Supporting Information

Reprogramming Exosomes as Nanoscale Controllers of Cellular Immunity

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Experimental Materials and Methods.

Materials. Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), Minimum essential medium (MEM) were purchased from Corning Inc. (Corning, NY). Fetal bovine serum (FBS) was purchased from VWR International (Radnor, PA). Opti-modified Eagle's medium (Opti-MEM), Expi293 expression medium and ExpiFectamine 293 transfection reagent were purchased from Thermo Fisher Scientific (Waltham, MA). MitoSpy Red and carboxyfluorescein succinimidyl ester (CFSE) were purchased from BioLegend (San Diego, CA). Pierce Coomassie Plus (Bradford) assay kit and QuantaBlu fluorogenic peroxidase substrate were purchased from Thermo Fisher Scientific (Waltham, MA).

Cell lines. Breast cancer cell lines (MDA-MB-468, MDA-MB-453, and MDA-MB-231) and Jurkat cell line were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C in 5% CO_2. BT20 cell line was kindly provided by Dr. Julie E. Lang (University of Southern California, Los Angeles, CA) and cultured in MEM with 10% FBS at 37°C in 5% CO_2. Expi293F cells were purchased from Thermo Fisher Scientific (Waltham, MA) and maintained in Expi293 expression medium with shaking at a speed of 125 rpm min^{-1} at 37°C in 8% CO_2. Human peripheral blood mononuclear cells (PBMCs) were purchased from HemaCare (Van Nuys, CA).

Animals. Six to eight-week female NOD.*Cg-Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal studies

were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Southern California.

Molecular cloning and expression of SMART-Exos in mammalian cells. Synthetic genes encoding scFv fragments of αEGFR cetuximab and αCD3 UCHT1 antibodies were purchased from Integrated DNA Technologies, Inc. (Skokie, IL), in which a $(GGGGS)_3$ linker was inserted between V_H and V_L regions. To generate single polypeptide encoding dual scFv antibodies, overlap extension polymerase chain reactions (PCR) were performed by placing a $(GGGGS)_3$ flexible linker between two individual scFv fragments. The orientations of variable regions for each construct were arranged as follows: $V_{H\text{-}\alpha EGFR}$-$V_{L\text{-}\alpha EGFR}$ (αEGFR scFv), $V_{L\text{-}\alpha CD3}$-$V_{H\text{-}\alpha CD3}$ (αCD3 scFv), $V_{H\text{-}\alpha EGFR}$-$V_{L\text{-}\alpha EGFR}$-$V_{L\text{-}\alpha CD3}$-$V_{H\text{-}\alpha CD3}$ (αEGFR/αCD3 scFv), and $V_{L\text{-}\alpha CD3}$-$V_{H\text{-}\alpha CD3}$-$V_{H\text{-}\alpha EGFR}$-$V_{L\text{-}\alpha EGFR}$ (αCD3/αEGFR scFv). Primers used for PCRs to amplify these gene fragments are listed in Table S1 with indicated restriction enzyme sites of BglII and SalI. The amplified inserts were ligated in-frame using T4 DNA ligase (New England Biolabs, Ipswich, MA) between the N-terminal signal peptide and the transmembrane (TM) domain of human platelet-derived growth factor receptor (PDGFR) in pDisplay vector (Thermo Fisher Scientific, Waltham, MA). A hemagglutinin (HA)-tag was fused at the N-terminus of the inserted fusion protein. The generated expression vectors were confirmed by DNA sequencing provided by GENEWIZ (South Plainfield, NJ).

Transfection-grade plasmids for the sequence-verified expression constructs were prepared using ZymoPURE II Plasmid Kits (ZYMO Research, Irvine, CA). Transient transfections for the expression constructs of SMART-Exos were performed with

Expi293F cells cultured in chemically-defined Expi293 expression medium using ExpiFectamine 293 transfection kits by following manufacturer's instruction. Media were collected on day 3 and day 6 post transfection through centrifugation.

Exosome purification. Expressed exosomes were purified from the collected culture media through differential centrifugation. Harvested cell cultures containing exosomes were subjected to centrifugation at 4°C for 10 min at 100 ×g and for 30 min at 4000 ×g, followed by 14,000 ×g for 50 min. Collected supernatants were then centrifuged at 371,000 ×g (60,000 rpm) in a Type 70 Ti rotor (Beckman Instruments, Indianapolis, IN) for 2 h at 4°C. After decanting supernatant, exosomes were washed twice with PBS, followed by filtration using 0.2 μm syringe filters. Protein concentrations of the purified exosomes were determined by Bradford assays.

Nanoparticle tracking analysis (NTA) and zeta potential of the purified exosomes. Particle concentration and size distribution of the purified exosomes were determined through NTA using a Nanosight LM10 (Malvern Instruments, U.K.) according to the manufacturer's instruction. Zeta potential of the generated exosomes was measured using a Zetasizer Nano ZS (Malvern Instruments, U.K.).

Transmission electron microscopy (TEM) of the generated exosomes. TEM grids were pretreated by placing 20 μL of the 0.1% poly-lysine solution on the grid and incubating for 10 min. Residual solvent was removed with filter paper. Exosomes were prepared for TEM analysis by placing 20 μL of the sample solution on 200 μm mesh grids and incubating for 15 min. Residual solvent was subsequently removed from the

grids with filter paper. Then, 20 μL of 2% uranyl acetate solution was placed on the grids for 5 min, followed by removal of residual solution with filter paper. The exosome samples were loaded to a JEOL 2010F TEM (JEOL, Peabody, MA) for analysis.

Immunoblot analysis. Purified exosomes (4 μg of protein) were reduced with 10 mM dithiothreitol and incubated at 95°C for 5 min in loading buffer (60 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 0.05% bromophenol blue, 10% glycerol). The samples were then separated in 4-20% ExpressPlus-PAGE gels (GeneScript, Piscataway, NJ) and subsequently transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories, Inc, Hercules, CA) at 15 V for 30 min using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc, Hercules, CA). After incubation in PBST with 5% BSA for 1 h, the membranes were incubated with appropriate primary antibodies (anti-HA (clone: 2-2.2.14) from Thermo Fisher Scientific, anti-CD63 (clone: H5C6) from BioLegend, anti-CD81 (clone: 1.3.3.22) from Thermo Fisher Scientific, and anti-CD9 (clone: D8O1A) from Cell Signaling Technology) for 1 h at room temperature. The membranes were further incubated with appropriate secondary antibodies (anti-mouse IgG-HRP (catalog number: 62-6520) from Thermo Fisher Scientific or anti-rabbit IgG-HRP (catalog number: 65-6120) from Thermo Fisher Scientific) for 1 h at room temperature, followed by additions of SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) and imaging with a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc, Hercules, CA).

Flow cytometric analysis. The binding of the generated SMART-Exos to TNBC cell lines (MDA-MB-453, MDA-MB231, BT20, and MDA-MB-468) and Jurkat cells

were evaluated by flow cytometry. Cells were incubated with 0.1 mg mL^{-1} exosomes in PBS with 0.2% FBS for 30 min at 4°C and washed twice with PBS containing 0.2% FBS, followed by incubation with the anti-HA antibody (clone: 2-2.2.14, from Thermo Fisher Scientific) for 30 min at 4°C. Cells were then washed twice with PBS with 0.2% FBS and subsequently incubated with the Alexa Fluor 488-labeled goat anti-mouse IgG H&L antibody (catalogue number: ab150113 from Abcam) for 30 min at 4°C. Following twice wash, samples were analyzed using an LSR II Flow Cytometer (BD Biosciences, San Jose, CA). Data were processed by FlowJo_V10 software (Tree Star Inc., Ashland, OR).

Confocal imaging of cell crosslinking mediated by the SMART-Exos. TNBC cells and Jurkat cells were stained with MitoSpy Red and CFSE, respectively by following the manufacturer's instructions. Jurkat cells (6×10^4) were incubated with α-CD3/α-EGFR SMART-Exos (0.1 mg mL^{-1}) in 100 μL PBS for 30 min at 4°C. As controls, Jurkat cells were incubated with a mixture (1:1) of αCD3 SMART-Exos and αEGFR SMART-Exos (0.1 mg mL^{-1}). Following washing with 1 mL of cold PBS, Jurkat cells were resuspended in 500 μL RPMI-1640 medium with 10% FBS, then mixed with TNBC cells (2×10^4) in the same medium. The cell mixtures were subsequently added into clear bottoms of 24-well plates and incubated for 5 hours at 37°C with 5% CO_2. The cells were then gently washed four times with PBS and imaged with a Leica SP8 confocal laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, IL) equipped with a 40×, 1.3 NA PLAPO oil immersion objective lens using

FITC (for CFSE) and rhodamine (for MitroSpy Red) filters. Images were processed using LAS X software (Leica Microsystems Inc., Buffalo Grove, IL).

Confocal imaging of SMART-Exos participating in cell crosslinking. MDA-MB-468 cells (3×10^4) were seeded into clear bottoms of 24-well plates one day before the experiment. Jurkat cells (3×10^4) were incubated with PKH67-labeled α-CD3/α-EGFR SMART-Exos (0.1 mg mL^{-1}) in 100 μL PBS for 30 min at 4°C. As controls, Jurkat cells were incubated with a mixture (1:1) of PKH67-labeled αCD3 SMART-Exos and PKH67-labeled αEGFR SMART-Exos (0.1 mg mL^{-1}). The mixture of Jurkat cells and PKH67-labeled SMART-Exos were then mixed with MitoSpy Red-labeled MDA-MB-468 cells and incubated in PBS for 1 h at 4°C. The cells were then gently washed four times to remove non-bound Jurkat cells and imaged with a Leica SP8 confocal laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, IL) equipped with a 40×, 1.3 NA PLAPO oil immersion objective lens using FITC (for PKH67) and rhodamine (for MitroSpy Red) filters. Images were processed using LAS X software (Leica Microsystems Inc., Buffalo Grove, IL).

In vitro **T-cell activation assays.** Non-activated human PBMCs from the same healthy donor were incubated for 20 hours with target TNBC cells in the absence or presence of αCD3/αEGFR SMART-Exos or a mixture (1:1) of αCD3 SMART-Exos and αEGFR SMART-Exos. The cell mixtures were then stained with FITC-labeled anti-human CD3 antibody (clone: UCHT1, from BioLegend), APC-Cy7-labeled anti-human CD25 antibody (clone: BC96, from BioLegend), and APC-labeled anti-human CD69 antibody (clone: CL50, BioLegend), followed by flow cytometric analysis. The levels

of secreted interleukin-2 (IL-2) and interferon gamma (IFN-γ) cytokines were determined by enzyme-linked immunosorbent assay (ELISA) kits for human IL-2 and IFN-γ (R&D Systems, Minneapolis, MN). Data are shown as mean ± SD.

***In vitro* cytotoxicity assays.** TNBC cells (target cells) (1×10^4 cells) were mixed with non-activated human PBMCs (effector cells) (1×10^5 cells) and incubated for 40 hours in the presence of various concentrations of SMART-Exos at 37°C. To evaluate the effects of E:T ratios on cytotoxicity, 1×10^4 target TNBC cells were incubated with various amounts of non-activated human PBMCs ($0.1\text{-}1\times10^5$) for 40 hours at 37°C in the presence of 1, 10, and 100 ng mL^{-1} SMART-Exos. Cells were then washed twice with PBS to remove PBMC suspensions, followed by additions of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. Following 3 hours incubation at 37°C and subsequent additions of 100 μL of lysis buffer (20% SDS in 50% dimethylformamide, pH 4.7), plates were incubated for 4 hours at 37°C and measured for absorbance at 570 nm using a BioTek Synergy H1 Hybrid Multi-Mode Microplate reader (BioTek, Winooski, VT). Cytotoxicities were calculated as the percentage of cell viability= $[(\text{absorbance}_{\text{experimental}} - \text{absorbance}_{\text{spontaneous average}})/(\text{absorbance}_{\text{maximal viability average}} - \text{absorbance}_{\text{spontaneous average}})] \times 100$.

Pharmacokinetics of αCD3/αEGFR SMART-Exos in mice. αCD3/αEGFR SMART-Exos (800 μg per mouse) were administrated by intravenous (i.v.) injection into BALB/c mice (*n*=4). Blood samples were collected using Multivette 600 LH-Gel tubes (order number:15.1675, SARSTEDT, Germany) at 5 min, 30 min, 60 min, 120 min, 180 min, 240 min and 360 min, immediately followed by centrifugation at 10,000

×g for 5 min at room temperature. The obtained plasma samples were diluted with PBS (25× dilution) and analyzed by sandwich ELISA with a mouse anti-HA monoclonal antibody (clone: 2-2.2.14; Thermo Fisher Scientific) as the capture antibody, a rabbit anti-HA polyclonal antibody (catalog number: 600-401-384, Rockland Immunochemicals Inc., Limerick, PA) as the detection antibody, and a goat anti-rabbit IgG (H+L) HRP (catalog number: 5220-0336, SeraCare, Milford, MA). Serial dilutions of αCD3/αEGFR SMART-Exos in PBS with 4% mouse plasma were used as standards to quantify the plasma concentrations of αCD3/αEGFR SMART-Exos. The half-life for the elimination phase of αCD3/αEGFR SMART-Exos was determined by fitting the last four data points into the first-order equation, $A = A_0e^{-kt}$, where A_0 is the initial concentration, t is the time, and *k* is the first-order rate constant.

***In vivo* efficacy studies.** The αCD3/αEGFR SMART-Exos were evaluated for *in vivo* efficacy using 6 to 8-week female immunodeficient NSG mice bearing tumors derived from EGFR-expressing human TNBC cell line MDA-MB-468. To establish human tumor xenograft model, 5×10^6 MDA-MB-468 cells in 50% matrigel (BD Biosciences) were subcutaneously implanted into the right flank of immunodeficient NSG mice, respectively. When tumor sizes reached 60 mm^3 approximately 13 days post tumor implantation, each mouse received 20×10^6 non-activated human PBMCs via intraperitoneal injection to generate the PBMC-humanized mouse. All human PBMCs purchased from HemaCare Corporation were from the same healthy donor. One day after PBMCs injection, mice (*n*=5) were administered intravenously with αCD3/αEGFR SMART-Exos (10 mg kg^{-1}; 2.8×10^{10} particles per mouse) or PBS every

other day for a total of six times. Tumors were measured three times per week by a caliper. Tumor volume was calculated as $mm^3=0.5\times(length)\times(width)^2$. All procedures were approved by Institutional Animal Care and Use Committee of the University of Southern California.

***In vivo* T cell infiltration studies.** 5×10^6 MDA-MB-468 cells in 50% matrigel were subcutaneously implanted into the right flank of 6 to 8-week female NSG mice (n=5), followed by intraperitoneal injection of 20×10^6 non-activated human PBMCs post 4-week tumor implantation. Two days after PBMCs injection, mice were treated intravenously with αCD3/αEGFR SMART-Exos (10 mg kg^{-1}; 2.8×10^{10} particles per mouse) or PBS every other day for a total of three times. On day 8 after PBMCs injection, mice were euthanized, and tumors were then collected. Tumors were cut into small pieces and subjected to mechanical disruption and separation, followed by passing through 70 μm strainers and treatment with the erythrocyte lysis solution (BioLegend) by following manufacturer's instruction. The resulting single-cell suspensions were stained with the FITC-labeled anti-human CD45 antibody (clone: HI30, from BioLegend) and the Pacific Blue-labeled anti-human CD3 antibody (clone: UCHT1, from BioLegend) and analyzed by a BD Fortessa X20 (BD Biosciences). Flow cytometry data were processed using FlowJo_V10 software (Tree Star Inc., Ashland, OR).

Statistical analysis. Two-tailed unpaired t tests were performed for comparison between two groups. One-way ANOVA with Tukey post-hoc tests were carried out for comparing multiple groups. Tumor growth curves were analyzed using two-tailed

unpaired *t* tests with repeated measures. A $P < 0.05$ was considered statistically significant. Significance of finding was defined as: ns = not significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Data are shown as mean ± SD. All statistical analyses were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA).

Table S1. List of primer sequences used for molecular cloning. Restriction enzyme sites of BglII and SalI are underlined and in italics.

Name	Sequence
αEGFR scFv Forward	5′-GGA*AGATCT*CAGGTGCAGCTGAAGCAGTCTGG-3′
αEGFR scFv Reverse	5′-ACGC*GTCGAC*GCTTCCGCCCCCGCCCTTCAGTTCCAGCTTG-3′
αCD3 scFv Forward	5′-GGA*AGATCT*GATATCCAGATGACACAGACAACCTCAAG TCTTAGTGC-3′
αCD3 scFv Reverse	5′-ACGC*GTCGAC*GCTTCCGCCCCCGCCGCTGCTAACGGTAA-3′
αEGFR-αCD3 scFv overlap Forward	5′-GAACTGAAGGGCGGTGGCGGATCAGGC-3′
αEGFR-αCD3 scFv overlap Reverse	5′-ATCCGCCACCGCCCTTCAGTTCCAGCTTGGTGCCAGCG-3′
αCD3-αEGFR scFv overlap Forward	5′-CGGAAGTGGAGGAGGTGGCTCTGGCGGTGGAGGAAGCC AGGTGCAGCTGAAG-3′
αCD3-αEGFR scFv overlap Reverse	5′-CCAGAGCCACCTCCTCCACTTCCGCCACCTCCGCTGCTAA CGGTAACGGTGGTACCTGC-3′

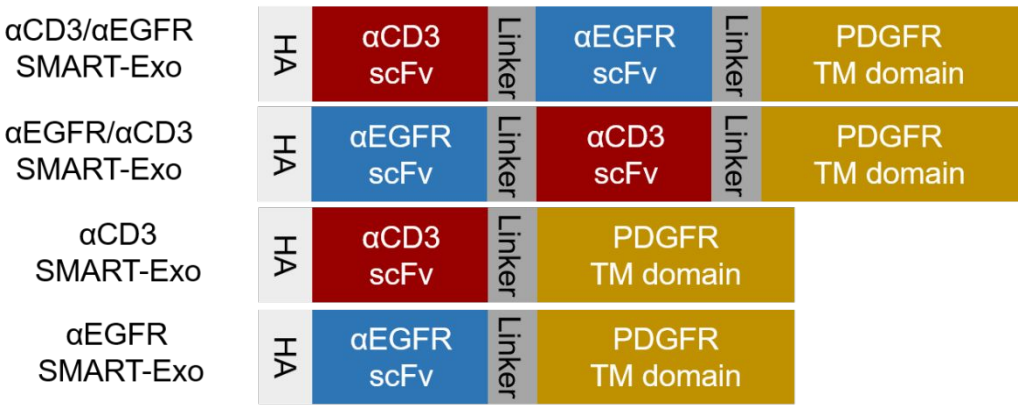

Figure S1. Molecular designs of antibody-PDGFR transmembrane (TM) domain fusions for the generation of SMART-Exos. Each fusion protein contains an N-terminal hemagglutinin (HA) epitope tag and flexible linkers.

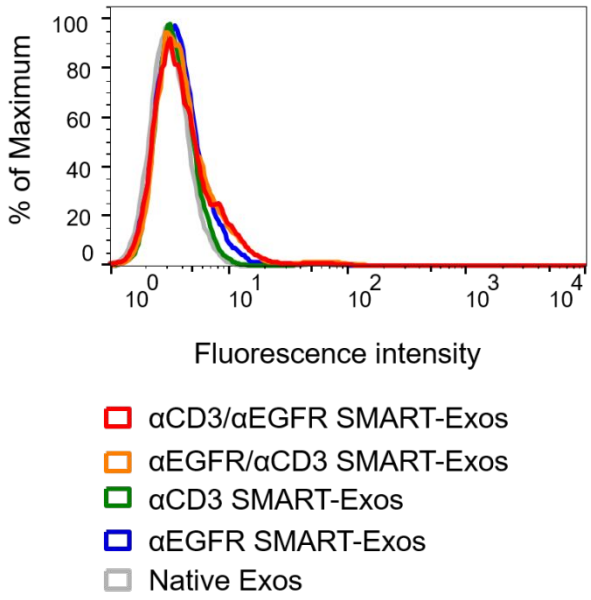

Figure S2. Flow cytometric analysis of the binding of the generated SMART-Exos to MDA-MB-453 cells ($EGFR^-$ $CD3^-$).

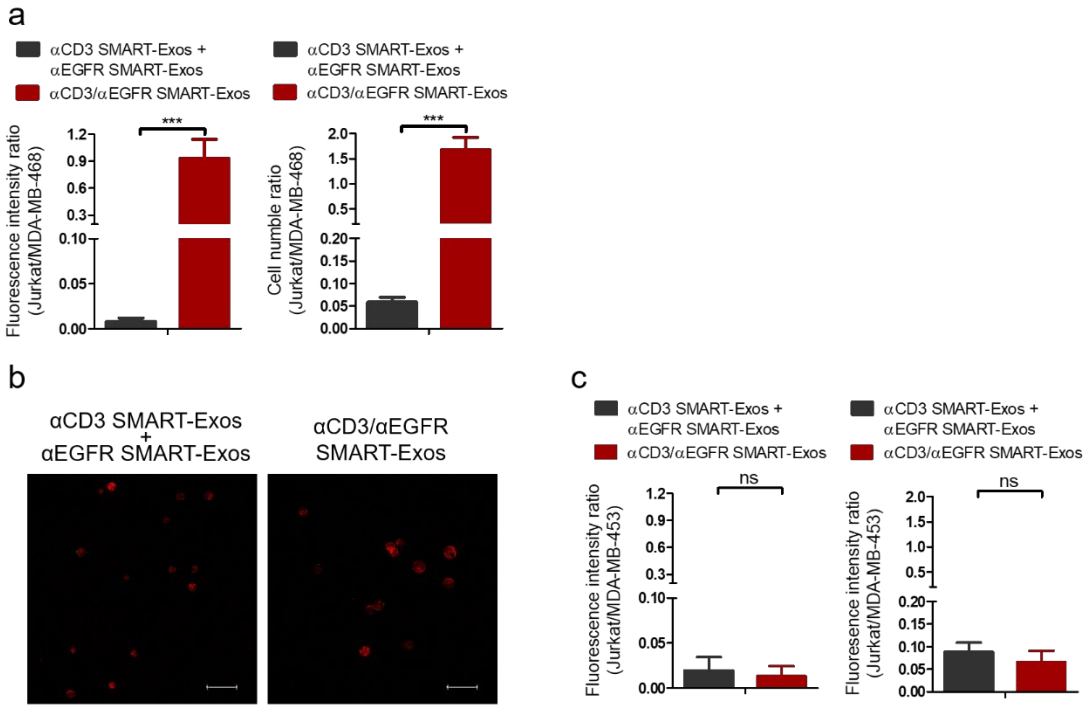

Figure S3. Quantitative and confocal microscopic analysis of cell-cell crosslinking induced by SMART-Exos. (a) Quantitative analysis of the crosslinking levels of MDA-MB-468 and Jurkat cells on the basis of the fluorescence intensity ratio (left) or cell number ratio (right) of Figure 2d. ***, $P < 0.001$. (b) Confocal microscopic analysis of the crosslinking of Jurkat and MDA-MB-453 cells induced by SMART-Exos. Fluorescently labeled MDA-MB-453 cells (red) and Jurkat cells (green) were mixed at a 1:3 ratio and incubated for 5 hours in the presence of αCD3/αEGFR SMART-Exos or a mixture (1:1) of αCD3 SMART-Exos and αEGFR SMART-Exos. Non-bound cells were washed away by PBS. Scale bars: 50 μm. (c) Quantitative analysis of the crosslinking levels of MDA-MB-453 and Jurkat cells on the basis of the fluorescence intensity ratio (left) or cell number ratio (right) of Figure S3b. ns = not significant, $P > 0.05$.

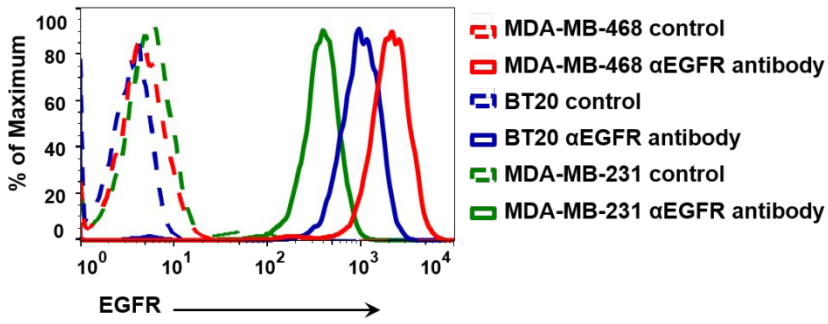

Figure S4. Flow cytometric analysis of EGFR expression for three TNBC cell lines, including MDA-MB-468 ($EGFR^{+++}$), BT20 ($EGFR^{++}$), and MDA-MB-231 ($EGFR^{+}$).

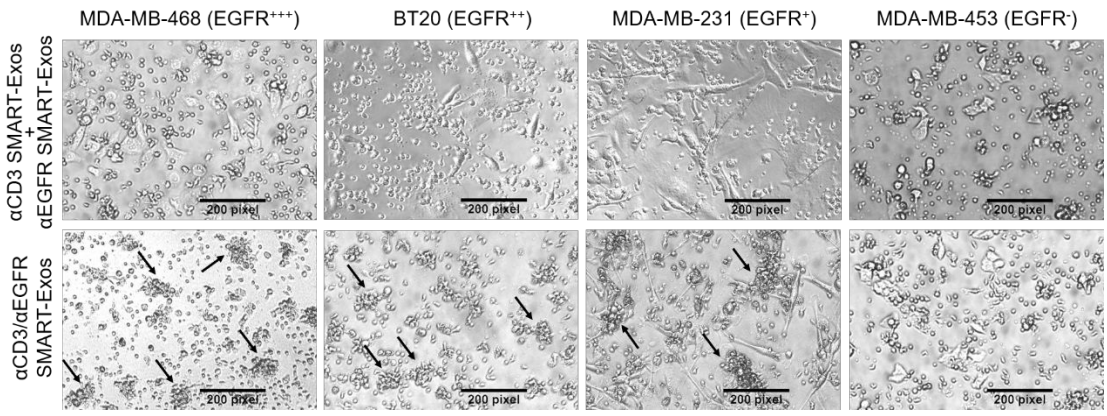

Figure S5. Brightfield microscopic analysis of TNBC cell lysis mediated by the αCD3/αEGFR SMART-Exos. TNBC cells (target cells) with various levels of EGFR expression were incubated with non-activated human PBMCs (effector cells) at an E:T ratio of 10 for 40 h in the presence of 100 ng mL^{-1} αCD3/αEGFR SMART-Exos. Mixtures (1:1) of αCD3 SMART-Exos and αEGFR SMART-Exos were included as controls. Arrows indicate clusters of T cells and TNBC cells induced by the αCD3/αEGFR SMART-Exos. Scale bar = 200 pixel (132.4 μm).

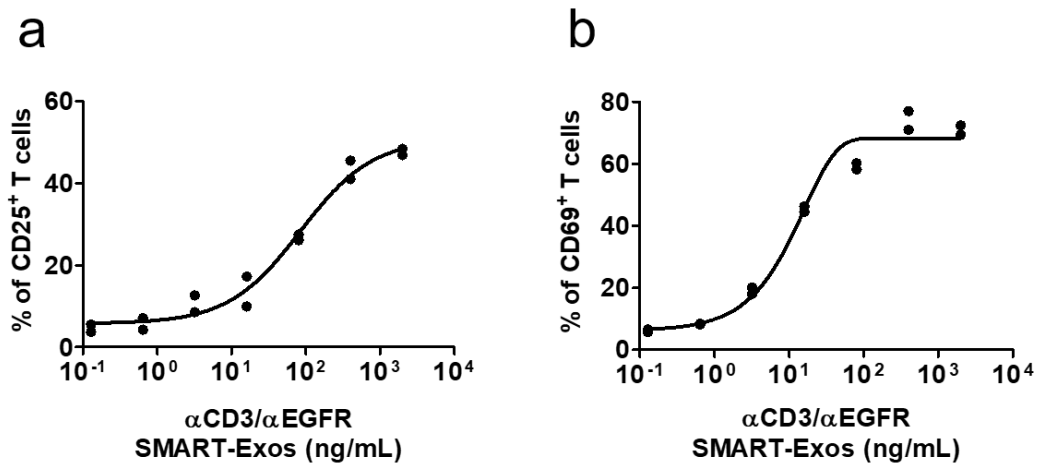

Figure S6. Dose-dependent activation of T cells by the αCD3/αEGFR SMART-Exos as evaluated by T-cell surface activation markers CD25 (a) and CD69 (b). Non-activated human PBMCs were incubated with MDA-MB-468 ($EGFR^+$) cells at an E:T ratio of 10 for 20 hours in the presence of αCD3/αEGFR SMART-Exos. Expression levels of CD25 and CD69 on T-cell surface were analyzed by flow cytometry.

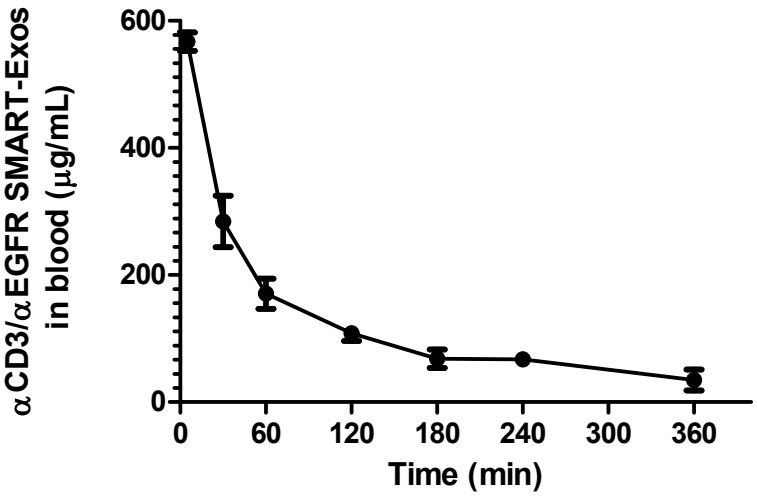

Figure S7. Pharmacokinetics of αCD3/αEGFR SMART-Exos in mice. Plasma concentrations of SMART-Exos were determined by sandwich ELISA post i.v. injection of αCD3/αEGFR SMART-Exos. Data are shown as mean ± SD (n = 4).

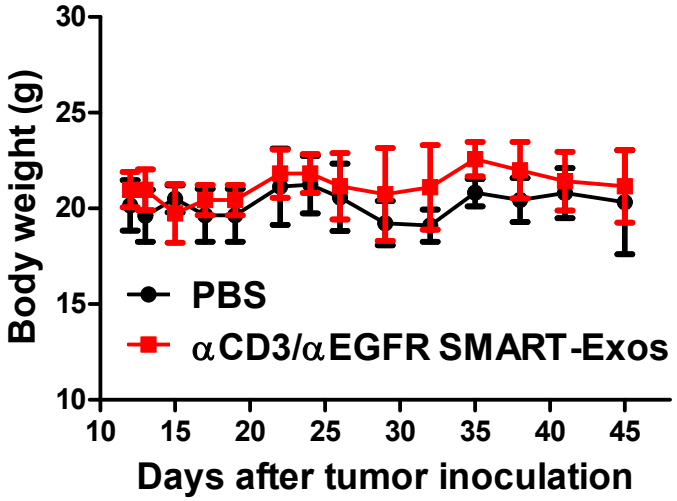

Figure S8. Body weights of mice during *in vivo* efficacy studies using the human MDA-MB-468 tumor xenograft model. Data are shown as mean ± SD.

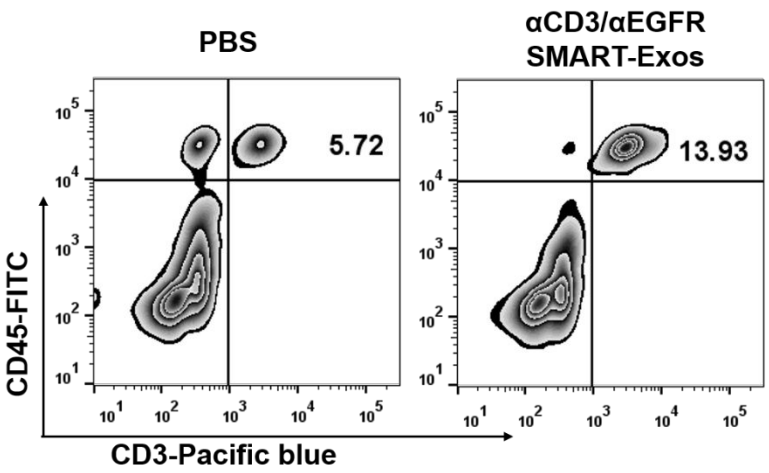

Figure S9. *In vivo* T-cell infiltration induced by the αCD3/αEGFR SMART-Exos. Single-cell preparations of residual tumors for PBS- and SMART-Exos-treated groups were analyzed by flow cytometry. Representative contour plots show the percentages of human $CD3^+CD45^+$ T cells in tumors of PBS- and SMART-Exos-treated groups.