Supplementary Material & Methods

^{89}Zr-Df-IAB22M2C: Production, chelation and radiolabeling

The anti-CD8α-Mb IAB22M2C was engineered and humanized from a murine hybridoma anti-human CD8 antibody. The IC_{50} value of Df-IAB22M2C was determined by ELISA. Briefly, a 96-well plate was coated with human CD8 antigen and then incubated with Df-IAB22M2C followed by the secondary antibody mouse Anti-Human IgG Fc-HRP (Southern Biotechnology, Birmingham, AL). The colorimetric substrate TMB BioTX slow kinetic one component (Surmodics, Eden Prairie, MN) was used as detection reagent. The Mb Df-IAB22M2C resulted in an IC_{50} of 0.4 nM.

The IAB22M2C Mb was conjugated with the chelator p-isothiocyanatobenzyl-desferrioxamine (p-SCN-Bn-Df; Macrocyclics, Plano, TX) by amine coupling to solvent exposed lysine as described (1). Here, a 5-fold molar excess of p-SCN-Bn-Df in a buffer containing 100 mM sodium borate, 300 mM sodium chloride, 5 mM EDTA (pH 8.5) resulted in a chelator to Mb ratio (CMR) of 1.6 - 2.1. The CMR was calculated by measuring the concentration of the chelator CDF (M), and the concentration of the protein CProt (M) from values of absorbance at 280 nm and 250 nm:

CProt (M)= (A280 * ε 250DF - A250 * ε 280DF) / (ε 280Prot * ε 250DF - ε 250Prot * ε 280DF)

CDF (M)= (A280 * ε 250Prot - A250 * ε 280Prot) / (ε 280DF * ε 250Prot - ε 250DF * ε 280Prot)

The CMR was than calculated as: CMR = CDF (M)/CProt (M).

Protein recovery of Df-conjugated Mb after purification was approximately 85%. Size exclusion chromatography using Phenomenex Yarra SEC-2000 and 20 mM Tris / 80 mM Arg / 0.05% NaN_3, pH 7.3 as running buffer revealed that less than 0.5% of the Df-conjugated Mb existed as high MW species. Endotoxins were removed to levels below 0.02 EU/mg in the final product using Pierce Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher Scientific Waltham, MA). For radiolabeling, ^{89}Zr-oxalate was provided by MIR (Washington University School of Medicine, St. Louis, MO) and 3D Imaging Drug Design & Development (Little Rock, AR). The radiolabeling reactions were performed as described previously (2). The Mb was radiolabeled at a ratio of 50-100 µCi ^{89}Zr-oxalate per 10 µg protein for 1 h. The labelling efficiencies were in the range of 96.3 – 99.5% (n = 4) as determined by instant thin layer chromatography. The mean specific activities from two radiolabeling reactions were 5.57 µCi/µg for the CEA-TCB/CEA 4-1BBL study and 9.11 µCi/µg for the FOLR1-TCB study.

In vitro cytotoxicity assay

Human peripheral blood mononuclear cells (PBMC) were purified from fresh blood of healthy donors by conventional Histopaque-1077 gradient (Sigma-Aldrich, Buchs, Switzerland). Tumor cell lines MKN-45 and HeLa were transduced to express fluorescent nuclear red protein (NLR) a(NLR) using NucLight Red Lentivirus Reagent (Essen Bioscience, Welwyn Garden City, UK) and stable clones were selectively expanded under selection of Puromycin (InvivoGen, San Diego, California, USA).

Adherent target tumor cells MKN-45-NLR or Hela-NLR were detached (0.05 % trypsin/EDTA; Gibco, Darmstadt, Germany) and seeded in sterile tissue-culture flat-bottom 96 well plates (TTP, Trasadingen, Switzerland) at a density of 5,000 and 10,000 cells per well respectively. After overnight adherence of MKN45-NLR tumor cells, CEA-TCB was added at 2 nM (0.0776 µg/well) or 20 nM (0.776 µg/well), CEA-4-1BBL was added at a fixed dose of 2 nM (0.0712 µg/well) and human PBMC effector cells were added at an effector to target (E:T) ratio of 2:1. Similarly, HeLa-NLR cells were treated with serial dilution (0.64 pM – 10 nM equivalent to 0.00002 µg – 0.386 µg) of FOLR1-TCB and human PBMC effector cells were added at an E:T of 5:1. To monitor apoptosis, IncuCyte® caspase-3/7 green apoptosis assay reagent (Essen Bioscience, Welwyn Garden City, UK) was added to all samples. Five hours after initiation of the cytotoxicity reaction, assay media containing fixed and saturating concentration of 125 nM (2 µg/200 µL/well = 10 µg/mL) anti-CD8 tracer per well was added to the samples. All samples were performed in triplicates. The assay plates were placed into the IncuCyte live-cell analysis system at 37 °C, 5% CO_2 and scanned (4 images/well) every 3 h for 4 days using a 10X objective. IncuCyte® image analysis software was used to quantify apoptotic tumor cells and target cell lysis or killing. Tumor cells count was quantified by setting an image mask determining the number of red objects per image (tumor cell nuclei). Apoptotic tumor cells were quantified by setting an image mask determining the number of double red/green positive objects/image (overlap of red nuclei and green nuclei of apoptotic cells). To quantify the percentage of tumor cell lysis, data was normalized to initial tumor cell count at t_0 of untreated control wells containing tumor cells and PBMCs in the absence of CEA-TCB, CEA-4-1BBL or FOLR1-TCB.

Ex vivo γ-counting and autoradiography

After the PET/CT imaging the mice were euthanized, and necropsy was performed to isolate the following organs/tissues for the CEA-4-1BBL/CEA-TCB study: blood, liver, kidneys, spleen, heart, lung, stomach, intestine, bone, carcass and muscle. For the FOLR1-TCB study, blood, liver,

kidneys, spleen, lung, femur bone, carcass and thigh muscle were isolated. The radioactivity in all samples was measured on a Wizard 3" automatic γ-counter (PerkinElmer) with a set of 2 counting standards prepared from a measured aliquot of the ^{89}Zr-Df-IAB22M2C stock solution. The percent of the injected dose per gram (%ID/g) was calculated by referencing the measured counts for each organ/tissue sample to the decay-corrected injected radioactive dose.

For autoradiography two freezing containers were prepared, one with a slurry of dry ice and isopentane (2-methylbutane) and the other with dry ice only. Tumors were embedded in Tissue-Plus™ O.C.T Compound in Shandon™ Peel-A-Way Disposable Embedding Molds (both from Fisher Scientific, USA) that were placed into the isopentane bath until samples were all but frozen in the center at which time they were transferred onto dry ice and allowed to freeze for additional 10 minutes. Frozen tumors were sectioned into 20 µm slices in a Leica CM3050 cryostat (Leica Microsystems, Nussloch, Germany). The slices were placed on Superfrost™ Plus Microscope Slides (Fisher Scientific) that were placed in an autoradiographic cassette. The phosphor Imaging plate was wrapped in plastic (for protection) and placed on top of the slides. The cassette was clamped tightly and placed in -20 °C over the weekend. The phosphor imaging plate was read in FujiBas-5000 (FujiFilm Medical Systems, Stamford, CT, USA).

Ex vivo immunohistochemistry

IHC staining of CD8+ cells in the tumor was prepared for four samples from the indicated CEA-TCB/CEA-4-1BBL treatment groups. Eight HeLa samples from the FOLR1-TCB group and six samples from the vehicle group were also analyzed by CD8-IHC. The tissue samples were harvested from animals after the PET scans, fixed in 10% formalin (Sigma, Germany) and later processed for FFPET (Leica 1020, Germany). Four µm paraffin sections were subsequently cut in a microtome (Leica RM2235, Germany). Human CD8 T cell immunohistochemistry was performed with anti-human CD8 antibody (Diagnostic Biosystem, Germany), human CEA tumor marker with anti-human CEA (Rb anti-huCEA T84.66, Roche-Glycart) and mouse CD31 vessel marker with anti-mouseCD31 (Dianova DIA-310, clone SZ31) in the Leica autostainer (Leica ST5010, Germany) following the manufacture's protocols. Routine H&E staining was performed in the Leica ST5010 Autostainer XL machine following the manufacturer's protocol. Slides were scanned with Olympus scanner. Quantification of human CD8 positive cells from scan images was performed with Definiens software (Definiens, Germany). For this, whole scans were uploaded in the tissue developer module and necrotic areas were excluded with segmentation analysis. Secondly, a threshold was set to recognize the brown staining of the targeted CD8 T cells and

subsequently the algorithm for cell quantification was automatically run. For vessel quantification a threshold for small (0-250 µm diameter long), medium (250-500µm diameter long), and large (≥500µm diameter long), CD31 positive vessels was set and subsequent the algorithm for vessel quantification was automatically run. The output data for CD8 and CD31 quantification was then transferred to Graphpad Prism version 7.04 (GraphPad Software, La Jolla, California, USA) for analysis of significance.

Statistics

All data are presented as mean ± SD (standard deviation) and mean ± SEM (standard error of mean). One-way ANOVA with multiple comparisons (Tukey Kramer, Fishers LSD) or Student´s t-test were calculated in GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla CA) to assess significant differences between the treatment groups which are presented as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

References

1. Vosjan MJ, Perk LR, Visser GW, Budde M, Jurek P, Kiefer GE*, et al.* Conjugation and radiolabeling of monoclonal antibodies with zirconium-89 for PET imaging using the bifunctional chelate p-isothiocyanatobenzyl-desferrioxamine. Nat Protoc **2010**;5:739-43
2. Tavare R, Escuin-Ordinas H, Mok S, McCracken MN, Zettlitz KA, Salazar FB*, et al.* An Effective Immuno-PET Imaging Method to Monitor CD8-Dependent Responses to Immunotherapy. Cancer Res **2016**;76:73-82