**Supplemental Information (SI) Materials and Methods**

**CEA TCB production and purification.** CEA TCB was captured from harvested and clarified fermentation CHO clone supernatant by Protein A affinity chromatography (MabSelect SuRe) followed by a bind-elute cation exchange chromatography (Poros 50 HS). The eluate was subjected to a bind-elute hydrophobic interaction chromatography (Butyl-Sepharose 4FF) at room temperature. To obtain flow through conditions for the subsequent anion exchange chromatography (Q-Sepharose FF), the buffer of the eluate had to be exchanged by ultrafiltration/diafiltration. The flow-through obtained from anion exchange chromatography was transferred to a 20 mM histidine/histidine-HCl, 140 mM sodium chloride buffer at pH 6.0 by a second ultrafiltration/diafiltration.

**Determination of affinity and avidity of CEA TCB by Surface Plasmon Resonance (SPR).** The binding affinities of the CEA TCB for human CD3ε and human CEA (CEACAM5) were determined by SPR. The KD affinity values of binding of CEA TCB to CEA (B3 domain of human CEA) were 132 nM, those of binding to the human CD3eε/CD3eε.dimer were 75 nM. The avidity of the CEA antibody is 220 pM with a half-life of the bivalent binding up to 26 minutes for CEA. The half-life of CEA TCB for the monovalent binding to the respective CD3ε antigen is 5.7 minutes. Since the half-life of CEA TCB binding to CEA is six-fold higher than the binding to CD3, the molecule will display a preferential binding to CEA-expressing tumor cells rather than CD3-expressing T cells.

**Characterization of mutations, RER status and Gene-Expression Microarrays on cell lines used in Fig. 3.** PCR amplicons were sequenced directly using the appropriate PCR primers and Big Dye Sequencing kit on an Applied Biosystems 377 sequencer. The *KRAS* (codons 12, 13, 61, 117, and 146), *NRAS* (codons 12 and 13), *BRAF* (codon 600), *PIK3CA* (codons 542, 545, and 1047), *APC* (mutation cluster region), *TP53*, *CTNNB1* (exons 3, 4, 5, and 6) and *FBXW7* genes were analyzed. The cell lines were characterized for mismatch repair deficiency (replication error status: RER status) and four microsatellite loci, *BAT25*, *BAT26*, *D17S250*, and *D18S69* were amplified and used to determine RER status. Gene microarray expression analyses were performed using the Affymetrix Human genome U133+2 chips.

**Binding of CEA TCB to target cells as assessed by flow cytometry.** Binding of CEA TCB was tested on CEA-expressing target cells (MKN-45 cells, A549 expressing human CEA (A549-huCEA) or cynomolgus monkey CEA (A549-cyCEA), as well as CD3-expressing cells (Jurkat cells, primary human T cells (isolated from human PBMCs by negative magnetic isolation using the human Pan T Cell Isolation Kit II, Miltenyi Biotec), and primary cynomolgus monkey CD4 T cell line HSC-F generated from a foetal splenocyte immortalized by infection with herpesvirus saimiri subtype C). Briefly, adherent tumor target cells were trypsinized (0.05 % trypsin/EDTA; Gibco) and washed with PBS. Suspension cells were harvested and washed with PBS. Human pan T cells were isolated from human PBMCs by negative magnetic isolation (human Pan T Cell Isolation Kit II, Miltenyi Biotec), according to the manufacturers’ instructions. CEA TCB binding was assessed using 0.1 – 0.2 Mio cells/96-round-bottom well plate and incubated with the indicated antibody concentrations for 30 min at 4°C. Cells were washed twice with cold FACS buffer (PBS, 0.5 % BSA) and incubated with goat anti-human IgG Fcγ-specific secondary antibody for further 30 min at 4°C (1:50 dilution of #109-096-098 or #109-116-170, Jackson Immuno Research). Cells were washed twice with 150 µl cold FACS buffer and either measured directly, using a BD FACS CantoII or BD FACS Fortessa, or fixed with 2 % PFA-containing FACS buffer for 20 min at 4°C. Cells were resuspended in 150 µl cold FACS buffer/well and analyzed using BD FACS Diva or FlowJo softwares (Tree Star Inc.).

**Assessment of T cell proliferation and activation after tumor cell lysis.** Tumor target cells (10 000 cells/well) were incubated with PBMCs (pre-labeled with 2 mM CFSE, Sigma-Aldrich) and CEA TCB for 5 days (E:T 10:1). PBMCs were harvested, transferred into 96-round-bottom well plates, washed once and stained for 30 min at 4°C with an antibody mix of CD4, CD8 and CD25 (BD or BioLegend) according to the suppliers’ indications. After two washing steps, T cell proliferation and up-regulation of CD25 on CD4+ and CD8+ T cell subsets was determined by assessment of CFSE dye dilution by flow cytometry.

**Jurkat-NFAT reporter assay.** The capacity of CEA TCB to induce T cell cross-linking and subsequently T cell activation was assessed using co-cultures of CEA-expressing tumor target cells and Jurkat-NFAT reporter cells (Promega). 20 000 MKN-45 tumor cells/white-wall 96-flat-bottom plate (Breiner bio-one) were incubated with CEA TCB and Jurkat-NFAT effector cells (E:T of 5:1).Cells were incubated for 2, 4 and 6 h in a humidified incubator at 37°C followed by addition of an equivalent volume of ONE-Glo solution (Promega) and incubation for 10 minutes in the dark. Luminescence was detected using an ELISA plate reader (WALLAC Victor3 ELISA reader, PerkinElmer2030) with 5 sec/well detection time.

**Antibody-Dependent Cellular Cytotoxicity (ADCC).** MKN-45 tumor cells (30 000 cells/flat-bottom-96-well-plates) were incubated with PBMCs (E:T 25:1) and increasing concentrations of CEA TCB or equimolar concentrations of anti-CEA IgGs for 4 h in a total volume of 200 µl/well using a humidified incubator at 37°C. Cytotoxicity was assessed by detection LDH released into cell supernatants (Roche Applied Science).

**Complement-Dependent Cellular Cytotoxicity (CDC).** MKN-45 tumor cells (50 000 cells/flat-bottom-96-well-plates) were incubated with low-tox Rabbit complement (Cedarlane Laboratories Limited) and increasing concentrations of CEA TCB or equimolar concentrations of anti-CEA IgGs (for 10 min at RT), followed by addition of 50 µl of the diluted rabbit complement to obtain a final volume of 150 µl. The mixture was incubated for 2 h in a humidified incubator at 37°C. CDC was assessed by detection LDH released into cell supernatants (Roche Applied Science). AlamarBlue (Invitrogen was added to cells and incubated overnight in a humidified incubator at 37°C. Absorbance was measured using Wallac Victor 1420 Multilabel Counter (ex 584 nm, em 612 nm). Z138 cells (mantle cell lymphoma, a kind gift from Martin Dyer, University of Leicester) were used as positive control, seeded in separate wells and treated with the indicated concentrations of MabThera.

**Correlation between CEA expression and CEA TCB activity on a panel of 110 CRC cell lines.** The panel of colorectal cancer (CRC) cell lines used in Fig. 3 consist of 110 cell lines, including a number of duplicate cell lines that have been isolated from the same patient (indexed with the same number), Table S1. Cell lines were tested for PCR amplicons by direct sequencing using the appropriate PCR primers and Big Dye Sequencing kit on Applied Biosystems 377 sequencer. The *KRAS* (codons 12, 13, 61, 117, and 146), *NRAS* (codons 12 and 13), *BRAF* (codon 600), *PIK3CA* (codons 542, 545, and 1047), *APC* (mutation cluster region), *TP53*, *CTNNB1* (exons 3, 4, 5, and 6) and *FBXW7* genes were analyzed. Cell lines were also characterized for mismatch repair deficiency (Replication Error (RER) status) and 4 microsatellite loci (BAT25, BAT26, D17S250, D18S69) were amplified and used to determine RER status ([19](#_ENREF_19)). In addition, gene microarray expression analyses were performed using the Affymetrix Human genome U133+2 chips.

The CRC cell lines used for the assays were seeded from cryovials. The method used to maintain the frozen stock was conducted as described (Bracht, Nicholls et al. 2010). Human PBMC were isolated from leukocyte cones (filter from a unit of fresh whole blood) obtained from single donors (Blood and Transplant Service, John Radcliffe Hospital, Oxford, UK). Cells obtained from the leukocyte cone were diluted with PBS1X (1:10) and layered on Lymphoprep™ (Stemcell Technologies) in 50 mL falcon tubes. After centrifugation at 1800 rpm for 25 min, the PBMC layer was withdrawn from the interface and washed 4 x PBS. PBMC were counted, frozen in 10% DMSO in FCS under controlled-rate freezing conditions at 40 x 106 cells/mL and stored in liquid nitrogen until use. CEA TCB activity on a large panel of colorectal cell lines was assessed by plating cells directly into 96-well plates from frozen stock as well as using cryopreserved PBMCs. Cells were warmed quickly and transferred immediately into pre-warmed medium, centrifuged, and re-suspended in complete medium (DMEM, Iscoves or RPMI-1640 all supplemented with 10 % FCS and 1% penicillin/streptomycin) and plated at a density of 2.5 x 104 cells per well. Plates were then incubated at 37°C in a humidified 10% CO2 incubator. The next day, the medium was replaced by 100 μL of RPMI-1640 2% FCS with 1% glutamine and CEA TCB concentrations (50 μL) were then added in duplicate wells for each condition. The antibody dilutions and PBMC thawing were performed in RPMI 2% FCS with 1% glutamine. Final concentrations of CEA TCB ranged from 6.4 pM to 100 nM (1:5 titration). Fresh-thawed PBMCs were used for the assay (thawed from frozen vials within 2 hours of the assay start) and 50 μL (3 x 105) was added to each well to give an effector: target ratio of 10:1. Plates were incubated at 37°C for 48 h, spun at 1200 rpm for 4 min and 100 μL of the supernatant transferred to 96-well plates. Triton X100 (50 μL of 4%) was added to 150 μL of target cells to obtain maximum release values. The activity of CEA TCB was determined using the LDH Detection Kit (Roche) in accordance with manufacturer’s instructions. Percentage of specific cell lysis was calculated as [sample release - spontaneous release]/[maximum release - spontaneous release] x 100.

**Quantitative assessment of CEA expression (Qifikit analysis).** For the analysis of CEA surface expression, the Qifikit (DakoCytomation, Glostrup, Denmark) was used to calibrate the fluorescent signals and determine the number of binding sites. Cells were harvested and incubated for 30 min at 4 °C with anti-human CEACAM5 monoclonal antibody (0.5μg for 5 x 105 cells, sc-23928, Santa Cruz Biotechnology) or a respective mouse IgG1 isotype control (BD). Cells were washed twice with cold FACS buffer, and bound antibodies were stained using goat anti-mouse IgG provided by the Qifikit. Dead cells were excluded from the analysis using 4',6-diamidino-2-phenylindole (DAPI) staining. Calibration beads were prepared in parallel using the same procedure. Samples were analyzed using BD FACS CantoII, BD FACS Fortessa or CyAn™ ADP Analyzer (Beckman Coulter). Mean fluorescence intensities (MFIs) were obtained after data analyses using BD Diva or Summit 4.3 software and used to determine the relative number of antibody binding sites/cell based on a calibration curve (Qifikit calibration beads). Negative values obtained by Qifikit (no CEA expression) were replaced by an arbitrary value of 1 (Table S1).

**Confocal microscopy.** 100 000 MKN-45 target cells were seeded on microscope coverslip wells (80826 µ-Slide 8 Well, ibiTreat: #1.5 polymer coverslip, tissue culture treated, sterilized) and incubated at 37°C overnight. For therapy, medium was removed, wells supplemented with 400 000 NLV peptide-specific CD8 T cells (in medium without FCS), CEA TCB (10 nM) and incubated for 20 min at 37°C. Cells were fixed by PFA 2.64% for 30 min at 4°C, washed 3x PBS and fixed with MeOH (only for MTOC staining) for 5 min at -20°C. Cells were washed 3x PBS and blocked with PBS+10% FCS for >30 min at r.t.. Primary antibodies (diluted in PBS+10% FCS+0.1% Triton) were added for 1 h at r.t., cells washed 3x PBS and incubated with secondary antibodies (diluted in PBS+10%FCS) for 45 min r.t.. Cells were washed 1x PBS and incubated in Hoechst 33342 solution (1:1000 dilution in PBS) for 6 min at r.t., washed 1x PBS, incubated in MilliQ water for >15 min and mounted using Thermo Scientific Shandon Immu-Mount media (#990402). Fluorescence z-stacks were measured with a Zeiss LSM 700 microscope. 3D reconstruction and image analysis were done with Imaris. Primary antibodies (all mouse): y-tubulin (GT4511; Abcam), talin (8DV; Sigma), CD8 (OKT-8; E-bioscience), perforin (DeltaG9; GeneTex). Secondary antibodies against IgG1 (AlexaFluor 568; Life Technologies), IgG2b (AlexaFluor 488; Life Technologies), IgG2a (AlexaFluor 647; Life Technologies). Confocal microscopy assessing the binding and internalization of CH1A1A IgG (the CEA binder included in CEA TCB) was performed by incubating 10 g/ml of the antibody with MKN45 cells for 45 min at 4°C (to allow binding). The excess of the antibody was washed using ice-cold phosphate buffer saline (PBS). One part of cells was immediately fixed on ice using ice-cold PFA, 4%. The rest of the cells were incubated in complete medium (DMEM + 10% FCS) for further 23 h in humidified cell incubator at 37°C, 5% CO2. At the end of incubation time, cells were fixed using PFA 4%, permeabilized using 0.03% Triton X-100 and subjected to staining. The anti-CEA antibody was detected using goat anti-human IgG secondary antibody conjugated to Alexafluor488 (green). Early endosomes were stained using the early endosome marker (EEA1) followed by incubation with rabbit anti-mouse IgG secondary antibody conjugated to Alexafluor594 (red).

**FRET competition assay for the assessment of binding in presence of shed CEA (sCEA).** 1000 Hek-EBNA cells expressing human CEA-SNAP, labeled with Lumi4Terbium (Cisbio) were transferred to each well of a 384 well plate and mixed with 12.5 nM CH1A1A IgG labeled with d2. Subsequently, soluble/shed human CEA (AbD Serotec) was added at concentrations ranging from 0.625 to 40 g/ml and incubated for 2 h at room temperature. Upon excitation at 340 nm and a delay time of 50 sec the emission signal of the donor fluorophor (Terbium) and the acceptor fluorophor (d2), at 620 and 665 nm respectively, was determined using an Infinite F200 Reader (Tecan). The ratio of 620 nm to 665 nm was calculated and the ratio of the control (consisting of 1000 cells/well) was subtracted.

**Single-dose pharmacokinetic analysis (SDPK).** SDPK of CEA TCB was evaluated in NOG mice after a single 0.5 mg/kg iv bolus administration. A biphasic decline was observed with a half-life of 5.9 days (NCA analysis) and clearance of 12 mL/d/kg (2-compartmental model). The Vc was 28 mL/kg with a Vss of 94 mL/kg. The clearance was faster than expected compared to a normal untargeted IgG and the Vc slightly lower than expected. A two-compartmental model was fit to the PK data and the higher dose level of 2.5 mg/kg as well as the repeat dose simulations were performed. Steady state was approximately reached around 5 doses. The Cmin remains above 7 ug/mL (0.5 mg/kg biweekly) and 34 ug/mL (2.5 mg/kg biweekly) after 4 doses and above 14 ug/mL (2.5 mg/kg weekly) after two doses of CEA TCB. Phoenix v6.2 from Pharsight Ltd was used for PK analysis, modelling and simulation.

**Preparation of tumor cells and of human PBMC for in vivo efficacy experiments.** LS174T human colon carcinoma cells, originally obtained from ATCC, were stably transfected with a plasmid coding for firefly luciferase to generate LS174T-fluc2 cells that were cultured in DMEM containing 10 %FCS (Gibco) and 3 ug/ml Puromycin (Gibco) at 37°C in humidified incubator at 5% CO2. Cells were trypsinized and resuspended in RPMI medium (InvitroGen) for injection in a total volume of 100µl. Buffy coats (Blutspende Zürich) from healthy volunteers were used to isolate human PBMCs by conventional Histopaque gradient (Sigma-Aldrich). Cells were resuspended in serum-free RPMI medium for in vivo injection.

**Ex vivo flow cytometry analysis.** Upon explant, one part of the tumor tissue was processed using gentleMACS C-tube filled with 5 ml of RPMI medium containing Collagenase V (1mg/ml), Dispase II (0.64mg/ml) and DNAse I (0.025mg/ml) (SIGMA Aldrich). C-tubes were placed into the gentle MACS dissociator and processed using the appropriate program. The suspension was incubated for 30 min at 37°C before applying a second step of dissociation using the gentleMACS dissociator. The suspension was filtered using a Cellstrainer (70 m) to remove clumps. Cells were resuspended in FACS buffer (PBS, 2% FCS, 2 mM EDTA) and an aliquot was used for subsequent flow cytometry analysis. Cells were stained with anti-huCD45, anti-huCD3, anti-huCD25 and anti-huPD1 (all from Biolegend) for 20 min at 4°C in a total volume of 100 l of FACS buffer (PBS, 2% FCS, 2mM EDTA). After a washing step, the tumor cell suspension was stained with DAPI (4',6-diamidino-2-phenylindole) to exclude dead cells and analyzed at the LSR Fortessa (BD biosciences). Blood samples were lysed for erythrocytes using the BD lysis buffer (BD Pharm Lyse) and stained following the same procedure used for the tumor single cell suspension.

**Isolation of splenocytes and flow cytometry analysis.** Murine splenocytes were isolated from spleens of C57BL/6, transferred in gentleMACS C-tubes (Miltenyi biotec) containing MACS buffer (PBS 0.5 % BSA, 2 mM EDTA) and dissociated using the GentleMACS Dissociator (Miltenyi biotec). After centrifugation, cells were passed through a 100 µm nylon cell strainer (Miltenyi biotec), washed, further passed through a 70 µm nylon cell strainer (Miltenyi biotec) and washed with RPMI1640, 10 % FCS (Invitrogen). Erythrocytes were lysed by incubation in ACK Lysis Buffer (0.15 M NH4CL, 10 mM KHCO3, 0.1 mM EDTA, pH 7.2-7.4) for 5 min at room temperature, splenocytes washed and used for subsequent FACS staining. For binding of CD3 antibodies to splenocytes, non-specific Fc-mediated interactions were blocked by incubating splenocytes with anti-mouse CD16/CD32 antibody mix (# 553142, BD) for 15 min on ice. Cells were washed and CD3 binding performed as described for CEA TCB. After incubation with the goat anti-human IgG Fcγ-specific secondary antibody, cells were washed and incubated for 30 min at 4°C with diluted FITC anti-mouse TCRβ antibody (BioLegend), according to manufacturers’ instructions. Cells were washed and fixed in 2% PFA-containing FACS buffer (PFA, Sigma) and analyzed using a BD FACS CantoII equipped with FACS Diva software.

**Cell culture and preparation of cells for two-photon in vivo imaging.** LS174T human colon carcinoma cells stably expressing RFP (LS174T-RFP cells) were generated in house by transduction with lentiviral vectors. Cells were cultured in DMEM containing 10% FCS and 4 ug/ml Puromycin (Gibco) under standard cell culture conditions (37°C, 5% CO2). For live cell imaging experiments, tumor cells were harvested by trypsinization and mixed with CFSE-labeled T cells at an E:T ratio of 3:1. Cells were re-suspended in RPMI without further supplements to a final concentration of 1x106 tumor cells, 5x106 CFSE-labeled T cells and 5x106 PBMC (without T-cells) per 30 ul (injection volume).

**T cell isolation from PBMC and CFSE staining for in vivo two-photon imaging.** Isolated PBMCs were re-suspended in MACS buffer and T cell isolation performed using the pan T cell isolation kit (Mylteni Biotec). Isolated T cells and column eluted PBMC were re-suspended in PBS at a final concentration of 1x106/ml. For CFSE staining, T cells were incubated for 10 min at 37°C in the presence of 1uM CFSE. The staining reaction was stopped by diluting the solution in RPMI complete medium. Stained T cells were washed twice before being added to the previously collected PBMC at a 1:1 ratio.

**In vivo two-photon imaging.** 2P microscopy was performed on a Zeiss 710 combined confocal and multiphoton system. Mice were gas- anesthetized by isoflurane, placed on a heating pad and fixed onto the microscopic stage. During the time of an experiment, the body temperature of the study animals was monitored and kept constant at 37°C using a rectal temperature probe. Simultaneous excitation of CFSE, SHG and Alexa 645 was achieved at an excitation wavelength of 880 nm while dsRed was excited at 760 nm. For time-lapse imaging, sequential 3D stacks were acquired at step sizes of 5 um. Images were reconstructed and analyzed using ImageJ. Fluorescence reflectance imaging was employed to monitor tumor size and total CFSE signals in the skinfold chamber. For this purpose, experimental animals were gas-anesthetized with isolflurane and placed in the IVIS imaging chamber. Spectral scans were performed using excitation from 465 - 645 nm and detection from 520 – 780 nm. Quantitative analysis of the fluorescent signal was carried out using the Living Image (Perkin Elmer) software. See supporting information for cell culture and preparation of cells for imaging, T cell isolation from PBMC and CFSE staining and skinfold chamber surgery and tumor cell injection.

**Skinfold chamber surgery and tumor cell injection.** The skinfold chamber surgery was performed according to the procedures described by Koehl et al. ([41](#_ENREF_41)). Briefly, mice were anesthetized using isoflurane in combination with an air oxygen mixture as carrier gas and placed on a heating pad. During the entire surgery, their body temperature was monitored through a rectal probe and kept constant at 37 °C. After formation of a dorsal skinfold on the back of the mice undergoing surgery, a round piece of skin of ca. 10 mm diameter was removed from the first layer in the fold, leaving a facial plane with associated vasculature on the opposing side. The titanium frame of the skinfold chamber was subsequently sutured onto the skin, tightening the dorsal window at the upper edge. Screws were then used to pierce the skinfold and fix the skinfold chamber onto the animal. After mounting screws and nuts, the superficial layer of fat was removed under the stereomicroscope using fine forceps, the exposed skin layer was rinsed with saline and finally a coverslip was fixed onto the chamber by a spanning ring thereby closing the window. Mice were allowed to wake under a warming infrared lamp. 24 h after the skinfold chamber surgery, cells were injected subcutaneously into the window in anesthetized mice (30 ul injection volume).

**Assessment of tumor targeting in vivo.** Fluorescence imaging (FLI) experiments were performed using high CEA-expressing MKN45 gastric carcinoma model. Tumors were around 150mm3 at the time of treatment and the groups were randomized in order to have comparable tumor size. Mice were shaved at the location of tumor injection (s.c.) to allow better detection of the fluorescence signal. At the time of in vivo FLI acquisition, mice were anesthetized by isofluorane inhalation and placed on the side to allow access to the tumor. At termination, liver and tumor were excised and imaged ex vivo on black tissue culture plates. The signal was followed over time and detected using the IVIS® Spectrum (PerkinElmer). The Regions of interest (ROI) around the tumor were drawn and quantified using the Living Image software. The fluorescent signal coming from the ROI was normalized by the background signal. The resulting value was further normalized by the number of molecules labeled, labeling efficiency and injected dose for each mouse (calculated by ELISA of the serum collected 1 h post therapy injection). Data analysis was done using Microsoft excel, the statistical significance calculated using GraphPad Prism.

**Detection of tumor binding and retention in vitro using Ligand Tracer technology.** The Ligand Tracer technology measures the binding kinetics on live cells in a physiologically relevant concentration range and provides a setup to measure actual k(on), k(off), KD values on live cells. Briefly, ~3x105 HPAF-II cells (pancreatic adenocarcinoma, high CEA expression) were seeded one day prior to measurement in cloning cylinders (Ø 15 mm). The measuring protocol was performed at room temperature with disk rotations 0.6 cycles/min and passing the fluorescence reader spot once every round. Calibration was performed for 30 min in PBS (buffer). Two concentrations of Alexa-labeled TCB antibodies (CEA TCB\_2+1 and CEA TCB\_1+1) were added sequentially directly into the buffer. Each association phase was monitored for 1 – 2 h; after that, the buffer was exchanged to a new one not containing TCBs to measure the dissociation phase for 2 h.

**Supplemental Information (SI): Figure Legends**

**Fig. S1.** Assessment of binding of CEA TCB to human and Cynomolgus monkey CEA- and CD3-expressing cells. Median fluorescence intensities of binding of CEA TCB to (A) human lung adenocarcinoma cells (A549) stably transfected with plasmid coding for human CEA (A549-huCEA, circles) or Cynomolgus monkey CEA (A549-cyCEA, empty squares). (B) Binding of 12.5 nM of CH1A1A IgG-d2 (the CEA binder included in CEA TCB) to HEK EBNA cells expressing human CEA-SNAP, assessed by FRET in presence of increasing concentrations of shed CEA (sCEA). (C) Confocal microscopy assessing the binding and eventual internalization of CH1A1A IgG to MKN45 cells over time. CH1A1A IgG displayed strong plasma membrane staining after 45 min on binding at 4°C. The same intensity and plasma membrane localization was found after incubation of pre-bound cells for 23 h at 37°C indicating the absence of internalization of the CEA binder included in CEA TCB (green, CH1A1A IgG antibody; red, early endosome marker (EEA1)). (D) Binding of CEA TCB to human CD3-expressing Jurkat cells, (E) binding of CEA TCB to Cynomolgus monkey CD3-expressing HSC‑F cells, (F) Binding of CH2527 IgG (the anti-human/cyno CD3 binder included in CEA TCB) to murine splenocytes in comparison to the positive reference 2C11 antibody (anti-murine CD3 binder). Whereas 2C11 antibody binds well to murine splenocytes (dotted line, squares), there is no binding of CH2527 IgG to the same cells (bold line, circles), further confirming the absence of mouse cross-reactivity of the CH2527 antibody included in CEA TCB. The error bars depict SD based on triplicates. (G) Assessment of complement-dependent cellular cytotoxicity (CDC) after 2 h (LDH readout) and 22 h (Alamar blue readout) of incubation of MKN45 and the following molecules: CEA TCB (empty square), CH1A1A IgG (the anti-CEA binder included in CEA TCB) as wild-type (circle), glycoengineered (filled square) or P329G LALA Fc IgG1 variant (triangle). MabThera (rituximab) tested on Z138 cells, was used as positive control. The results of the CDC assay show that none of the molecules mediate CDC activity. On the contrary, MabThera (the positive reference) mediated good CDC activity, validating the assay conditions. (H) Assessment of antibody-dependent cellular cytotoxicity (ADCC) after 4 h (LDH readout) of incubation of MKN45 using the same molecules as in (H). Whereas the glycoengineered version of the parental anti-CEA binder (CH1A1A IgG glyco Fc) mediated weak ADCC, the other molecules were devoid of any activity.

**Fig. S2.** Pharmacokinetic profiles of CEA TCB in NOG mice. (A) PK profile of 0.5 mg/kg i.v. bolus administration of CEA TCB fit to a 2-compartmental model using Phoenix v6.2 (Pharsight). (B) Predicted PK profile assuming linear kinetics of 2.5 mg/kg i.v. administration. (C) Predicted repeat dose administration of 2.5 mg/kg i.v. biweekly. (D) Analysis of CD3 downstream signaling pathway detected as in Fig.2 A using CEA TCB and untargeted TCB. No activity with untargeted TCB. (E) Kinetic analysis of early T cell activation detected as in Fig.2 D-E using CEA TCB and untargeted TCB. No activity with untargeted TCB. (F) Dose and time-dependent tumor cell lysis detected as in Fig.2 F using CEA TCB and untargeted TCB. No activity with untargeted TCB. (G) Quantification of cytokines and cytotoxic granules released into culture supernatants detected as in Fig.2 G. No activity with untargeted TCB, only minor secretion of IL-6, IL-10 and granzyme B at the highest TCB concentration.

**Fig. S3.** (A-D) Detection of tumor targeting by in vivo imaging (fluorescent intensity, FLI) of several Alexa647-labeled TCB formats. (A) Schematic of the experimental schedule. The time points indicate the time of FLI acquisition post-therapeutic injection. Bleeding was performed 1 h post TCB injection to calculate the injected dose. (B) Representative images during the FLI acquisition 96 h post injection. The signal intensity is represented in bright yellow, scaling progressively down to a dark red, which represents low intensity of FLI. (C) Chart representation of the FLI signal over time at tumor location of the different TCB formats. Stars represent significance of the CEA TCB\_2+1 over all other groups (n=6). The detailed significance is illustrated in the table below. (D) At termination tumor and liver were extracted and analyzed ex vivo for FLI signal (n=6). \*=p<0.05 \*\*=p<0.01 \*\*\*=p<0.001 \*\*\*\*=p<0.0001. The data analysis was performed with 2-way ANOVA with Tukey correction. Data represent combined results of two independent experiments. CEA TCB\_2+1 corresponds to the molecule used through the manuscript (ie the one currently in the clinics) having bivalent binding for CEA and monovalent for CD3; CEATCB\_1+1\_HT corresponds to CEA TCB having monovalent binding for CEA and for CD3, fused together in a Head to Tail (HT) orientation. (E) Detection of tumor binding and retention in vitro using Ligand Tracer technology and HPAF-II cell line (high CEA expression level). Mono- and bivalent binding is differentiated by the shape of the curves; a higher avidity results in a higher total binding signal. Binding curves of the Ligand Tracer experiments show large portion of bivalent binding for CEA TCB\_2+1 and monovalent binding for CEA TCB\_1+1. The bivalent binding mode of CEA TCB\_2+1 increases the KD value by 100x as compared to monovalent binding, ie from 2.7x10-9 to 1.8x10-7, caused by its slower dissociation rate further demonstrating better tumor retention obtained via the bivalent binding to tumor antigen.

**Supplemental Information (SI): Videos**

**Video 1.** Representative videos of intravital two-photon (2P) microscopy of tumors treated with untargeted TCB. LS174T-RFP cells (RFP, red fluorescence protein, red) were co-grafted with human PBMCs at E:T 5:1 (T cells labeled with CFSE, green), injected into the skinfold chamber and allowed to grow for 4 days. Representative videos were obtained 24 h after single injection of untargeted control (MCSP TCB) at 2.5 mg/kg.

**Video 2.** Representative videos of intravital two-photon (2P) microscopy of tumors treated with CEA TCB. LS174T-RFP cells (RFP, red fluorescence protein, red) were co-grafted with human PBMCs at E:T 5:1 (T cells labeled with CFSE, green), injected into the skinfold chamber and allowed to grow for 4 days. Representative videos were obtained 24 h after single injection of CEA TCB at 2.5 mg/kg.