

Supplementary Information for

The SHREAD gene therapy platform for paracrine delivery improves tumor localization and intratumoral effects of a clinical antibody

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Supplementary Methods

Redesign of pShuttle vector. The AdEasy™ Adenoviral Vector System^{1,2} (Agilent Technologies) was adapted to allow for the rapid generation and exchange of modular expression cassettes encoding a variety of payloads. The multiple cloning site (MCS) of the pShuttle vector was replaced with a synthetic MCS module, named MCS1, via Gibson Assembly (New England Biolabs). The synthetic MCS1 module contained from 5' to 3', the CMV promoter, *NheI* restriction site, *XhoI* restriction site, an IRES from EMCV, the eGFP gene, the polyA site from BGH. The MCS1 module was synthesized by GeneArt (Life Technologies Europe BV) containing the N-terminal flanking DNA 5'- GAA TAA GAG GAA GTG AAA TCT GAA TAA TTT TGT GTT ACT CAT AGC GCG TAA -3,' and C-terminal flanking DNA 5'- TAA GGG TGG GAA AGA ATA TAT AAG GTG GGG GTC -3' for Gibson Assembly into pShuttle to generate the plasmid pShuttle-MCS1.

Construction of antibody and reporter constructs. Antibody heavy and light chains were codon-optimized and synthesized by GeneArt (Life Technologies Europe BV) in a single ORF containing the FMDV 2A self-processing peptide, which allows for bicistronic expression of heavy and light chains via a previously described a ribosomal skipping mechanism (heavy_chain-furin-2A_peptide-light_chain)^{3,4}. The 2A peptide (APVKQTLNFDLLKLAGDVESNPGP)⁵ contained an N-terminal furin cleavage site RKRR⁶ to remove excess 2A amino acids from the antibody heavy chain following translation. The N-terminal flanking DNA contained a *NheI* restriction site for cloning into MCS1 followed by a mammalian Kozak consensus sequence (gccRccAUGG)⁷; the C-terminal flanking DNA contained two stop codons followed by a *XhoI* restriction site for cloning into MCS1.

The heavy and light chains of the humanized IgG1k antibody trastuzumab were synthesized from the known protein sequences^{8,9} of trastuzumab (Herceptin®) and cloned into pShuttle-MCS1 to generate pShuttle-MCS1_TZB. As an isotype control, a humanized variant of the murine IgG1k D1.3 specific for hen egg lysozyme (HEL) was synthesized using a previously described Fab fragment sequence¹⁰ on the same human IgG1k framework as trastuzumab and cloned into pShuttle-MCS1 to generate pShuttle-MCS1_huD1.3. Additionally, a reporter virus containing the gene for TdTomato was generated for *in vitro* studies by Gibson assembly of the PCR-amplified TdTomato gene (GenBank: AMO27245.1) into a variant of pShuttle-MCS1 that lacks the IRES-eGFP, called pShuttle-MCS1', to generate pShuttle-MCS1'_TdTomato.

Virus production. The plasmid containing the adenoviral genome, pAdEasy-1, from the AdEasy™ Adenoviral Vector System^{1,2} (Agilent Technologies) was previously modified to include a mutation to the hypervariable loop 7 (HVR7) of the hexon, which prevents blood factor X binding to virions and thus reduces liver infection¹¹. To generate viral constructs, the modified pAdEasy-1_HVR7 plasmid was co-transformed with the pShuttle-MCS1 variant encoding the antibody and/or reporter into *recA*-proficient *E. coli* BJ5183 cells, from which the desired recombinants, obtained by homologous recombination, could be isolated for virus production. For the generation of Ad_HVR7-MCS1_TZB (Ad-TZB) and Ad_HVR7-MCS1_huD1.3 (Ad-D1.3), subsequent procedures were done according to the AdEasy™ Adenoviral Vector System manual (Agilent Technologies)^{1,2} using HEK293 cells (ATCC® CRL-1573™) as a packaging cell line and purifying viral particles on two consecutive cesium chloride density gradients, subsequently dialyzed and stored at -80°C in 20 mM HEPES pH 8.1, 150 mM NaCl, 1 mM MgCl₂, 10 % glycerol. For the Ad_HVR7-MCS1'_TdTomato (Ad-TdTomato) reporter virus, packaging and amplification were performed by Vector Biolabs (Malvern, PA, USA) on two consecutive cesium chloride density gradients and provided directly in PBS with 5% glycerol.

Cell culture (continued). All cell lines were confirmed to be free from mycoplasma contamination. FreeStyle™ Chinese Hamster Ovary Cells (CHO-S, Thermo Fisher R80007) were used for antibody expression, and were cultured in CHOgro® expression medium (Mirus, MIR6200) supplemented with 20 mM glutamine and 0.3% Poloxamer 188 (Mirus, MIR6230). HEK293 and A549 cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Sigma D6429), and BT474 and SKOV3ip cells were cultured in RPMI 1640 medium containing GlutaMAX™ supplement (Gibco 61870-010). Both media were supplemented with 1% (v/v) penicillin/streptomycin solution (5,000 U/mL; Thermo Fisher) and 10% (v/v) fetal calf serum (FCS;

Amimed 2-01F10-I) to generate the complete formulations. For cell culture experiments where IgG concentrations were determined or IgG was purified, cells were adapted to medium containing ultra-low IgG FCS (Pan Biotech P30-2802) at least two passages beforehand. Cells were maintained at 37°C, 5% CO₂ and >90% humidity and passaged according to the manufacturer's instructions no more than 15 times from the provided aliquot.

Electrospray ionization mass spectrometry (ESI-MS). Prior to analysis, samples were deglycosylated with 9 units of PNGase F (Sigma P7367) per µg protein for 3 hours at 37°C, reduced in 50 mM dithiothreitol (DTT), desalted using C4 ZipTips (Millipore) and analyzed in MeOH:2-PrOH:0.2% formic acid (30:20:50). The solutions were infused through a fused silica capillary (ID 75 µm) at a flowrate of 1 µL/min and sprayed through a PicoTip (ID30 µm; New Objective, Woburn, MA). Nano ESI-MS analyses of the samples were performed on a Synapt G2_Si mass spectrometer, and the data were recorded with the MassLynx 4.2 Software (both Waters, UK). Mass spectra were acquired in the positive-ion mode by scanning an m/z range from 100 to 5000 Da with a scan duration of 1 s and an interscan delay of 0.1 s. The spray voltage was set to 3 kV, the cone voltage to 50 V, and the source temperature 80°C. The recorded m/z data were then deconvoluted for single peaks into mass spectra by applying the maximum entropy algorithm MaxEnt1 (MaxLynx) with a resolution of the output mass 0.5 Da/channel and the Uniform Gaussian Damage Model at the half height of 0.7 Da. Data were acquired at the Functional Genomics Center Zurich.

Antibodies used. The recombinant therapeutic antibody Herceptin® (trastuzumab) was obtained from Genentech. Antibodies used for flow cytometry included mouse anti-CAR (clone RmcB, Millipore 05-644), goat anti-human IgG-AlexaFluor 647 (Invitrogen A-21445) and goat anti-mouse IgG AlexaFluor 488 (Invitrogen A-11001). Antibodies used for ELISA included goat anti-human IgG (Invitrogen 31119) and rabbit anti-goat IgG-alkaline phosphatase (Sigma A4187). Reagents used for Western blotting included goat anti-human κ-chain F(ab')₂-biotin (Sigma SAB3701413), anti-GAPDH (Santa Cruz sc-32233), avidin-alkaline phosphatase (Sigma A7294) and goat anti-mouse IgG-alkaline phosphatase (Sigma A3562).

The primary antibodies used for IHC of cleared tissues were rat anti-F4/80 (Thermo MA516630), rabbit anti-vWF (Thermo PA516634), rabbit anti-vimentin (Thermo MA516409), rabbit anti-human ErbB2 (Thermo PA5-14635) and mouse anti-GFP-biotin (clone: GF28R; Thermo MA5-15256-BTIN). The secondary reagents used for IHC of cleared tissues included goat anti-rabbit - AlexaFluor 405 (Thermo A31556), goat anti-rat IgG-AlexaFluor 405 (Abcam ab175671) and streptavidin-AlexaFluor 405 (Thermo S32351).

Flow cytometry. For reporter analyses of transduced cells, the cells were washed with 0.5 mL Dulbecco's PBS/well, then detached by addition of 0.5 mL trypsin-EDTA solution (Sigma T3924) per well and incubated at 37°C for 3-5 minutes. The trypsinized cells were transferred into 0.5 mL complete medium, centrifuged at 300 × g for 3 minutes at 4°C, and washed with 1.5 mL cold PBS containing 1% BSA (bovine serum albumin; PBS/BSA).

To determine cell surface receptor expression, 80-100% confluent T150 flasks of cells were harvested in bulk by trypsinization (5 mL per flask for 3-5 minutes). Cells were transferred into conical tubes containing complete medium, centrifuged at 300 × g for 3 minutes at 4°C, washed in cold PBS/BSA, resuspended in cold PBS/BSA and passed through a 70 µm (SPL Life Sciences 93070) cell strainer. For each staining condition, 1 × 10³ cells were added to each well of V-bottom flow cytometry plates (Greiner 651201), centrifuged at 300 × g for 3 minutes, and supernatants were aspirated. The cells were resuspended in 80 µL titrated dilutions of primary antibody in PBS/BSA (100 nM to 160 pM for TZB, or 1:62,500 to 1:100 for anti-CAR) and incubated in plates at 4°C for 1 hour with gentle orbital shaking. Cells in each well were washed twice with 150 µL PBS/BSA pelleting cells at 1,000 × g for 5 minutes, 50 µL of a 1:200 dilution of the corresponding fluorophore-conjugated secondary antibody was added, and plates were incubated for 30 minutes at 4°C with gentle orbital shaking. Cells were washed twice with 150 µL PBS/BSA, then resuspended in 100 µL PBS/BSA per well. Sample readings were acquired without cell fixation on an LSRII Fortessa flow cytometry with FACSDiva software (Becton Dickinson) using a high-throughput sampler, and data were analyzed with FCS Express 5 (De Novo Software).

Enzyme-linked immunosorbent assay (ELISA). Nunc MaxiSorp flat-bottom plates (Thermo Fisher 44-2504-21) were washed in PBS, and then coated with 100 μ L per well of 50 nM HER2 extracellular domain (Sino Biological 10004-HOSH) or 1 μ M hen egg lysozyme (Sigma L6876) for 12-16 hours at 4°C with gentle rocking. Following coating, plates were blocked in 10% nonfat dry milk (Applichem A0830) in PBS for 1 hour at 25°C with gentle rocking. Plates were washed five times with PBS-T (1 \times PBS pH 7.4 + 0.1% Tween) using a Biotek ELx405 plate washer (each cycle: aspirate, shake for 5 s at intensity level 3, soak for 15 s), then titrated standards (Herceptin® or purified D1.3), blanks or sample dilutions (cell culture supernatants or mouse plasma) were added in triplicate in 100 μ L volumes per well. For mouse plasma ELISAs, naïve Fox Chase SCID beige plasma was spiked into wells for obtaining the standard curve at the same dilution as the samples that were interpolated from it (1:5, 1:10, 1:20, 1:100 or 1:2,500 in PBS). For cell culture supernatants, samples were analyzed undiluted and diluted 1:50 in PBS. Samples were incubated at 25°C for 2 hours with gentle rocking, then plates were washed 5 times as before. Goat anti-human IgG (Invitrogen 31119) was diluted 1:2,000 in 2% nonfat dry milk made in PBS-T and 100 μ L was added to each well. Plates were incubated at 25°C for 1 hour with gentle rocking, then washed 5 times as before. For detection, rabbit anti-goat IgG-alkaline phosphatase was diluted 1:10,000 in 2% nonfat dry milk made in PBS-T and 100 μ L was added to each well. Plates were incubated at 25°C for 30 minutes with gentle rocking, then washed 5-times again. Signal was detected following addition of 100 μ L/well 3 mM para-nitrophenyl-phosphate (4-NPP) substrate in 4-NPP buffer (50 mM NaHCO₃, 50 mM MgCl₂) by reading absorbance at 405 nm using 540 nm as a reference wavelength until an optimal signal was reached, typically 1-4 hours after incubation at 25°C with gentle orbital shaking. Absorbance was normalized to the reference wavelength and blank wells, and concentrations were determined by interpolation of a corresponding standard curve using a sigmoidal 4-parameter logistic fit [Sigmoidal, 4PL, x is log(concentration)] using GraphPad Prism 8 software. For *in vitro* transductions, ELISA data were further normalized to transduction efficiency (*i.e.*, pg of antibody produced in each well per GFP-positive cell).

Viral titer determination. Viral physical titers were determined by measuring absorbance at 260 nm on a Nanodrop One Microvolume UV Spectrophotometer (ThermoFisher) using the following equation: Virus concentration [vp/mL] = $1.1 \times 10^{12} \times A_{260} \div (\text{path length})$, where the path length is 0.1 cm and the absorptivity for purified virus is 1.1×10^{12} particles per OD unit (ref.¹²). Infectious titers were determined via quantitative PCR (qPCR). A549 cells were seeded with 5×10^4 cells per well in 0.5 mL DMEM medium supplemented with 10% fetal calf serum (Amimed, Bioconcept) in 24-well plates 24 hours pre-infection. Purified virus (3 μ L) was added to each well and cells were incubated at 37°C, 5% CO₂ for two hours before the medium was aspirated, cells were washed with 1 mL DPBS (Sigma) per well and genomes were extracted from cells using a DNA isolation kit (Genkam cat no. SB0072) according to the manufacturer's protocol. The number of adenoviral genomes was then determined by qPCR with the following adenovirus serotype-5-specific primers (0.5 μ M each) in 20 μ L volumes using PrimeTime® Gene Expression Master Mix (IDT Cat No. 1055770) with a Stratagene Mx3005P (Agilent) instrument: Ad_fwd: 5'-GTGATAACCGTGTGCTGGAC-3' and Ad_rev: 5'-CAGCTTCATCCCATTGCAA-3' as well as a hexon sequence-specific HEX™ (Hexachloro-Fluorescein)-labeled probe: 5'-TCCGCGCGTGCTGGACAGG-3'. The following program was used: 10 min at 95°C, 0.5 min at 95°C, 1 min at 56°C and 1 at 72°C for 50 cycles, each cycle starting at the second step with DNA quantification after the third step. Ct values were determined using the MxPro Mx3005P software with the automated single-threshold mode.

Electron microscopy. Prior to imaging, Zebra Spin Desalting Columns (7K MWCO, Thermo Scientific Cat. No. 89877) were used to exchange the adenoviral storage buffer into 20 mM HEPES, 150 mM NaCl, pH 6.8. Viral particles were irradiated under a UV light for 1 min, then samples were negative-stained by spotting 10 μ L droplets on copper grids (300 mesh, glow discharged) for 1 min, then overlaid with 10 μ L droplets of 1% uranyl acetate for 1 min. The excess uranyl acetate was removed with filter paper, and the grid was allowed to air dry. Grids were examined with a CM100 transmission electron microscope (Thermo Fisher Scientific, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV using an Orius 1000 digital camera (Gatan, Munich, Germany).

Samples were negatively stained and imaged by the Center for Microscopy and Image Analysis (ZMB) at the University of Zurich.

Stable cell line generation. The BT474-TdTomato stable cell line was generated by transduction of BT474 cells with a lentivirus encoding TdTomato (rLV.EF1.tdTomato-9; Takarabio, 0036VCT). Following transduction, cells were passaged three times, then harvested by trypsinization and washed 2-3 times with PBS containing 1% BSA (bovine serum albumin). Cells were sorted once by fluorescence-activated cell sorting (FACS) using a BD FACSMelody cell sorter and cells expressing medium-high levels of TdTomato were further expanded for experiments.

Mice housing conditions. Animals were housed according to Federation of European Laboratory Animal Associations (FELASA) recommendations under BSL2 conditions: mice were placed in individually ventilated type III plastic cages (425 × 266 × 150 mm, floor area 820 cm², 2-5 animals per cage) with sterile dust-free wooden bedding (80–90 g per cage; Schill AG, Muttenz, Switzerland), a red translucent plastic mouse house and paper tissues (2 per cage) for nesting. Cages were individually ventilated with filtered air (15 complete air changed per hour, 50 Pa, HEPA H 14 filter, Vokes-Air, Uster, Switzerland). Mice were provided a pelleted mouse diet (Kliba No. 3431, Provimi Kliba, Kaiseraugst, Switzerland) and sterilized drinking water *ad libitum*. The room was kept at 21 ± 1 °C, 50 ± 5% relative humidity with a 12 h light / 12 h dark cycle (40 lux artificial light from 07:00 to 19:00 h). All animal experimentations were approved by the Cantonal Veterinary Office (Nr.221/2014 and 237/2017, Zurich, Switzerland) and carried out in accordance with Swiss animal protection laws and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe no. 123 Strasbourg 1985).

Tumor lysis. Upon xenograft study termination, tumors from mice not used for CLARITY analysis were excised, frozen on dry ice and stored at -80°C. Samples were thawed on ice and 200-250 mg sections were cut into eighths and transferred into 2 mL microcentrifuge tubes (Eppendorf 0030.120.094) containing 5 mm stainless-steel beads (Qiagen 69989) and a 5× volume-to-weight ratio of lysis buffer [50 mM Tris-phosphate pH 7.8, 4 mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (DCTA), 20% glycerol, 2% Triton X-100] was added (*i.e.*, 1 mL for every 200 mg). Tissue was homogenized using a Qiagen TissueLyser with cassettes pre-cooled to -20°C (25 Hz for 2 cycles of 10 minutes, rotating the cassette 180° between cycles). Tubes were centrifuged at 3,200 × *g* for 30 minutes at 4°C, supernatants were transferred into 1.5 mL microcentrifuge tubes and further cleared by a second centrifugation step. The protein concentration of cell lysates was determined with a MicroBCA™ Protein Assay Kit (Pierce 23235) according to the manufacturer's protocol with lysates diluted 1:200 interpolated from a standard curve generated from a BSA standard diluted in PBS containing 1:200 lysis buffer. Lysates were sub-aliquoted, frozen on liquid nitrogen, and stored at -20°C.

Western blots. For each gel lane, 20 µg of tumor lysate was diluted in 1× TGX running buffer (BioRad 161-0772) containing 60 mM DTT and a cocktail of protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 48 µg/mL; pepstatin-A, 1 µg/mL; and leupeptin, 5 µg/mL). Laemmli sample buffer 4x concentrate was added to a final concentration of 1× (Bio-Rad 161-0747), and samples were heated to 99°C for 10 minutes. Samples were cleared by centrifugation at 16,100 × *g*, and 20 µg total protein was loaded into each well of a Criterion TGXTM Precast Midi Protein Gel (4-20%, Bio-Rad 5678095) in 1× TGX buffer after incubation at room temperature for 30 minutes. PageRuler™ Protein Ladder (3 µL) was used as a molecular weight marker (Thermo 26616). Samples were transferred on to a methanol-activated Immobilon-P PVDF transfer membrane (Merck IPVH00010) using a Bio-Rad Trans-Blot Turbo Transfer system (2.5 A, 7 min). The membrane was blocked in 1× Sigma Casein Blocking Solution (Sigma Cat. No. B6429) in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.5) for 30 min at room temperature with gentle orbital shaking. After blocking, the membrane was cut just below 35 kDa, and incubated with either a goat anti-human κ-chain (1:3,000; bottom piece of blot) or a mouse anti-GAPDH mAb (1:3,000; top piece) in antibody dilution buffer (1× Sigma Casein Blocking Solution in TBS-T buffer) for 12-16 hours at 4°C with gentle orbital shaking. Membranes were washed 4× over 20 minutes with TBS-T (1X TBS with 0.05% Tween-20), then incubated with either avidin-alkaline phosphatase (1:100,000;

bottom piece) or a goat anti-mouse IgG AP-conjugated antibody (1:10,000; top piece) in antibody dilution buffer for 1 hour at room temperature. Membranes were washed 4× over 20 minutes with TBS-T, then imaged on a Vilber Fusion Fx Spectrometer after addition of CDP-Star® Chemiluminescent Substrate (Sigma C0712).

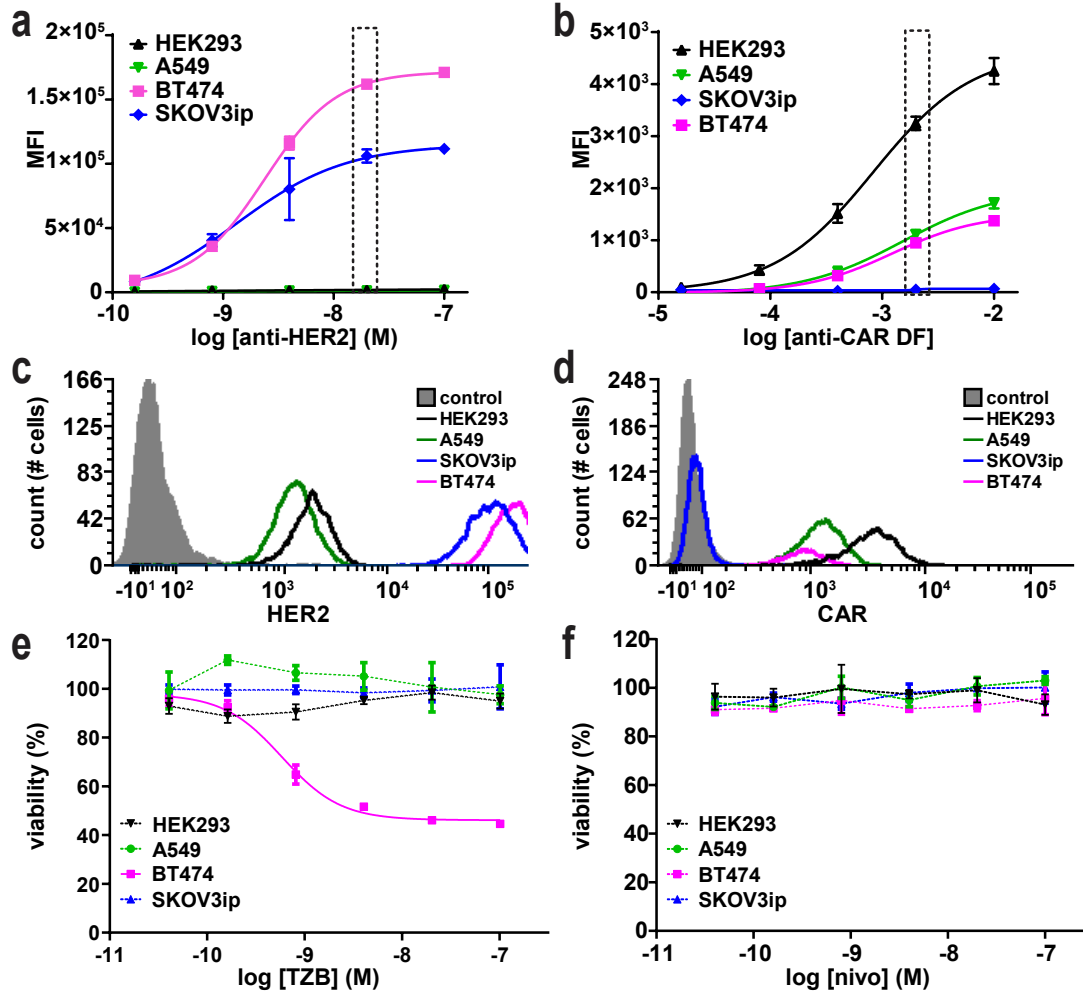


Fig. S1 | Characterization of surface marker expression levels and trastuzumab sensitivity in HER2-positive tumor cell lines. (a) Cell surface levels of human epidermal growth factor receptor 2 (HER2) by flow cytometry staining with the therapeutic anti-HER2 antibody trastuzumab: human embryonic kidney cells (HEK293), human epithelial lung carcinoma (A549), human ovary adenocarcinoma (SKOV3ip) and human mammary gland ductal carcinoma (BT474). Mean fluorescence intensity (MFI) is normalized to cells stained with only secondary antibody. Error bars represent standard deviation for $n=3$. Curves were fit using GraphPad Prism software (version 8.3.1) (non-linear regression four parameter logistic, model where x is $\log(\text{concentration})$). The dotted box indicates the staining concentration where flow cytometry histograms are shown. (b) Cell surface levels of the native primary receptor for Ad5, CAR, of various cell lines by flow cytometry staining with an anti-CAR antibody. Mean fluorescence intensity (MFI) is normalized to cells stained with only secondary antibody. Error bars represent standard deviation for $n=3$. Curves were fit using GraphPad Prism software (version 8.3.1) (non-linear regression four parameter logistic, model where x is $\log[\text{dilution factor}]$). The dotted box indicates the staining concentration where flow cytometry histograms are shown. (c) Representative flow cytometry histograms of cell lines stained with a concentration of 20 nM trastuzumab (from the titration above). (d) Representative flow cytometry histograms of cell lines stained with a 1:500 dilution of the anti-CAR antibody. (e) Cell viability assay (XTT) of cell lines treated with recombinant trastuzumab (Herceptin[®], Genentech), or (f) a control antibody (anti-human PD1, nivolumab Opdivo[®], BMS). Error bars represent standard deviation for $n=3$ replicates. Only the curve for BT474 cells treated with TZB (solid magenta line) was fitted using GraphPad Prism software (version 8.3.1) (non-linear regression four parameter logistic, model where x is $\log[\text{dilution factor}]$).

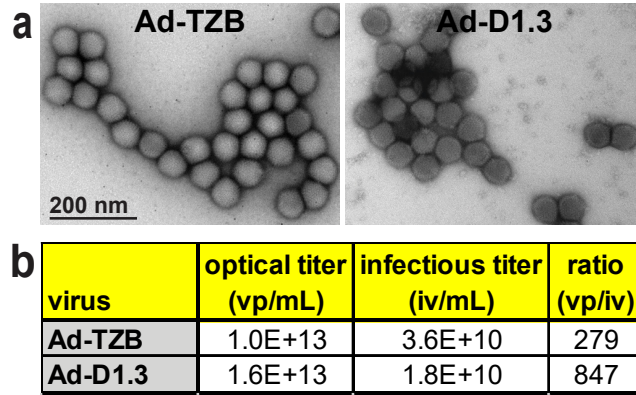


Fig. S2 | Generation of antibody-encoding Ad5. (a) Transmission electron micrographs (TEM) of uranyl acetate negative-stained Ad-TZB (*left*) and Ad-D1.3 (*right*) viral particles. (b) Table showing physical and infectious viral titers for the Ad-TZB and Ad-D1.3.

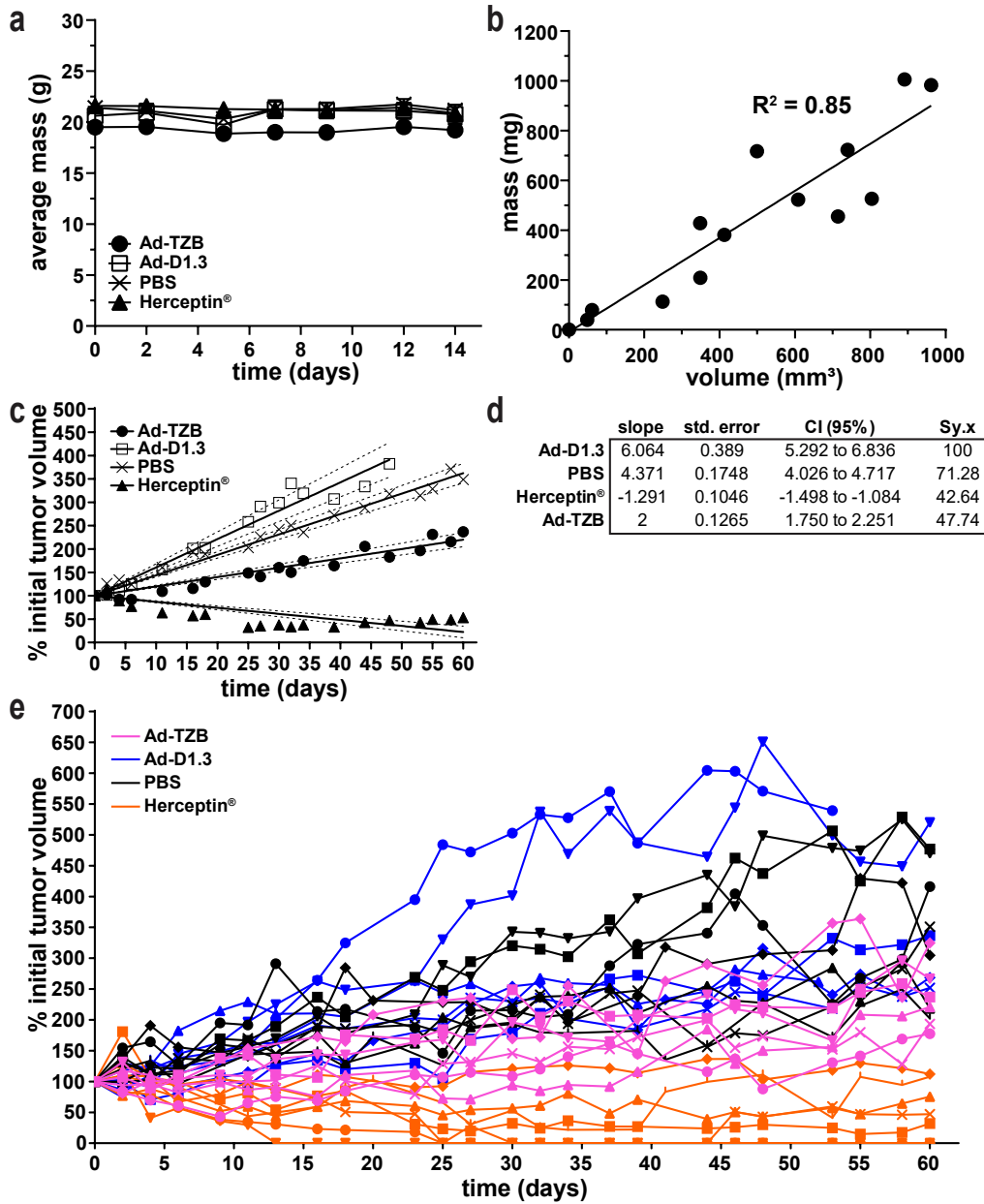


Fig. S3 | Additional metrics from animal xenograft experiments. (a) The body masses of mice in all treatment groups were monitored for two weeks post treatment initiation to track any acute toxicity of therapy. No significant drop in weight was observed in any treatment group. (b) A linear correlation plot is shown for tumor volumes measured pre-euthanasia with calipers versus the mass of excised tumors post euthanasia. Data were fit with a linear regression model using GraphPad Prism software (version 8.3.1) and gave $R^2 = 0.853$. (c) Tumor outgrowth shown in Fig. 2b was analyzed by applying a linear regression analysis using a mixed effects model using GraphPad Prism software (version 8.3.1). Dotted lines show the 95% confidence bands of the fit (solid line). (d) Summary table of the regression analysis. The slopes of the fits show non-overlapping confidence intervals across all treatment groups. (e) Tumor outgrowth for individual mice from averaged data shown in **Fig. 2b** 60 days post-initiation of treatment for Ad-TZB ($n=6$, magenta), Ad-D1.3 ($n=7$ until day 16, then $n=6$ until day 48 when mice reached euthanasia criteria, blue), PBS ($n=7$, black), and Herceptin® ($n=7$, orange).

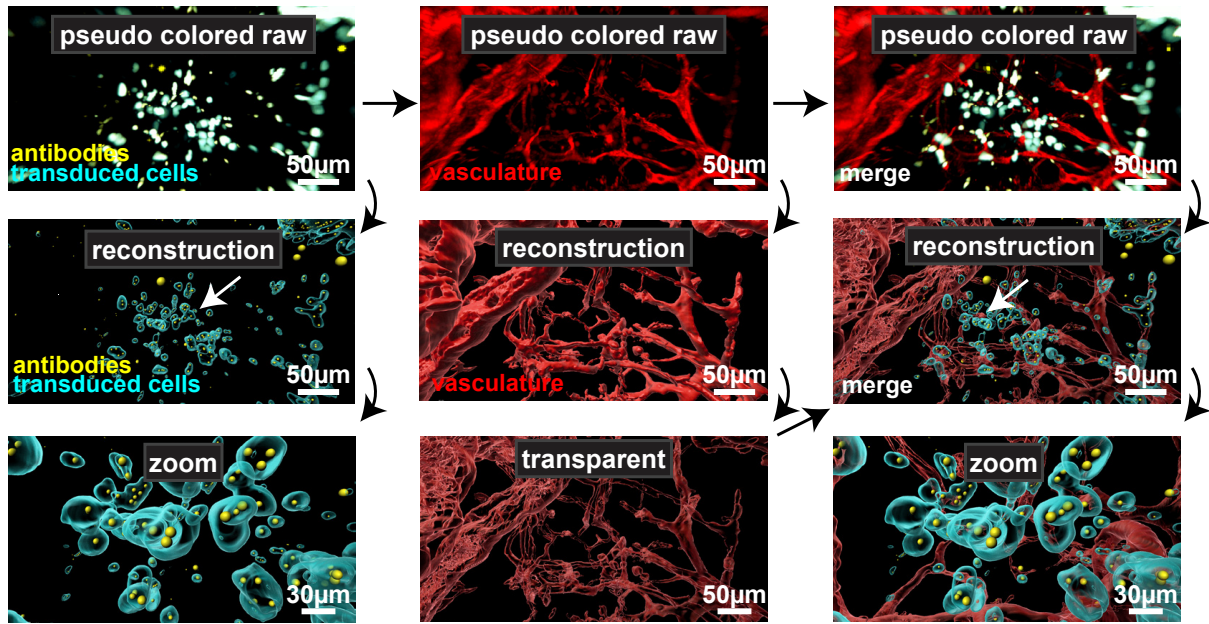


Fig. S4 | 3D rendering profiles of confocal images of vasculature, cells and secreted antibody. The top row shows pseudo-colored raw fluorescent images of transduced cells (*cyan*), secreted antibodies (*yellow*) and vasculature, and their merge. The image conversion from raw fluorescence signal to digital reconstruction is shown from top to middle row in each column. The bottom-most row shows the rendered zoom views in the left and right column, and a transparent view of the rendered vasculature in the middle column (vasculature). The arrows indicate the workflow.

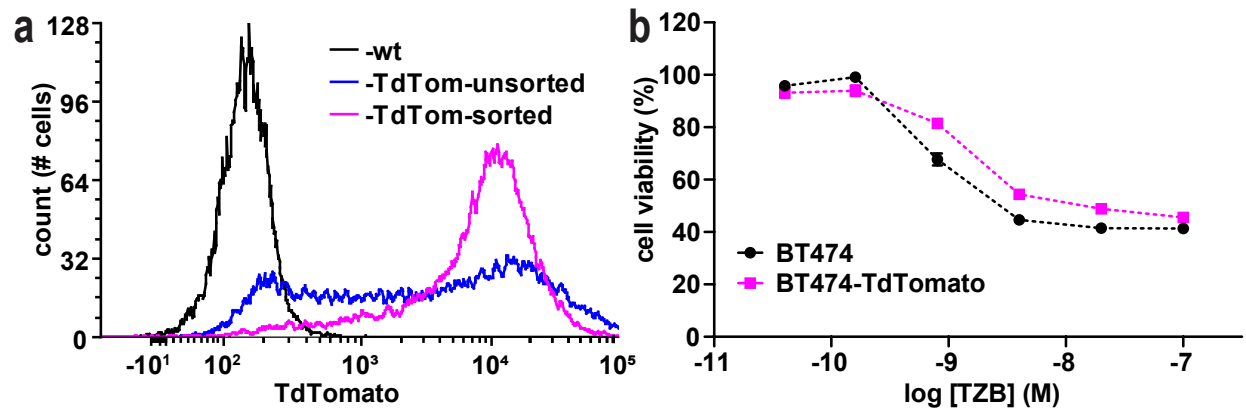


Fig. S5 | Generation of the BT474-TdTomato cell line and sensitivity to TZB. (a) The BT474-TdTomato cell line was generated to track transduction biodistribution in mouse tumors. BT474 cells were transduced with a lentivirus that encodes TdTomato (rLV.EF1.tdTomato-9). Cells were sorted for a population that expresses moderate TdTomato immediately prior to transduction. Histograms show the TdTomato reporter expression in the cell line pre- (*blue*) and post- (*magenta*) sorting in comparison to the parental cell line, BT474 (*black*), which does not express TdTomato. (b) Cell viability assay (XTT) of BT474 (*black*) and BT474-TdTomato treated with recombinant trastuzumab (Herceptin[®], Genentech). Both cell lines show similar responsiveness to TZB. Error bars represent standard deviation for n=3 replicates.

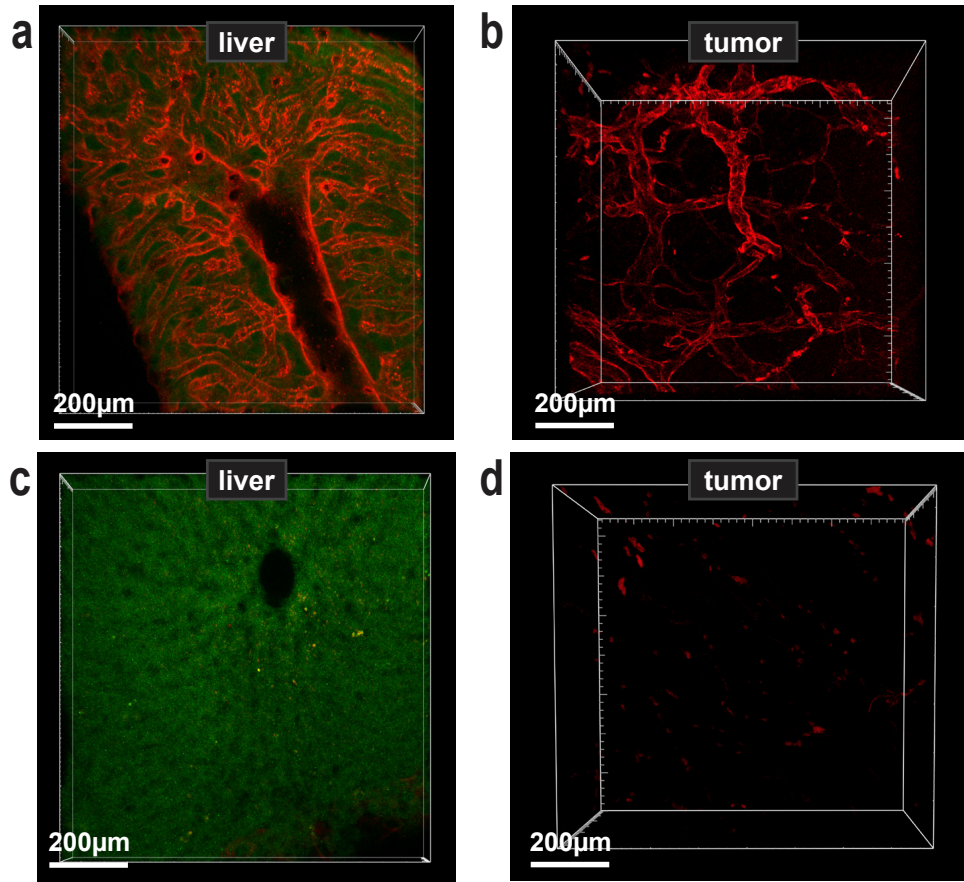


Fig. S6 | Perfusion-fixation quality control criteria. For all tumor samples analyzed throughout this study, livers from corresponding mice were harvested following vasculature labelling and perfusion-fixation. Mice which had inadequate tissue processing (*e.g.*, poor vasculature labelling or fixation) were excluded from image analysis and therapeutic effect characterization to ensure that differences between treatment groups were indeed due to the therapeutic intervention rather than tissue preparation. **(a)** An example liver staining from a mouse with adequate liver vasculature staining (*red*) and tissue quality (autofluorescence in *green*). **(b)** The corresponding tumor vasculature staining (*red*) to the liver in **(a)**. Vasculature aberrations here can be assessed as a result of the therapeutic intervention delivered since adequate quality is achieved in distal tissues. **(c)** An example of liver staining from a mouse with inadequate liver vasculature staining (*red*) and tissue quality (autofluorescence in *green*). **(d)** The aberrations in the corresponding tumor vasculature staining (*red*) to the liver in **(c)** could be the result of the poor tissue preparation rather than the therapeutic intervention; thus, these mice were excluded from the imaging analysis.

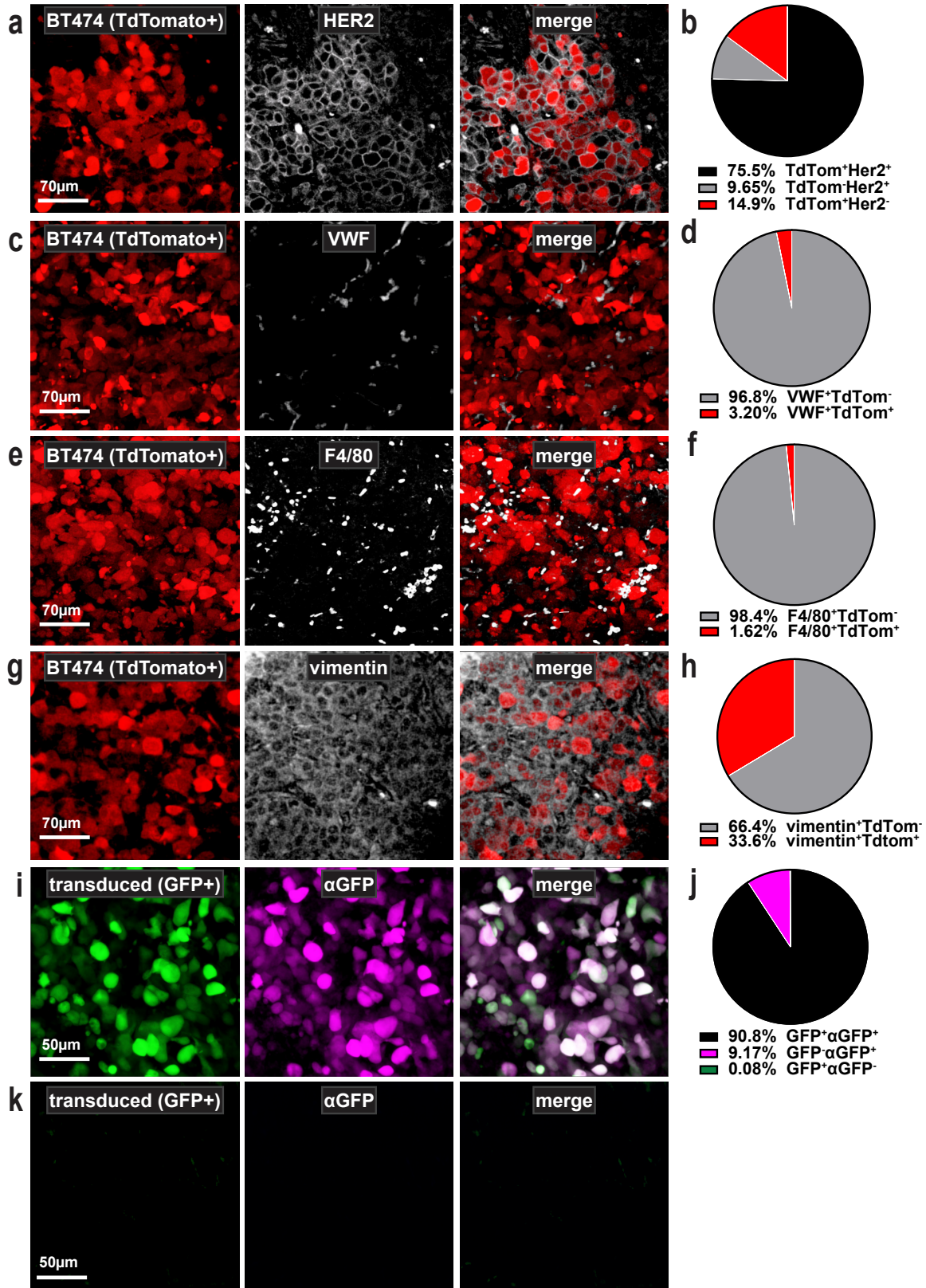


Fig. S7 | Characterization of endogenous fluorophore expression and IHC staining at day 11. (a) Untreated BT474-TdTomato tumors were stained with fluorescently labeled antibodies for

HER2 expression (*white*). Tumor cells were defined as cells that showed signal for the endogenous TdTomato and/or staining for human HER2. **(b)** The average composition of BT474 tumor cells by biomarker expression. Although the majority of tumor cells express both markers, expression becomes more heterogenous *in vivo*, thus requiring a multi-parameter characterization. Pie chart values represent the average from percentages calculated in n=3 mice. **(c-h)** Confocal imaging and quantification of PBS-treated tumor slices stained via IHC for various stromal markers. Pie chart values represent the average from percentages calculated in n=3 mice. **(c)** Overlay of TdTomato with vWF staining (*white*). **(d)** The percentage of vWF-positive cells in PBS tumors which co-express the TdTomato reporter. **(e)** Overlay of TdTomato with anti-F4/80 staining (*white*). **(f)** The percentage of F4/80-positive cells in PBS tumors which co-express the TdTomato reporter. **(g)** Overlay of TdTomato with anti-vimentin staining (*white*). **(h)** The percentage of vimentin-positive cells in PBS tumors which co-express the TdTomato reporter. **(i)** Overlay of the endogenous eGFP expression signal of transduced cells (*green*) with the fluorescence signal from an anti-GFP mAb (*magenta*) from an Ad-D1.3-treated mice. **(j)** Percent coverage of GFP signal to characterize Ad transduction via endogenous fluorescence. Pie chart values represent the average from percentages calculated in n=3 mice. Over 90% of GFP signal is sufficiently above the detection threshold and does not require amplification with a fluorophore-conjugated antibody. **(k)** No GFP signal (*green*) and anti-GFP signal (*magenta*) can be detected in mock-treated mice.

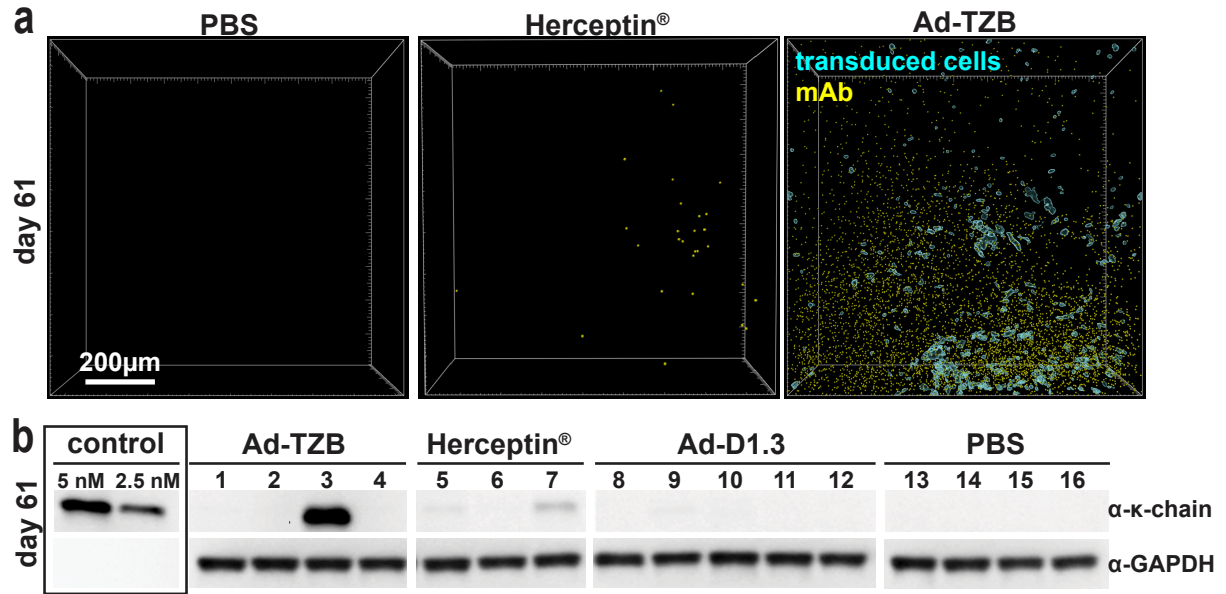


Fig. S8 | Detection of TZB-production at day 61. (a) A representative confocal image of PBS- (n=3), Ad-TZB- (n=3) and Herceptin-treated (n=2) tumors analyzed by confocal microscopy at the endpoint of the experiment (day 61). Remaining transduced cells (cyan; for Ad-TZB) and antibody (yellow) are shown. (b) Western blots of tumor lysates prepared from tumors extracted from a subset of mice on day 61. TZB is detected with an antibody that detects the κ -light chain (top row). The loading control shows staining for GAPDH (bottom row). The control lane shows recombinant TZB diluted in lysis buffer.

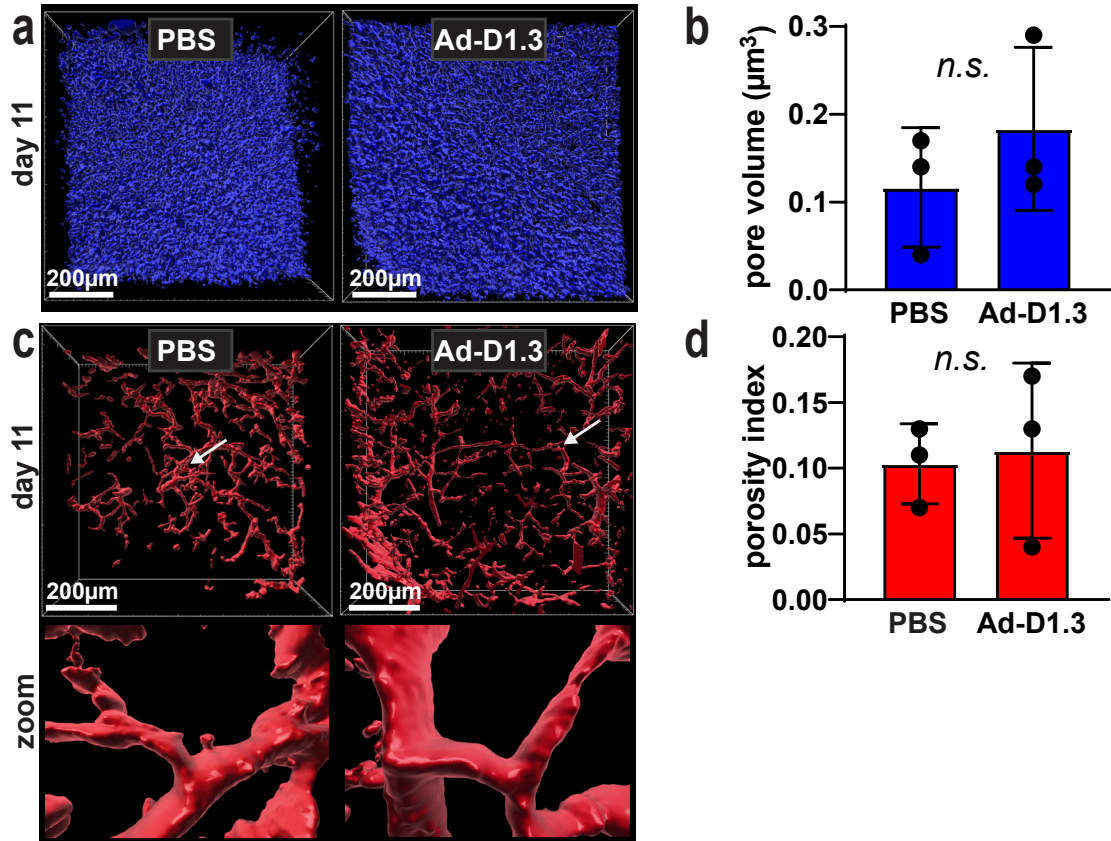


Fig. S9 | Therapeutic effects of the isotype control virus, Ad-D1.3. (a) Representative DAPI rendering (*blue*) showing tumor density of PBS (*left panels*) versus Ad-D1.3 (*right panels*) at day 11. As nearly all Ad-D1.3-treated mice reached euthanasia criteria prior to day 61, only day 11 could be analyzed. (b) Quantification of total pore volume (μm^3) in tumors as determined by measuring the total area within the tumor that does not stain positively for nuclear DAPI staining, excluding tissue margins. Bars represent the means, and error bars indicate standard deviation; data points from individual mice are shown as black dots. Data were analyzed by an unpaired t-test using GraphPad Prism software (version 8.3.1) (*n.s.*; $p > 0.05$). (c) Representative vasculature rendering (*red*) of PBS- (*left panels*) versus Ad-D1.3-treated (*right panels*) tumors at day 11. The region indicated by the white arrows is shown at 10 \times higher magnification in the zoom panels below each overview image. The vasculature images shown correspond to the same frames of samples shown in panel (a) above. (d) Quantification of vasculature porosity index (vessel pore volume/total volume) for PBS versus Ad-D1.3 11-days post treatment for $n=3$ mice. Bars represent the means, and error bars indicate standard deviation; data points from individual mice are shown as black dots. Data were analyzed by an unpaired t-test using GraphPad Prism software (version 8.3.1) (*n.s.*; $p > 0.05$).

Legends for Movies S1 to S5

Movies S1-4 (separate file) | Reconstruction of nuclei, infected cells, secreted antibody and vasculature: Visualization of the reconstruction procedure of raw confocal imaging data in IMARIS. The videos show full-resolution reconstruction of the 4 features quantified in paracrine delivery experiments: nuclei (**Movie S1**), vasculature (**Movie S2**), transduced cells (**Movie S3**), and secreted antibody (**Movie S4**). All features except for antibodies were rendered for visualization using the surfaces tool, while antibodies were rendered using the spot detector; however, for quantification, all features were measured using the surfaces tool (**Figures 6-7, S9**). The experiment was performed on 9 mice total for all samples.

Movie S5 (separate file) | 3D reconstruction of the SHREAD platform for paracrine delivery. Detailed visualization of vasculature (red), transduced cells (cyan), and secreted antibody (yellow) feature extraction using IMARIS from confocal data in a representative Ad-TZB-treated mouse at day 11. The video shows segmentation and feature demonstration on a subset of the whole dataset. Transduced cells and antibody can be seen distributed throughout the imaged section. Our PACT-confocal imaging enables reliable feature extraction down to the surface morphology for vasculature. The experiment was performed on 9 mice total for all samples.

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