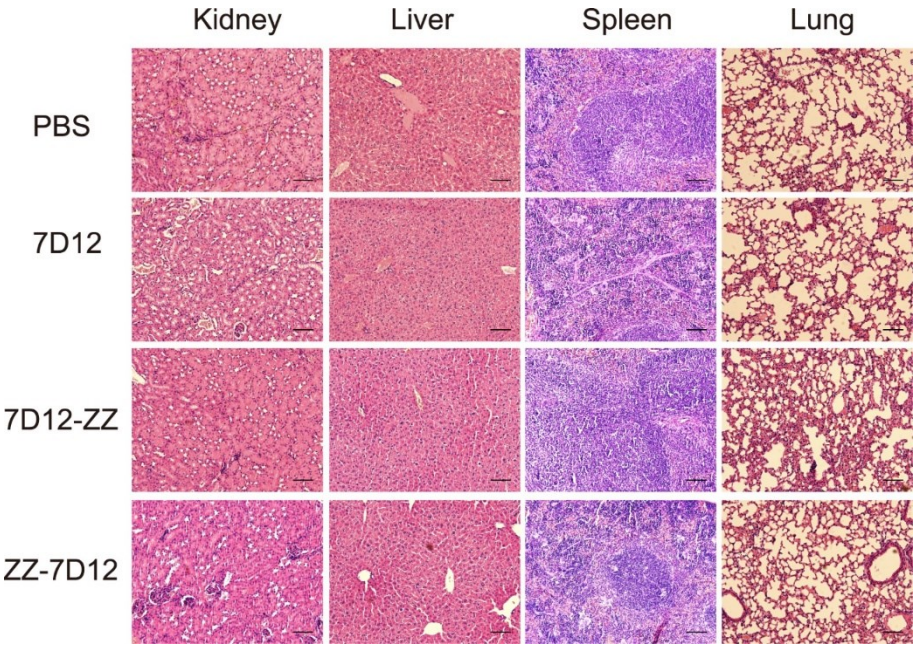

Fig. S10. Histological analysis of different tissues from MDA-MB-468 xenograft mice treated with PBS, 7D12, 7D12-ZZ or ZZ-7D12. Scale bar = 500 μm.

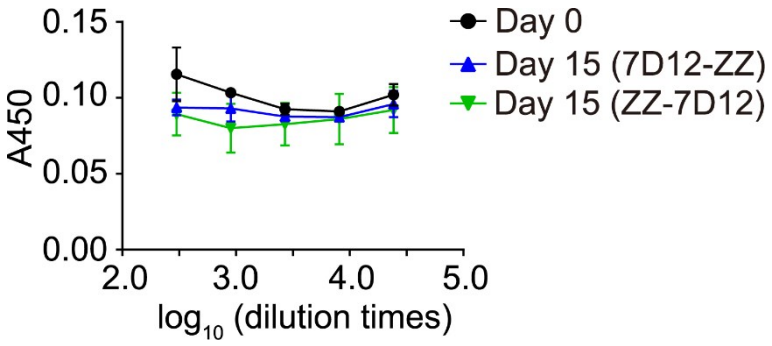

Fig. S11. ELISA evaluation of the nanobody-specific IgE level in C57BL/6J mice treated with 7D12-ZZ or ZZ-7D12. Treatments were given at day 0, followed by day 7 to 15 with a frequency of every two days. Error bars represent the SD (n=2-3).

References

1. H. F. Hong, Z. F. Zhou, K. Zhou, S. Z. Liu, Z. W. Guo and Z. M. Wu, *Chem. Sci.*, 2019, **10**, 9331-9338.
2. R. Kariya, K. Matsuda, K. Gotoh, K. Vaeteewoottacharn, S. Hattori and S. Okada, *In Vivo*, 2014, **28**, 779-784.
3. V. Vetvicka, L. Fornusek, M. Holub, J. Zidkova and J. Kopecek, *Eur. J. Cell Biol.*, 1984, **35**, 35-40.
4. F. A. Guhad, H. E. Jensen and J. Hau, *FEMS Microbiol. Lett.*, 2000, **192**, 27-31.
5. K. Sasaki, M. Harada, Y. Miyashita, H. Tagawa, A. Kishimura, T. Mori and Y. Katayama, *Chem. Sci.*, 2020, **11**, 3208-3214.