Chimeric antigen receptor T cells engineered to recognize the P329G-mutated Fc part of effector-silenced tumor antigen-targeting human IgG1 antibodies enable modular targeting of solid tumors

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SUPPLEMENTARY FILES

SUPPLEMENTARY TABLES

Supplementary table 1: Cell lines and culture method

Cell line	Origin	Type	Culture medium*
SUIT-2-mesothelin	Human	Pancreatic cancer	Dulbecco's Modified Eagle's Medium (DMEM)
Mia PaCa-2-mesothelin			
MSTO-211H-mesothelin-luciferase		Pleural mesothelioma	Roswell Park Memorial Institute (RPMI) 1640
HCC1569 wild type		Breast cancer	
MCF-7 HER2 overexpression			

*All cell lines were cultured in medium containing 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were grown at 37°C in a humidified incubator with 5% CO_2. Cells were frozen in freezing medium containing 90% FBS and 10% DMSO and stored at -80°C or in liquid nitrogen for long-term storage.

SUPPLEMENTARY METHODS

Fluorescence resonance energy transfer (FRET) competition assay

An antibody with an Fc bearing the P329G mutation was labeled with the FRET acceptor d2 (labeling kit #62D2DPEA; Cisbio/Perkin Elmer, USA) and mixed with the biotinylated Fab fragment of the anti-P329G binder and the FRET donor Streptavidin-Terbium (SA-Tb) (#610SATLF; Cisbio/Perkin Elmer, USA) to obtain a FRET signal (assay buffer: PBS, 0.1% Tween20, 0.2% BSA, pH 7.2). For the competition assay, 3 nM of d2-labeled antibody were mixed with 5 nM anti-P329G Fab, 1.8 nM of SA-Tb and decreasing concentrations of unlabeled IgG with and without the P329G mutation or with BSA (from 10 mg/mL down to 2.4 x 10^{-6} mg/mL in 4-fold dilution steps) and incubated for 16 hours at 4°C. Fluorescent signal A was measured at 620 nm and 665 nm for Tb and d2 respectively (M1000 Pro; TECAN, Schweiz), the fluorescence ratio R (A_{665}/A_{620}*10000) was calculated for each dilution and the background signal was subtracted to obtain ΔR.

CAR T cell generation and expansion

Human T cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors by density gradient centrifugation. T cells were enriched by anti-CD3 microbeads (Miltenyi Biotec, Germany) and activated by Dynabeads human T-Activator CD3/CD28 (Life Technologies, Germany) for two days before transduction. T cells were expanded in human T cell medium (hTCM) containing RPMI 1640 with 2.5% human serum, 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% sodium pyruvate and 1% non-essential amino acids solution supplemented with recombinant human IL-2 (Novartis, Switzerland) and IL-15 (PeproTech, Germany).

Cytotoxicity assay

For co-culture experiments, 2.5 x 10^4 tumor cells were plated in flat bottom 96-well plates. T cell numbers transduced with the indicated CAR constructs or untransduced T cells were added at different effector cell to target cell ratios (E:T ratios). Co-cultures were performed in hTCM without cytokines or DMEM-based culture medium supplemented with 1% sodium pyruvate and 1% non-essential amino acids solution depending on the tumor cell line (supplementary table 1). Medium was supplemented with different antibodies, recombinant mesothelin (MSLN) (ACROBiosystems, USA) in different concentrations as indicated or human IgG (Sigma-Aldrich, USA) at a concentration of 1 mg/mL. Killing was assessed by impedance-based real-time killing assays using an xCELLigence system (Agilent, USA) or luciferase-based killing assays using Bio-Glo™ Luciferase Assay System according to manufacturer's protocol (Promega Corporation, USA).

T cell stimulation assay using tumor cells

Co-cultures were performed as described above. After an incubation time of 24 hours, 48 hours or 72 hours, supernatants were obtained, and T cell phenotype was assessed by flow cytometry as described below.

Modularity experiment

CAR T cells targeting P329G, HER2 or MSLN and untransduced T cells were expanded as described above and frozen on day 12-14 of CAR T cell production. Dynabeads human T-Activator CD3/CD28 (Life Technologies, Germany) were removed prior to freezing. CAR T cells were thawed, resuspended in human T cell medium (hTCM) supplemented with cytokines and incubated overnight at 37°C in a humidified incubator with 5% CO_2. T cells were subsequently washed twice in PBS and resuspended in hTCM without cytokines at a concentration of 10^6 cells/mL. Thus, 2 x 10^5 T cells were transferred to every well of the first plate coated with recombinant HER2/ErbB2 (SinoBiological, China) or MSLN (ACROBiosystems, USA). Cell suspension was supplemented by no binder, HER2 binder or MSLN binder. Cells were incubated overnight at 37°C in a humidified incubator with 5% CO_2. After 24 hours, cells were transferred to a U-bottom 96-well plate, and supernatants were taken. After one washing step with PBS to remove any residual binder, cells were resuspended in 200 µl hTCM without cytokines per well and transferred to the second coated plate. Either the same binder as on the previous day (repeated binder), the opposite binder (switched binder) or no binder (depleted binder) were added. After 24 hours, cells were transferred to a U-bottom 96-well plate, and supernatants were taken. T cells were washed once in PBS to remove residual binder, resuspended in 200 µl hTCM without cytokines per well and transferred to the third coated plate. The same binder was added as on the previous day, and cells were incubated overnight at 37°C in a humidified incubator with 5% CO_2. After 24 hours, the cells were transferred to a U-bottom 96-well plate, and supernatants were taken.

Flow cytometry

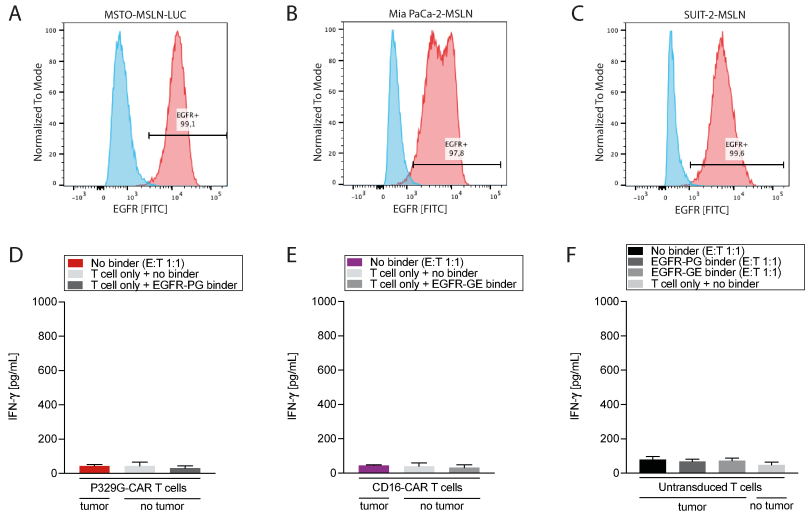
For staining, cells were transferred into U-bottom 96-well plates. Cells were washed with ice cold PBS and centrifuged at 400 g for 5 min at 4°C. Blocking of Fc receptors with TruStain FcX (BioLegend, USA) was performed for 15 min. Dead cells were excluded after staining with a fixable viability dye (eFluor™ 780) in all experiments (eBioscience, USA). Cell surface proteins were stained for 20 min at 4°C. The following fluorophore-conjugated antibodies reactive to human antigens were used for flow cytometry analysis: anti-CCR7 (G043H7), anti-PD-1 (EH12.2H7), anti-CD8 (SK1), anti-TIM-3 (F38-2E2), anti-CD45RA (HI100), anti-CD3 (HIT3A), anti-CD62L (DREG-56), anti-CD69 (FN50), anti-CD45 (HI30) and anti-CD4 (A161A1) (all from BioLegend). Anti-c-Myc (SH1-26E7.1.3) from Miltenyi Biotec or anti-CD16 (3G8) from BioLegend were used for detection of CAR constructs. Quantification of absolute cell counts was carried out by using Count Bright™ Absolute Counting Beads (Thermo Fisher Scientific, USA). Antigen density was assessed using the Quantitative Analysis Kit

(QIFIKIT) and calculated according to the manufacturer's protocol (Agilent Dako, USA). Cells were analyzed on a BD FACSCanto™ or BD LSRFortessa™ II flow cytometer, and data were analyzed with FlowJo software (version 10.7.2).

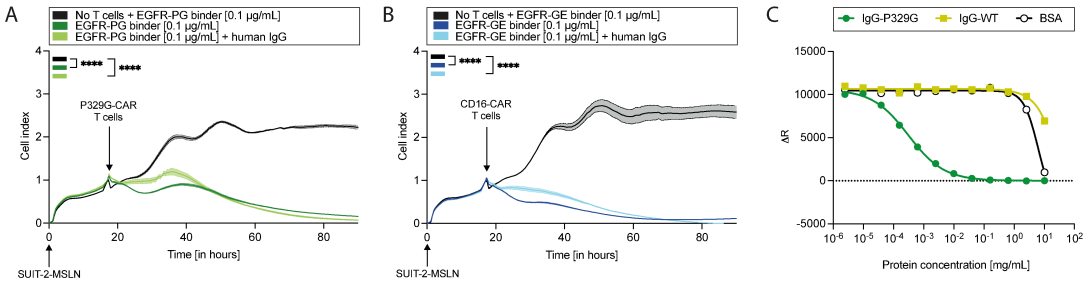
Preparation of single-cell suspensions

Blood was diluted with PBS followed by erythrocyte lysis. Spleens were passed through 30 µm cell strainers, followed by erythrocyte lysis. Tumors were digested with collagenase IV and deoxyribonuclease I for 30 min at 37°C under agitation, followed by passage through 30 µm cell strainers. Flow cytometry analysis is described above.

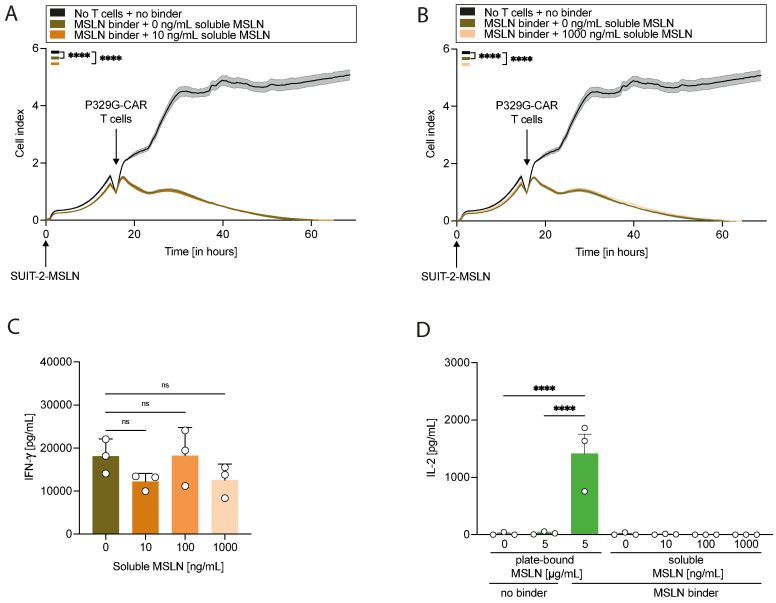
SUPPLEMENTARY FIGURES



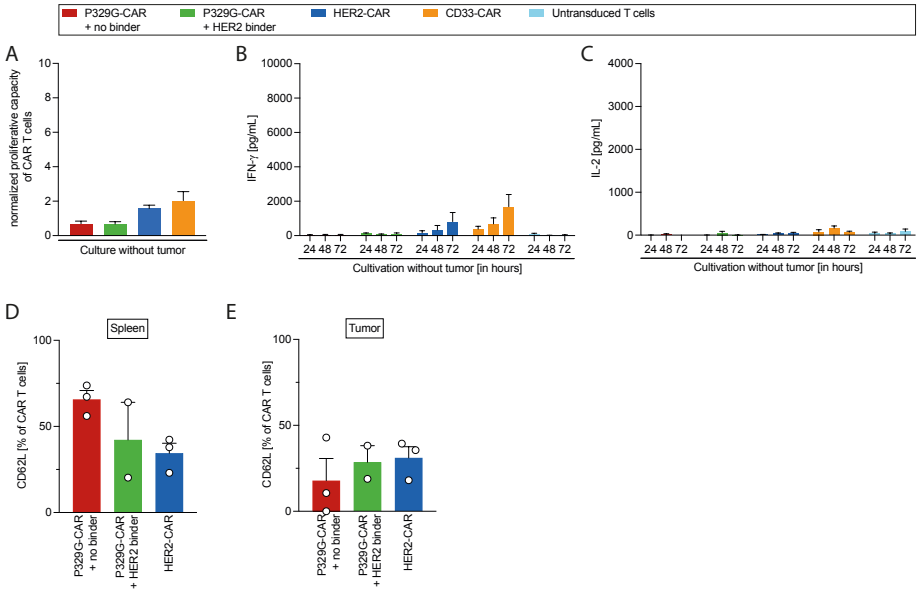
Supplementary figure S1. Comparison of P329G-targeting CAR T cells with CD16-ECD-CAR T cells. (**A-C**) EGFR expression (**A**) on MSTO-211H-mesothelin-luciferase (MSTO-MSLN-LUC) cells (**B**) on MIA PaCa-2-mesothelin (MIA PaCa-2-MSLN) cells and (**C**) on SUIT-2-mesothelin (SUIT-2-MSLN) cells. (**D-F**) IFN-γ ELISA after 48 hours co-culture of (**D**) P329G-targeting CAR (P329G-CAR) T cells, (**E**) CD16-ECD-CAR (CD16-CAR) T cells and (**F**) untransduced T cells (UT) without or with SUIT-2-MSLN cells and different binder conditions for three independent donors (n=3). P329G-LALA mutated EGFR (EGFR-PG) and glycoengineered EGFR (EGFR-GE) binders were used. Each experiment **(D-F)** was performed in triplicates. Values in all graphs represent means±SEM (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).



Supplementary figure S2. Influence of immunoglobulin G on P329G-targeting CAR T cells. SUIT-2-MSLN tumor cell killing by **(A)** P329G-targeting CAR (P329G-CAR) T cells and **(B)** CD16-ECD-CAR (CD16-CAR) T cells in different P329G LALA-mutated EGFR (EGFR-PG) or glycoengineered EGFR (EGFR-GE) binder conditions measured over time through xCELLigence**. (C)** Comparison of competition with unlabeled antibody with P329G mutation, competition with unlabeled wild type IgG and competition with BSA in a fluorescence resonance energy transfer (FRET) competition assay. Each experiment was performed in (**A-B**) triplicates or **(C)** duplicates. Subfigures (**A-B**) show data from one representative donor out of three independent experiments. Subfigure **(C)** represents the average values of duplicates for one experiment out of two independent experiments. Values in subfigures (**A-B**) represent means±SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). P values for xCELLigence data in (**A-B**) are shown for last time point. Only selected p-values are shown.



Supplementary figure S3. Influence of soluble recombinant MSLN on P329G-targeting CAR T cells. (A+B) SUIT-2-MSLN tumor cell killing by P329G-CAR T cells and mesothelin (MSLN) binder in absence or presence of soluble MSLN (10 or 1000 ng/mL). (**C**) IFN-γ ELISA after 48 hours co-culture for three independent donors (n=3). P329G-CAR T cells and MSLN binder (1 µg/mL) were cultivated with SUIT-2-MSLN in absence or presence of soluble MSLN (0, 10, 100 or 1000 ng/mL). (**D**) IL-2 ELISA on 24 hours co-culture supernatants of three independent donors (n=3). P329G-CAR T cells and MSLN binder were cultivated in presence or absence of plate-bound MSLN [5 µg/mL] or soluble MSLN [0, 10, 100, or 1000 ng/mL]. Each experiment was performed in triplicates. Subfigures (**A-B**) show data from one representative donor out of three independent experiments. Values in all graphs represent means±SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). P values for xCELLigence data in (**A-B**) are shown for last time point. Only selected p-values are shown.



Supplementary figure S4. *In vitro* and *in vivo* characterization of P329G-targeting CAR T cells with the HER2 binder. (**A**) Proliferative capacity of P329G-targeting CAR (P329G-CAR), HER2-targeting CAR (HER2-CAR), and CD33-targeting CAR (CD33-CAR) T cells after 72 hours of co-culture normalized to CAR T cells per beads measured by FACS after 24 hours co-culture in absence of tumor cells of three independent donors (n=3). Human IFN-γ (**B**) and IL-2 (**C**) ELISA after 24 hours, 48 hours, and 72 hours co-culture in absence of tumor cells for three independent donors (n=3). (**D+E**) Expression of CD62L on CAR T cells detected in spleens (**D**), and tumors (**E**) of treated mice 10 days after CAR T cell injection of the experiment shown in figure 6A. Each experiment in subfigures (**A-C**) was performed in triplicates. Values in all graphs represent means±SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).