Chimeric antigen receptor (CAR) T cell-based targeting of CD317 as a novel immunotherapeutic strategy against glioblastoma

Lena Hänsch[1], Matthias Peipp[2], Maximilian Mastall[1], Danielle Villars[1], Renier Myburgh[3], Manuela Silginer[1], Tobias Weiss[1], Dorothee Gramatzki[1], Flavio Vasella[1,4], Markus G. Manz[3], Michael Weller[1], Patrick Roth[1]*

[1]Department of Neurology and Brain Tumor Center, University Hospital Zurich and University of Zurich, Switzerland; [2]Division of Antibody-Based Immunotherapy, Department of Internal Medicine II, University Hospital Schleswig-Holstein, Campus Kiel, and Christian-Albrechts-University, Kiel, Germany; [3]Department of Medical Oncology and Hematology, University Hospital Zurich and University of Zurich, Switzerland; [4]Department of Neurosurgery, University Hospital Zurich and University of Zurich, Switzerland

*Corresponding author: Dr. Patrick Roth, Department of Neurology, University Hospital and University of Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland, Tel.: +41 (0)44 255 5511, Fax: +41 (0)44 255 4380, E-mail: patrick.roth@usz.ch

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ABSTRACT

Background: Chimeric antigen receptor (CAR) T cell therapy has proven to be successful against hematological malignancies. However, exploiting CAR T cells to treat solid tumors is more challenging for various reasons including the lack of suitable target antigens. Here, we identify the transmembrane protein CD317 as a novel target antigen for CAR T cell therapy against glioblastoma, one of the most aggressive solid tumors.

Methods: CD317-targeting CAR T cells were generated by lentivirally transducing human T cells from healthy donors. The anti-glioma activity of CD317-CAR T cells towards various glioma cells was assessed *in vitro* in cell lysis assays. Subsequently, we determined the efficacy of CD317-CAR T cells to control tumor growth *in vivo* in clinically relevant mouse glioma models.

Results: We generated CD317-specific CAR T cells and demonstrate strong anti-tumor activity against several glioma cell lines as well as primary patient-derived cells with varying CD317 expression levels *in vitro*. A CRISPR/Cas9-mediated knockout of *CD317* protected glioma cells from CAR T cell lysis, demonstrating the target specificity of the approach. Silencing of *CD317* expression in T cells by RNA interference reduced fratricide of engineered T cells and further improved their effector function. Using orthotopic glioma mouse models, we demonstrate the antigen-specific anti-tumor activity of CD317-CAR T cells, which resulted in prolonged survival and cure of a fraction of CAR T cell-treated animals.

Conclusions: These data reveal a promising role of CD317-CAR T cell therapy against glioblastoma, which warrants further evaluation to translate this immunotherapeutic strategy into clinical neuro-oncology.

Keywords: CAR T cell, BST2, adoptive immune cell transfer, immunotherapy, glioma

Keypoints:

- CD317 is stably expressed by glioma cells and represents a novel antigen for targeted immunotherapy
- CD317-specific CAR T cells display strong anti-glioma activity *in vitro* and prolong the survival of glioma-bearing mice

Importance of the Study

One of the major obstacles in targeting glioblastoma with CAR T cells is the lack of suitable target antigens. We identified CD317 as a stably expressed target antigen for CAR T cell-based therapy and developed CD317-specific CAR T cells, which display potent activity against various glioma cell lines *in vitro*. Importantly, these newly generated CAR T cells reduce the growth of experimental gliomas *in vivo*, prolong the survival of glioma-bearing mice and cure a fraction of these animals. Targeting CD317 with CAR T cells is a promising and novel immunotherapeutic approach against glioblastoma and this dataset provides a scientific rationale to translate this therapeutic strategy into clinical neuro-oncology.

INTRODUCTION

Immunotherapy with T cells genetically engineered to target tumor antigens with a chimeric antigen receptor (CAR) has demonstrated strong clinical activity against hematologic malignancies. In contrast to immune checkpoint inhibitors and cancer vaccines, CAR T cells combine antigen detection and T cell activation, which allows them to elicit an anti-tumor immune response independently from the endogenous immune system [1-3]. Solid tumors represent a particular challenge as CAR T cells may only partially reach and infiltrate the tumor site [4]. Glioblastoma is the most common malignant primary neoplasm in the central nervous system and among the most aggressive solid cancers [5]. Despite standard treatment consisting of surgery, radiotherapy and temozolomide chemotherapy, the prognosis remains poor with a median survival of approximately 16 months within clinical trials [6].

In the context of gliomas, tumor-derived immunosuppression as well as frequent use of steroids may represent hurdles for immunotherapeutic strategies requiring an adaptive immune response [7,8]. Therefore, there is a strong interest in exploiting CAR T cells also for the treatment of glioblastoma [9]. Currently explored CAR T cell strategies target the tumor antigens EGFR variant III (EGFRvIII) [10], Her2 [11] or interleukin (IL)-13 receptor subunit alpha 2 (IL13Ra2) [12]. However, the heterogeneous expression of these antigens led to antigen escape and tumor relapse. Therefore, a major goal is to identify glioma-associated antigens that can be targeted by engineered T cells to ideally cover all tumor cells and prevent immune escape.

The bone marrow stromal antigen CD317 (also known as HM1.24, BST-2 or tetherin) is a lipid raft-associated type II transmembrane protein with a unique topology that was initially discovered as an anti-viral restriction factor limiting the release of enveloped viruses from the surface of infected cells [13-15]. CD317 is directly inducible by type 1 interferons (IFN)

through the IFN response elements IRF-1/2 and ISGF3 [16]. While CD317 is not expressed by normal neurons and naïve microglia in the brain, it is overexpressed on malignant plasma cells and in certain solid tumor types including breast, lung, liver, and kidney cancer [16-21]. The functional role of CD317 in cancer remains largely unknown, but CD317 induces the proliferation, migration and tumorigenicity of hepatocellular carcinoma, breast cancer and glioma cells [19,22,23] and is involved in tumor immune escape mechanisms [24]. Targeting CD317 has therefore been regarded a promising anti-cancer strategy and an anti-CD317 antibody was already investigated in non-human primates with good tolerability [25]. Since CD317 expression is associated with poor survival and correlates with a higher WHO grade of malignancy in glioma, it might be a suitable target for CAR T cell therapy against glioblastoma [26]. The immunotoxin HM1.24-ETA' consisting of a CD317-targeting scFv coupled to Pseudomonas aeruginosa exotoxin A has been explored for its anti-glioma activity *in vitro* [26]. Compared to an immunoconjugate, which may not sufficiently cross the blood-brain barrier, CAR T cells targeting CD317 may have several advantages and represent a more attractive and therapeutically more powerful strategy [27].

Accordingly, we aimed at exploiting the expression of CD317 by glioma cells for a novel immunotherapeutic strategy using CD317-specific CAR T cells. Upon engineering of T cells to express a CD317-specific CAR, we provide proof-of-concept that these CAR T cells exert strong anti-tumor activity *in vitro* and *in vivo*.

MATERIALS and METHODS

Cell lines and reagents

The human long-term glioma cell (LTC) lines A172, LN-18, LN-229 and T98G and human embryonic kidney (HEK) 293T cells (Open BioSystems, Huntsville, AL) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mmol/L L-glutamine (all from Gibco, Life Technologies Limited, Paisley, UK). The glioma initiating cell (GIC) lines ZH-161, S-24, T-325, GS-2 and GS-5 were established from freshly dissected tumor tissue [28,29]. They were maintained in Neurobasal medium (Gibco, Life Technologies Corporation, Grand Island, NY) supplemented with 20 ng/ml fibroblast growth factor-2 and EGF (PeproTech, Rocky Hill, PA), 20 µl/ml B-27 (Gibco, Life Technologies Corporation) and 2 mmol/L L-glutamine. The generation of primary tumor cells from freshly resected brain tumor samples (ZH1054, ZH1055 and ZH1092) has been described [30]. Normal human astrocytes (NHA, Lonza, Visp, Switzerland) were cultured in DMEM/F12 (Gibco) supplemented with 10% FCS. Primary human macrophages and the microglia cell line CHME-5 were cultured in DMEM containing 10% FCS and 2 mmol/L L-glutamine. The human endothelial cell line hCMEC/D3 was cultured in endothelial cell growth medium (Promocell, Heidelberg, Germany). All cells were cultured in a humidified 37°C incubator with 5% CO_2 and regularly tested for mycoplasma contamination by PCR and authenticated at the German Collection of Microorganisms and Cell Cultures GmbH (DMSZ Leibniz Institute, Braunschweig, Germany) by short tandem repeat analysis. In some experiments, cells were exposed to IFN-β as previously described [31]. Briefly, 100'000 LN-18 or LN-229 or 400'000 ZH-161 cells were seeded per well in a 6-well plate. Twenty-four hours later, the medium was exchanged to serum-free medium and the cells were exposed to 50-100 IU/ml IFN-β (Biogen, Cambridge, MA) for 48 h or left untreated. RNA sequencing data were exported

from the publicly available Ivy Glioblastoma Atlas project (IvyGAP; glioblastoma.alleninstitute.org) to assess CD317 expression levels [32].

CAR design

The CD317-targeting single-chain variable fragment (scFv) was derived from the immunotoxin HM1.24-ETA' [33] and cloned into a second-generation CAR backbone vector containing 4-1BB and CD3ζ endodomains and a CD8α hinge and transmembrane domain [34]. The CAR was co-expressed with the marker gene RQR8 and separated by a self-cleaving T2A peptide. Additional expression of RQR8 allowed determining the transduction efficiency and potential purification of positively transduced T cells using anti-CD34 antibodies [35]. The lentiviral envelope-expressing plasmid pMD2.G (#12259) and the packaging plasmid pCMV-dR8.91 (#2221) were purchased from Addgene (Teddington, UK). pMD2.G, pCMV-dR8.91 and EF1α-human-CD317-scFv-T2A-RQR8 were used to transfect HEK 293T cells at a 1:1.75:2.25 ratio in full DMEM medium without antibiotics. Twenty-four hours later, the medium was exchanged and 2 days later the supernatant was collected and the lentiviruses were concentrated using Peg-it virus precipitation solution (System Biosciences, Palo Alto, CA).

Generation of CAR T cells

Human peripheral blood mononuclear cells (PBMC) from buffy coats from healthy donors (Blutspende Zurich, Switzerland) were separated by Percoll (Stemcell Technologies, Cologne, Germany) density gradient centrifugation. T cells were isolated using the EasySep Human T Cell Isolation Kit (Stemcell Technologies). Isolated T cells were stimulated with CD3/CD28 beads (Dynabeads; Gibco, Thermo Fisher Scientific, Waltham, MA) for 72 h at a 1:1 ratio.

Activated T cells were lentivirally transduced [34]. Briefly, 10^6 activated T cells were transduced with CD317-CAR, CD317-shRNA-CAR, empty-CAR-shRNA, empty-CAR (mock) or control-CAR vectors by spinoculation at 1000 xg for 1.5 h at 32°C in a 24-well flat bottom plate in a final concentration of 8 µg/ml hexadimethrine bromide (Sigma-Aldrich, St. Louis, MO). Twenty hours later, the medium was exchanged. T cells were cultured at a density of 10^6 cells/ml in advanced RPMI1640 medium supplemented with 10% FCS, 2 mmol/L glutamax (all from Gibco, Life Technologies Limited), 1% penicillin/streptomycin (Sigma-Aldrich) and 100 IU/ml recombinant human interleukin (IL)-2 (PeproTech). Transduced T cells were expanded for 10 days before use.

Isolation and cultivation of tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes were isolated by cutting freshly removed glioblastoma samples into small fragments (1-2 mm^3). Subsequently, 1-4 fragments were transferred into a 24-well plate and cultured in IMDM medium (Gibco) supplemented with 2 mmol/L L-glutamine, 7.5% heat-inactivated human AB serum, 0.5% penicillin/streptomycin and 1000 IU/ml IL-2 [36].

RNA interference-mediated *CD317* silencing in T cells

To silence *CD317* in transduced T cells, a shRNA sequence targeting *CD317* (Sigma-Aldrich) was incorporated together with the U6 promoter into the CAR backbone vector and upstream of the CAR construct to avoid disrupting the Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) that enhances the expression of its upstream sequence. The shRNA sequence was: CCGGCCCAGGAAGCTGGCACATCTTCTCGAGAAGATGTGCCAGCTTCCTGGGTTTTTG.

CRISPR/Cas9-based generation of *CD317* knockout cells

LN-229 *CD317* knockout (KO) cells were generated using CRISPR/Cas9 editing. The sgRNAs targeting human *CD317* (5-TGACTATTGCAGAGTGCCCA-3 and 5-CCTGCAACAAGAGCTGACCG-3) were designed using the Synthego CRISPR design tool (https://www.synthego.com/products/bioinformatics/crispr-design-tool)[37] and were separately inserted into lentiCRISPR v2. LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid #52961). LN-229 wildtype (WT) cells were transfected with 2 µg total DNA using the TransIT-X2 system (Mirus Bio, Madison, WI). Twenty-four hours later, positively transfected cells were selected using 6 µg/ml puromycin (Gibco Life Technologies) for 3 days. Single cells were seeded and expanded in a 96-well plate. The DNA of 5 clones for each condition was isolated with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and amplified by standard PCR in a Biometra thermocycler (Göttingen, Germany) using the 2X PCR Master Mix from Thermo Fisher Scientific and the following primers from Microsynth (Balgach, Switzerland) forward 5'-ATAAAGGGGTGGCCCGTAGA-3', reverse 5'-TTCCCCTACCAGGGTTTGTGG-3'. The conditions were 40 cycles at 95°C/30 s, 59°C/30 s and 72°C/1 min. The KO was confirmed by Sanger DNA sequencing (Microsynth) using the following primer 5'-AGATGCAGGGGAGGTCCTGAAA-3'.

***CD317* overexpression in human glioma cells**

The *CD317* coding sequence was synthesized by Genscript (Leiden, Netherlands) and cloned into pLenti-puro. pLenti-puro was a gift from Ie-Ming Shih (Addgene plasmid #39481). Lentiviruses were generated with the packaging and envelope-expressing plasmids pCMV-dR8.91 and pMD2.G as described in the CAR design section. Human glioma cells were lentivirally transduced by spinoculation at 700 rpm for 1.5 h at 32°C with a final

concentration of 8 µg/ml polybrene (Santa Cruz, Dallas, TX). Twenty-four hours later, the medium was exchanged and 48 hours later, positively transduced cells were selected with 3-10 µg/ml puromycin (Gibco, Life Technologies Corporation) for 3-10 days.

Clonogenic survival and migration assay

Determination of clonogenic survival and migration ability of glioma cells has been described in detail [38].

Flow cytometry

For flow cytometry analysis, the following antibodies were used: anti-human CD317-PE (clone 26F8; eBioscience, Thermo Fisher Scientific), CD317-APC (clone RS38E), CD8-PerCP, CD4-APC, CD3-PE, IFN-γ-BV421, CD223-APC (LAG-3), CD366-FITC (TIM-3), CD279-PerCP/Cyanine5.5 (PD-1) (all from Biolegend, San Diego, CA) and CD34-FITC (Thermo Fisher Scientific) with their appropriate isotype controls. Surface presentation of the CAR was confirmed by binding to a CD317-Fc fusion protein (Sino Biological, Beijing, China) and detection with PE labeled anti-human IgG (Biolegend). Live/dead staining was performed using the Zombie NIR, Zombie Aqua or Zombie Violet Fixable Viability Kit (Biolegend). For intracellular cytokine stainings, the cells were incubated for 6 h with GolgiPlug/GolgiStop (BD Biosciences, Allschwil, Switzerland) prior to antibody staining. To block Fc receptors (FcR), samples were pre-incubated with a FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Acquisition was performed on a BD FACSVerse Analyzer (BD Biosciences). Data were analyzed with FlowJo (Tree Star, Stanford, CA).

Lysis assay

Glioma cells were labeled using the PKH26 Red Fluorescent Cell Linker Kit for general cell membrane staining (Sigma-Aldrich) and co-cultured for 17 h with CD317-CAR T cells, CD317-shRNA-CAR T cells or non-transduced T cells at varying effector:target (E:T) ratios. The percentage of lysed glioma target cells was assessed by flow cytometry and valuated by subtracting the amount of dead target cells alone [39].

IFN-γ ELISA

IFN-γ secretion was measured in 100 µl supernatant from 17 h (co-)cultures of CAR or non-transduced T cells with or without tumor cells in a 1:1 E:T ratio using the IFN-γ DuoSet ELISA kit (R&D Systems, MN) as described [40].

Animal experiments

All animal experiments were executed according to the general guidelines of the Swiss federal law on animal protection and complementary with the Institutional Animal Care and Use Committee of the cantonal veterinary office (license no. ZH073_2018). Crl:CD1-Foxn1nu mice were purchased from Charles River and used at 6 to 12 weeks of age. NMRI-nude:Rj mice were purchased from the Janvier Laboratory and used at 4 to 6 weeks of age. To establish orthotopic gliomas, 75'000 or 100'000 LN-229 or ZH-161 cells were implanted stereotactically into the right striatum on day 0. Mice were sacrificed when developing neurologic symptoms or as indicated. For local administration, mice received up to $2x10^6$ CD317-CAR T cells, CD317-shRNA-CAR T cells, non-transduced or mock-transduced T cells intratumorally as indicated as a single treatment at day 5 or 7 upon tumor cell implantation. For systemic administration, up to 10^7 CAR T cells or non-transduced T cells were injected

intravenously into the tail vein up to 3 times on days 7, 10 and 14 upon tumor cell implantation as indicated. Some mice were treated subcutaneously with $8x10^7$ U/kg human peg-IFN-β1a as indicated (Biogen) [31].

Histology and immunohistochemistry

Anti-human-BST-2 polyclonal antibody was obtained from Thermo Fisher Scientific (PA5-82572, 1:500). For the analysis of CD317 expression, healthy human tissues (liver, skin, testis, tonsille, uterus, portio, brain) and glioblastoma specimens were analyzed on a tissue microarray [41]. The intensity of the CD317 staining was quantified using the histo-score (H score), ranging from 0 – 300 [41]. For mouse studies, cryosections from tumor-bearing mice were generated and stained as described [42].

Statistical analysis

All experiments were repeated at least 2 times if not differently indicated and data are represented as means +/- standard deviation. Statistical analyses were performed using GraphPad Prism by multiple t tests with the two-stage step-up method of Benjamini, Krieger and Yekutieli [43]. To assess survival differences in the animal experiments, Kaplan-Meier survival analysis was performed and p values were calculated using the log-rank test. Significance levels are *p<0.05; **p<0.01; ***p<0.001.

RESULTS

CD317 is expressed in glioma cells *in vitro* and *in vivo*

We determined the expression of CD317 on various human glioma cell lines *in vitro* by flow cytometry (Figure 1A) and *ex vivo* on orthotopically growing LN-229 glioma xenografts (Figure 1B) by immunohistochemistry and fluorescence confocal microscopy, respectively. CD317 was heterogeneously expressed in the tested glioma cell lines *in vitro* and also detectable *in vivo*. Importantly, also the GIC lines T-325, GS-2 and GS-5 expressed CD317. To explore CD317 expression *in vivo*, we performed immunohistochemical stainings demonstrating high and homogeneous expression of CD317 in different areas of glioblastoma tissue specimens. No or only minimal immunoreactivity was observed in healthy brain tissue sections whereas some organs in the periphery stained positive (Figure 1C, Suppl. Figure S1A-D).

CD317-CAR T cells lyse CD317-expressing glioma cells *in vitro*

We generated human CD317-CAR T cells by lentiviral transduction of T cells from healthy control donors with a second-generation CAR vector encoding the CD317-targeting scFv derived from the immunotoxin HM1.24-ETA'[33] and a 4-1BB co-stimulatory domain (Figure 2A). RQR8 staining revealed that more than 90% of the T cells were successfully transduced. The presence of the CAR construct on the surface of T cells was confirmed using a CD317-Fc fusion protein, which binds to the CD317-specific CAR (Figure 2B). To assess the lytic activity of CD317-CAR T cells *in vitro*, we co-cultured CD317-CAR T cells or non-transduced T cells as effector cells together with different human glioma cells including GIC lines as target cells at varying E:T ratios. CD317-CAR T cells lysed significantly more tumor target cells than non-transduced T cells (Figure 2C). Furthermore, we visualized the strong anti-glioma activity of

CD317-CAR T cells by time-lapse imaging (Suppl. Figure S1E). Importantly, CD317-CAR T cells were also active against glioma cells isolated from freshly resected tumor tissue (Suppl. Figure S2A). Non-transduced and mock-transduced T cells did not show a difference in their anti-glioma activity (Suppl. Figure S2B). In addition, we determined the activation status of T cells upon co-culture with tumor cells. Both $CD4^+$ and $CD8^+$ CD317-CAR-transduced T cells displayed higher intracellular IFN-γ expression than non-transduced T cells and IFN-γ levels in the co-culture supernatant were higher in the presence of CAR T cells (Figure 2D, E).

CD317-recognizing CAR T cells are target-specific

CD317 expression may be upregulated by type I IFN [44]. Exposure of various glioma cells, including freshly isolated tumor cells, to IFN-β increased CD317 cell surface levels up to 240-fold (Figure 3A, suppl. Figure S2C,D). On a functional level, increased CAR T cell-mediated cytotoxicity was observed against IFN-β-pretreated cells. Importantly, CD317 expression was induced by IFN-β also in glioma cells, which lack constitutive CD317 expression such as ZH-161 cells, thereby rendering them sensitive to lysis by CD317-specific CAR T cells (Figure 3B). To verify that the increased cytotoxicity of the CAR T cells towards IFN-β-exposed target cells depended on the up-regulation of CD317 and was not due to other CD317-independent effects, we generated *CD317*-overexpressing (OE) LN-229 and ZH-161 sub-cell lines (Figure 3C, suppl. Figure S2E). CD317-CAR T cells lysed significantly more LN-229 or ZH-161 *CD317* OE cells than WT cells (Figure 3D, suppl. Figure S2F).

To further confirm the target specificity of CD317-CAR T cells, we generated a *CD317* knockout (KO) in LN-229 cells using CRISPR/Cas9 gene editing and confirmed the KO efficiency by sequencing (Suppl. Figure S3A). On the protein level, CD317 expression was no longer detectable and not inducible by IFN-β in *CD317* KO cells (Figure 3E). Lack of target

antigen expression in *CD317* KO cells abrogated the increased cytolysis of CD317-CAR T cells compared to non-transduced T cells (Figure 3F). *CD317* was also described to induce the proliferation and migration of hepatocellular carcinoma, breast cancer and some glioma cells [19,22,23]. In contrast to these findings, we did not observe a reduced proliferation or migration rate of LN-229 cells with a *CD317* KO compared to CRISPR control or LN-229 WT cells (Suppl. Figure S3B,C).

Down-regulation of CD317 in T cells reduces target-driven fratricide of CD317-CAR T cells

Since CD317 is a viral restriction factor, we hypothesized that its expression might be induced in T cells upon viral transduction [45]. Viral-based engineering of human T cells increased CD317 surface levels up to 4-fold (Suppl. Figure S4A), independent of the gene encoded by the virus. CD317-CAR transduced T cells proliferated less, accumulated more cell debris and their viability was also significantly decreased compared to untreated or control-CAR transduced T cells (not shown), suggesting that the CD317-CAR T cells killed each other due to expression of the target antigen. Mutual killing of CD317-CAR T cells, also known as fratricide, may reduce their expansion rate as well as lytic activity, ultimately resulting in limited therapeutic activity, particularly when administered *in vivo*. Therefore, we aimed at down-regulating *CD317* expression in CAR-transduced T cells and their progeny and inserted a *CD317*-specific shRNA sequence into the CAR vector (Suppl. Figure S4B). To determine the efficiency of the shRNA-mediated *CD317* knockdown, we also generated an empty-CAR construct containing the shRNA and the CAR backbone only, but not the CD317 binding domain. Silencing of *CD317* in CAR T cells significantly increased their viability and expansion rate. The empty-CAR-shRNA transduced T cells had reduced CD317 expression but no total loss compared to non-transduced T cells (Figure 4A, suppl. Figure S4C,D).

Silencing of *CD317* in T cells triggers a transition from exhaustion to an activated phenotype and improves their cytolytic activity

We observed that CAR-transduced T cells expressed IFN-γ in the absence tumor cells, but CD317-CAR T cells expressed higher levels of IFN-γ than CD317-shRNA-CAR T cells alone (Figure 4B, suppl. Figure S5A). Upon co-culture with glioma cells, CD317-shRNA-CAR T cells produced significantly higher levels of IFN-γ than in the absence of target cells, whereas co-culturing of standard CD317-CAR T cells with tumor cells had only a minor impact on their activation status. Furthermore, CD317-CAR T cells expressed high levels of the exhaustion markers TIM-3 and LAG-3 in the absence of tumor cells, whereas CD317-shRNA-CAR T cells displayed constitutively low expression of these exhaustion markers, which were only induced upon co-culture with tumor cells (Figure 4C). To confirm these findings *in vivo*, we treated glioma-bearing mice with different CAR T cells and analyzed tumor-infiltrating CAR T cells 5 days later *ex vivo*. Again, CD317-shRNA-CAR T cells showed the lowest expression of exhaustion markers (Suppl. Figure S5B). On a functional level, CD317-specific CAR T cells had a significantly higher killing efficacy than non-transduced T cells and the highest anti-tumor activity was observed for CD317-shRNA-CAR T cells (Suppl. Figure S5C,D). Furthermore, we compared the capacities of CD317-CAR versus CD317-shRNA-CAR T cells to maintain long-term anti-tumor activity *in vitro* in a repetitive co-culture assay [46]. Only CD317-shRNA-CAR T cells retained their cytolytic activity through multiple rounds of re-challenge with glioma cells (Figure 4D).

Treatment with CD317-CAR T cells prolongs the survival of orthotopic glioma-bearing mice

To explore the activity of CD317-CAR T cells against experimental gliomas *in vivo*, we injected CD317-CAR T cells or non-transduced T cells intratumorally once at day 7 after

orthotopic tumor implantation. CD317-CAR T cells significantly prolonged the survival of LN-229 glioma-bearing mice compared to non-transduced T cells and cured 33% of the animals (Figure 5A). Non-transduced T cells, which showed the same activity as mock-transduced T cells (Suppl. Figure S6A), had a minor anti-tumor effect compared to PBS-treated animals. We observed persisting CD317 expression on glioma cells following CAR T cell treatment *in vivo*. Furthermore, CD317 was also stably expressed on human glioblastoma tissue specimens that were collected at the initial diagnosis and at tumor recurrence (Suppl. Figure S6B,C).

To investigate whether the efficacy of the CAR T cell treatment *in vivo* can be enhanced towards tumors with increased CD317 expression, we combined CAR T cell administration with IFN-β, which significantly induced CD317 expression *in vivo* (Suppl. Figure S7A). However, combinatorial treatment of CAR T cells and IFN-β did not improve the survival outcome and even abrogated the survival benefit of the CAR T cell treatment alone (Suppl. Figure S7B). *In vitro*, we observed that IFN-β exposure also induced CD317 expression in T cells (Suppl. Figure S7C) and interfered with CAR T cell expansion and their ability to control tumor growth over time (Suppl. Figure S7D).

Implanted LN-229 *CD317* KO tumor cells were resistant to CAR T cell treatment, demonstrating the antigen-specificity of the newly generated CAR T cells *in vivo* (Figure 5B). Similar to our *in vitro* findings, LN-229 *CD317* KO cells did not exhibit altered growth *in vivo*. Finally, we confirmed the anti-glioma activity of the newly generated CAR T cells in a second *in vivo* model using a glioma model with stem cell properties. Here, treatment with CD317-CAR T cells significantly prolonged the survival of ZH-161 glioma-bearing mice and cured 29% of the animals (Figure 5C). Overexpression of *CD317* in ZH-161-derived tumors resulted

in shorter survival when control T cells were administered. In contrast, treatment with CD317-specific CAR T cells resulted in a cure of 85% of these animals.

DISCUSSION

CAR T cell immunotherapy is a promising strategy that demonstrated substantial success against B cell neoplasms and is under investigation against several other malignancies [1,2,10,12]. In many solid tumors including glioblastoma a major challenge of this approach is the high level of tumor heterogeneity and the rarity of tumor-specific antigens that can be targeted by CAR T cell therapy.

We characterized CD317 as a novel tumor-associated antigen that is expressed by glioma cells *in vitro* and *in vivo,* while it is largely absent in healthy brain, making it an attractive target for local immunotherapy (Figure 1). We generated second-generation CD317-targeting CAR T cells and demonstrated their anti-tumor activity against glioma cell lines *in vitro* by cell lysis assays and by time-lapse imaging (Figure 2, suppl. Figure S1E, S2A). Prior exposure of glioma cells to IFN-β further increased this effect due to an induced expression of CD317 (Figure 3A,B, suppl. Figure S2C,D). The cytotoxicity of non-transduced T cells towards some IFN-β-treated glioma cells was also enhanced compared to untreated target cells, possibly by increasing the immunogenicity of these cells through an up-regulation of MHC molecules and subsequent antigen presentation [47]. CD317 expression was even induced in glioma cells that do not constitutively express CD317, which made them sensitive for CAR T cell treatment. This might be of interest for a clinical application in patients whose tumors express only low levels of CD317.

To verify that the increased cytotoxicity of the CAR T cells was antigen-dependent, we generated sub-cell lines overexpressing *CD317*. Similar to the IFN-β-mediated up-regulation

of CD317, the cytotoxicity of CD317-CAR T cells towards *CD317* OE cells was significantly higher compared to wild-type cells (Figure 3C,D, suppl. Figure S2E,F). Furthermore, we demonstrated the specificity of the CAR T cells for CD317 by generating *CD317* CRISPR KO cells that abrogated the increased lysis (Figure 3E,F). These findings suggest that the expression levels of the target antigen are an important predictor for response to CAR T cell therapy [48].

CD317 has been described to increase the proliferation and migration of hepatocellular carcinoma, breast cancer and some glioma cell lines [19,22,23]. *CD317* overexpression did not increase the proliferation of LN-229 cells *in vitro* and a knockout of *CD317* did not reduce their tumorigenicity *in vivo* (Suppl. Figure S3, Figure 5B). However, CD317 overexpression in the ZH-161 GIC model resulted in more aggressive tumors *in vivo* (Figure 5C).

A major challenge for a CAR T cell approach against glioblastoma is the rarity of tumor specific antigens. CD317 is not a neoantigen and is also expressed in some healthy tissues [44]. Since T cells also express CD317, transduction with a CD317-directed CAR caused T cell fratricide, which affected their viability and proliferation rate (Suppl. Figure S4A). Silencing of *CD317* in CAR T cells significantly increased their viability and proliferation (Figure 4A), which strengthened their effector function, especially in long-term conditions by reducing their exhaustion phenotype and overcoming chronic antigen stimulation and permanent activation even in the absence of tumor target cells (Figure 4B-D, suppl. Figure S5). High expression of immune checkpoint molecules renders effector cells susceptible to negative regulation by the immune system [49]. Thus, the anti-tumor activity of CD317-shRNA-CAR T cells *in vivo* might be even enhanced when combined with immune checkpoint blocking antibodies.

When administered intravenously to glioma-bearing mice, CD317-CAR T cells did not prolong their survival, probably because of an insufficient trafficking of T cells to the tumor site in the brain (Suppl. Figure S8A,B) as seen also in other studies with glioma xenograft models [50]. However, intratumoral administration of CD317-CAR T cells prolonged the survival and cured a significant fraction of glioma-bearing mice (Figure 5A). While no treatment-related toxicity was observed, a more comprehensive safety analysis was not feasible because of the lack of mouse CD317-specific sequences that would have allowed the assessment in a syngeneic setting. Of note, a humanized anti-CD317 antibody that cross-reacts with CD317 in monkeys was investigated in a study with non-human primates. This treatment was safe and well tolerated, which supports the assumption that CD317-specific CAR T cells can also be safely administered to human patients, particularly when given intratumorally [12,25]. Importantly, the antigen specificity of the newly generated CD317-CAR T cells was confirmed *in vivo* as mice bearing tumors with a *CD317* knockout had no survival benefit upon CAR T cell treatment (Figure 5B).

We also demonstrate the anti-tumor efficacy of CD317-CAR T cells against tumors derived from GIC *in vivo* (Figure 5C). GIC are considered as drivers of tumor growth and relapse due to their ability of pluripotency, self-renewal and tumor initiation [51]. Since ZH-161 glioma cells do not express CD317 *in vitro*, we generated *CD317* OE cells (Suppl. Figure S2E) and compared the anti-glioma activity of CAR T cells against ZH-161 *CD317* OE and WT *in vivo*. CD317-CAR T cells significantly prolonged the survival and cured the majority of ZH-161 *CD317* OE-bearing nude mice (Figure 5C). The CAR T cells also prolonged the survival of ZH-161 WT-bearing mice, because CD317 was upregulated *in vivo* on ZH-161 WT cells (Suppl. Figure S8C), rendering them sensitive to the CAR T cell treatment.

A major limitation for CAR T cell therapy against glioblastoma is target antigen loss by tumor editing [10]. In contrast to EGFRvIII, we observed stable expression levels of CD317 in glioblastoma tissue specimens independent of the time point of tumor evolution. Furthermore, CD317 was still detectable on glioma cells following CAR T cell therapy *in vivo* (Suppl. Figure S6B,C).

Since the application of CD317-CAR T cells did not cure all treated mice, we aimed at increasing CD317 expression *in vivo* in order to enhance the survival benefit of the CAR T cell treatment and to circumvent tumor immune escape by antigen loss. IFN-β treatment induced CD317 expression *in vivo* (Suppl. Figure S7A) but did not confer an additional survival benefit compared to CAR T cell treatment alone (Suppl. Figure S7B). We observed that the anti-tumor activity of CAR T cells was abrogated upon IFN-β treatment. IFN-β also induced the expression of CD317 in T cells (Suppl. Figure S7C), which might have triggered CAR T cell fratricide resulting in exhaustion and elimination of CAR T cells at the tumor site. Indeed, CAR T cells lost their ability to control tumor growth and failed to expand *in vitro* when the re-challenge with tumor cells was combined with concomitant IFN-β treatment (Suppl. Figure S7D).

A challenge for the development of CAR T cells has been the lack of antigens, which are exclusively expressed on tumors cells. While such tumor-specific antigens exist in some cancers [52], several strategies have been explored to reduce or avoid on-target/off-tumor toxicity if antigens are targeted that are also present on healthy tissue [53]. As tumor-specific antigens are largely absent in glioblastoma, there is a need to exploit antigens such as CD317 that are overexpressed on glioma cells but may also be present on some healthy tissues and bystander cells at the tumor site (Suppl. Figure S1A, S9, Erikson et al., 2011). Possibly, CD317-CAR T cells may also reduce the number of immunosuppressive cells in the

tumor microenvironment following intratumoral injection. Because of the risk of on-target/off-tumor toxicity of CD317-CAR T cells, at least upon systemic administration, we aimed at developing these cells for local application. In a clinical setting with CNS tumors, no CAR T cells were found in the periphery upon local administration of CAR T cells [12]. We also did not detect CAR T cells in spleens of mice that were treated intracranially (data not shown). CD317 expression levels in the brain are low (Figure 1C) and our CAR construct contains the suicide gene RQR8 as a safety switch allowing for a depletion of CAR T cells if needed [34]. As an alternative, mRNA-based transient CAR expression rather than stable genome engineering, which can be combined with the co-expression of a cytokine, represents a strategy to reduce the risk of toxicity [40].

In summary, our dataset demonstrates potent *in vitro* and *in vivo* activity of CD317-specific CAR T cells, which warrants further clinical evaluation of this novel therapeutic strategy in human glioblastoma patients.

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FIGURE LEGENDS

Figure 1. CD317 is expressed in glioma cells *in vitro* and *in vivo*.

(A) Determination of CD317 expression on the surface of long-term glioma cells and GIC lines by flow cytometry. HEK293T cells were used as a negative control. SFI values are indicated on the right. (B) Analysis of CD317 expression *ex vivo* in LN-229 xenografts using immunohistochemistry (upper panel, scale bars, 100 µm) or fluorescence confocal microscopy (lower panel, scale bars, 10 µm). (C) Representative CD317 immunohistochemical stainings of glioblastoma tissue specimens and healthy brain sections are shown. Size bars correspond to 20 µm or 200 µm (left). CD317 expression was quantified in 56 glioblastoma samples and 13 normal brain tissue sections by H score (right). *** indicates significance $p<0.001$.

Figure 2. CD317-specific CAR T cells lyse glioma cells in an effector:target-dependent manner. (A) Scheme of the CD317-specific CAR construct composed of the elongation factor-1 promoter (EF1), a GM-CSF signal peptide (SP), the CD317 targeting domain (CD317-scFv), a CD8 alpha hinge and transmembrane domain (CD8α TM), a 4-1BB cytoplasmic domain, a human CD3 zeta cytoplasmic domain (CD3ζ), a self-cleaving connecting peptide (T2A) and a marker suicide gene (RQR8). (B) Human T cells from healthy donors were transduced with the CD317-CAR construct or left untreated (non-transduced T cells). The expression of RQR8 was determined by flow cytometry (left panel). The presence of the CD317-specific CAR was determined by binding to a CD317-Fc fusion protein and detected by an anti-IgG antibody (right panel). Numbers indicate the percentage of RQR8-positive or CD317-CAR-positive T cells. (C) The human glioma cell lines A172, LN-18, LN-229, T98G or the human glioma-initiating cell lines GS-2, GS-5 or T-325 were used as target cells in 17 h

cytolysis assays. Human T cells were transduced with the CD317-CAR construct or not and used as effector cells at various effector:target (E:T) ratios. (D,E) The human glioma cell line LN-229 was co-cultured with CD317-CAR or control T cells at a E:T ratio of 1:1. After 17 h of co-culture, IFN-γ levels were determined in $CD4^+$ and $CD8^+$ T cells by flow cytometry or in the co-culture supernatant by ELISA. Numbers indicate the percentage of IFN-γ-positive T cells (D) or the IFN-γ concentration in the supernatant (E). Data are presented as mean ± SD (*p<0.05; **p<0.01; ***p<0.001).

Figure 3. CD317-CAR T cells are specific for their target antigen. (A) LN-18, LN-229 or ZH-161 glioma cells were exposed to 50-100 IU/ml IFN-β for 48 h or left untreated. The expression of CD317 on the cell surface was determined by flow cytometry. (B) Glioma cells were exposed to IFN-β or not and used as target cells in 17 h cytolysis assays. CD317-CAR or non-transduced T cells were used as effector cells at various E:T ratios. (C, D) *CD317* was overexpressed in LN-229 cells (OE) and the expression was determined by flow cytometry (C). LN-229 *CD317* OE cells were used as target cells and co-cultured with CD317-CAR or non-transduced T cells at various E:T ratios for 17 h (D). (E, F) *CD317* was knocked-out using CRISPR/Cas9 gene editing in LN-229 cells. CD317 expression was determined by flow cytometry (E). *CD317* knockout (KO) or control LN-229 cells were used as target cells in 17 h cytolysis assays at varying E:T ratios with CD317-CAR or non-transduced T cells as effectors (F). Data are presented as mean ± SD (*p<0.05; **p<0.01; ***p<0.001).

Figure 4. *CD317* gene silencing in T cells reduces fratricide and increases viability, expansion and cytotoxic function of CD317-CAR T cells. (A) The percentage of viable T cells transduced with the different constructs was determined by flow cytometry (left panel) and total numbers of T cells were identified manually (right panel) at the indicated days

following transduction. (B) Non-transduced T cells, CD317-CAR or CD317-shRNA-CAR T cells were co-cultured or not with LN-229 glioma cells at a E:T ratio of 1:1. After 17 h of co-culture, IFN-γ levels were determined in $CD4^+$ (left panel) and $CD8^+$ (right panel) T cells by flow cytometry. Numbers indicate percentage of IFN-γ-positive T cells. (C) CAR T cells or non-transduced T cells were co-cultured or not with LN-229 cells at a E:T ratio of 1:1 and the expression of the exhaustion markers PD-1, TIM-3 and LAG-3 was determined by flow cytometry after 17 h of co-culture. Numbers indicate percentage of positive T cells. (D) Re-challenge assay of CD317-CAR and CD317-shRNA-CAR T cells. Two thousand T cells were co-cultured with 2000 LN-229 cells and repetitively challenged with 3000 tumor cells every 48 h (arrows at day (D) 2, 4, 6 and 8). Numbers of remaining viable tumor cells were determined at indicated time points by flow cytometry (day 1, 3, 5, 7 and 9). Data are presented as mean ± SD (*p<0.05; **p<0.01; ***p<0.001).

Figure 5. Treatment with CD317-CAR T cells prolongs the survival and cures a fraction of orthotopic glioma-bearing mice. (A) LN-229 tumor-bearing mice were treated with a single intratumoral injection of 10^6 CD317-shRNA-CAR T cells, 10^6 non-transduced T cells or PBS control at day 7 after tumor implantation. (B) LN-229 wildtype (WT) or *CD317* KO cells were implanted into nude mice. Seven days later, mice were treated intratumorally with 10^6 CD317-shRNA-CAR T cells, non-transduced T cells or PBS control. (C) ZH-161 WT or *CD317* OE tumor-bearing mice were treated intratumorally with 10^6 CD317-shRNA-CAR T cells or 10^6 non-transduced T cells 5 days after tumor implantation. Survival data are presented as Kaplan-Meier plots and significance was determined with the log-rank test (*p<0.05; **p<0.01; ***p<0.001).

Figure 1

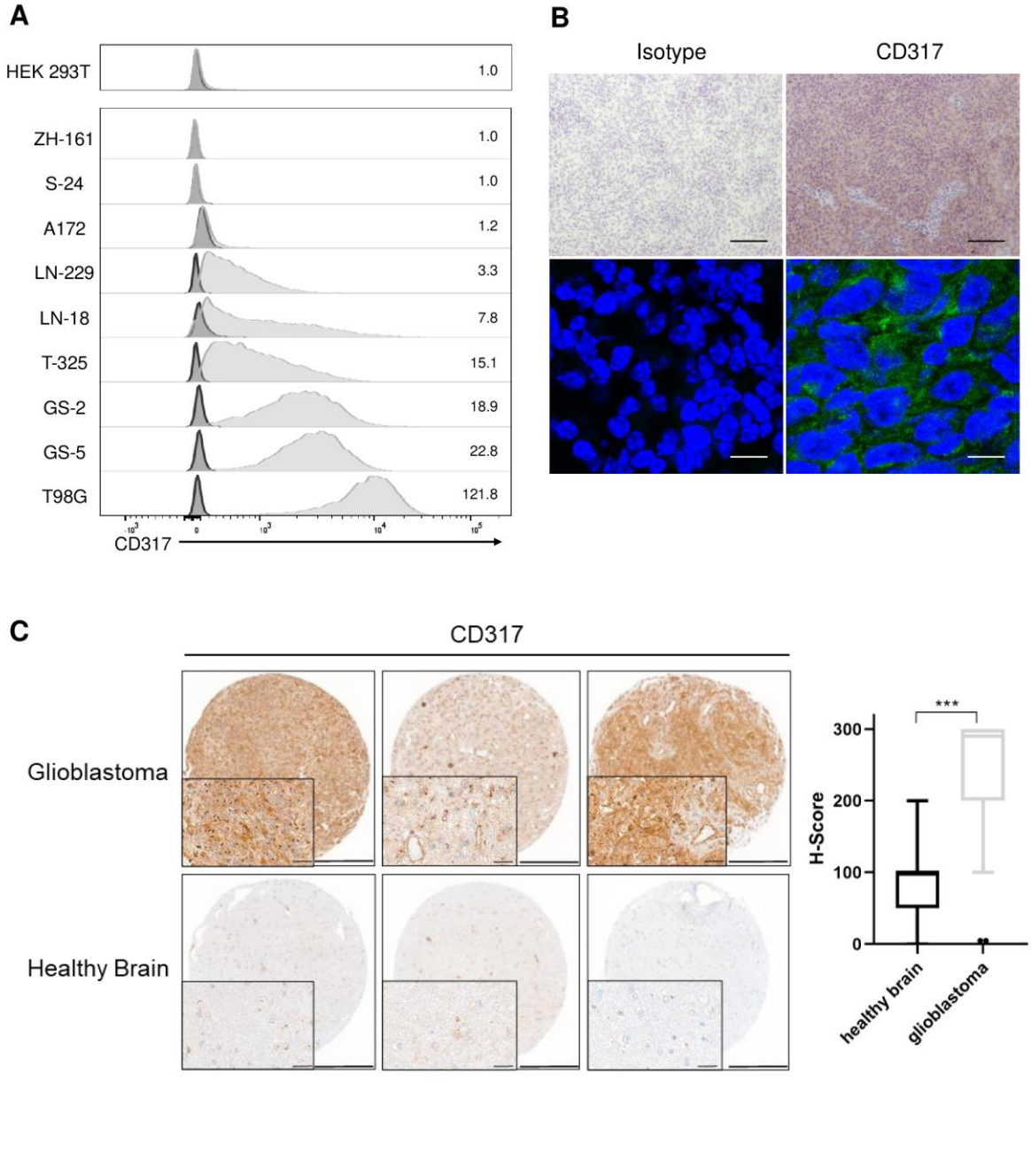

Figure 2

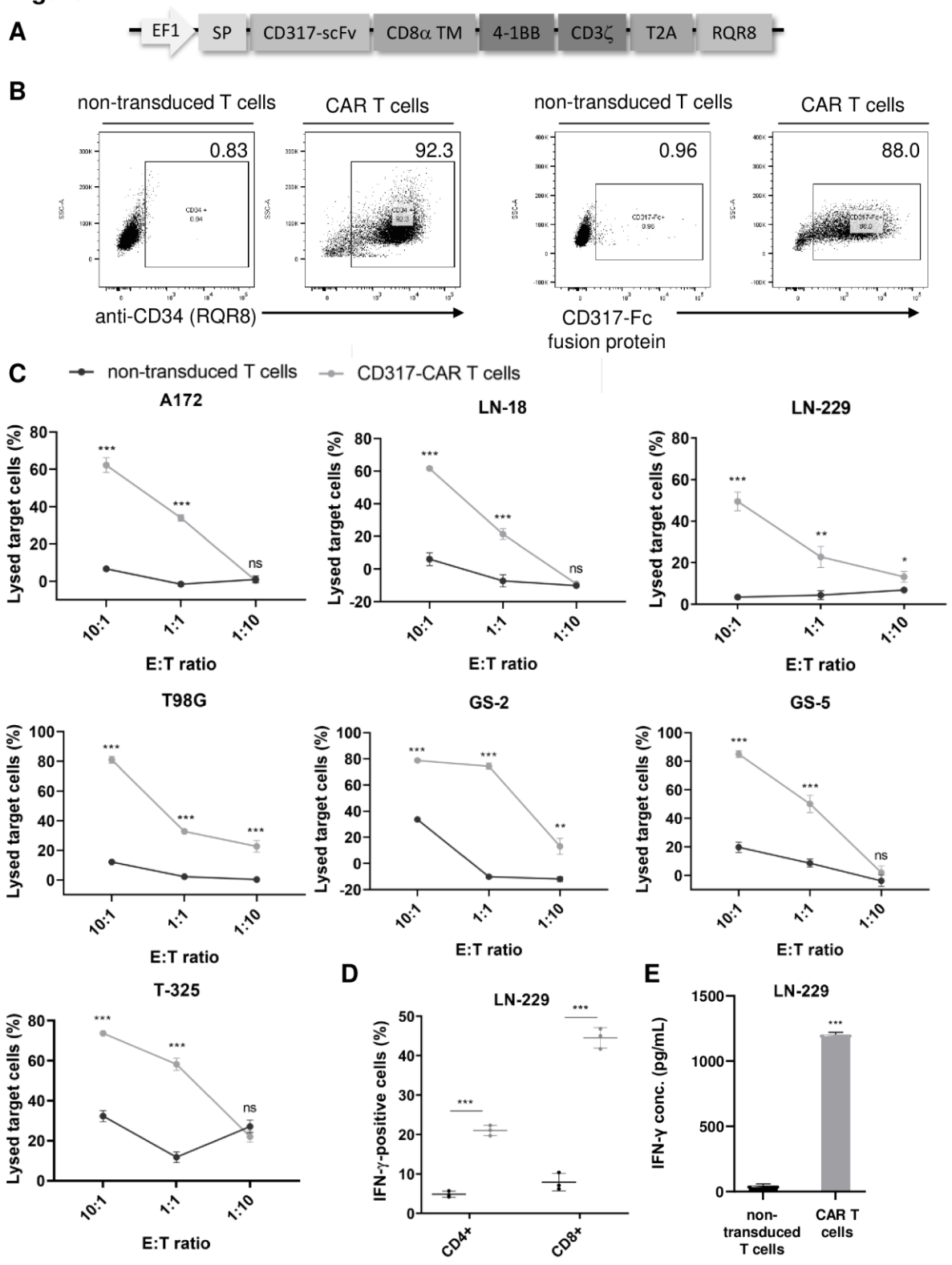

Figure 3

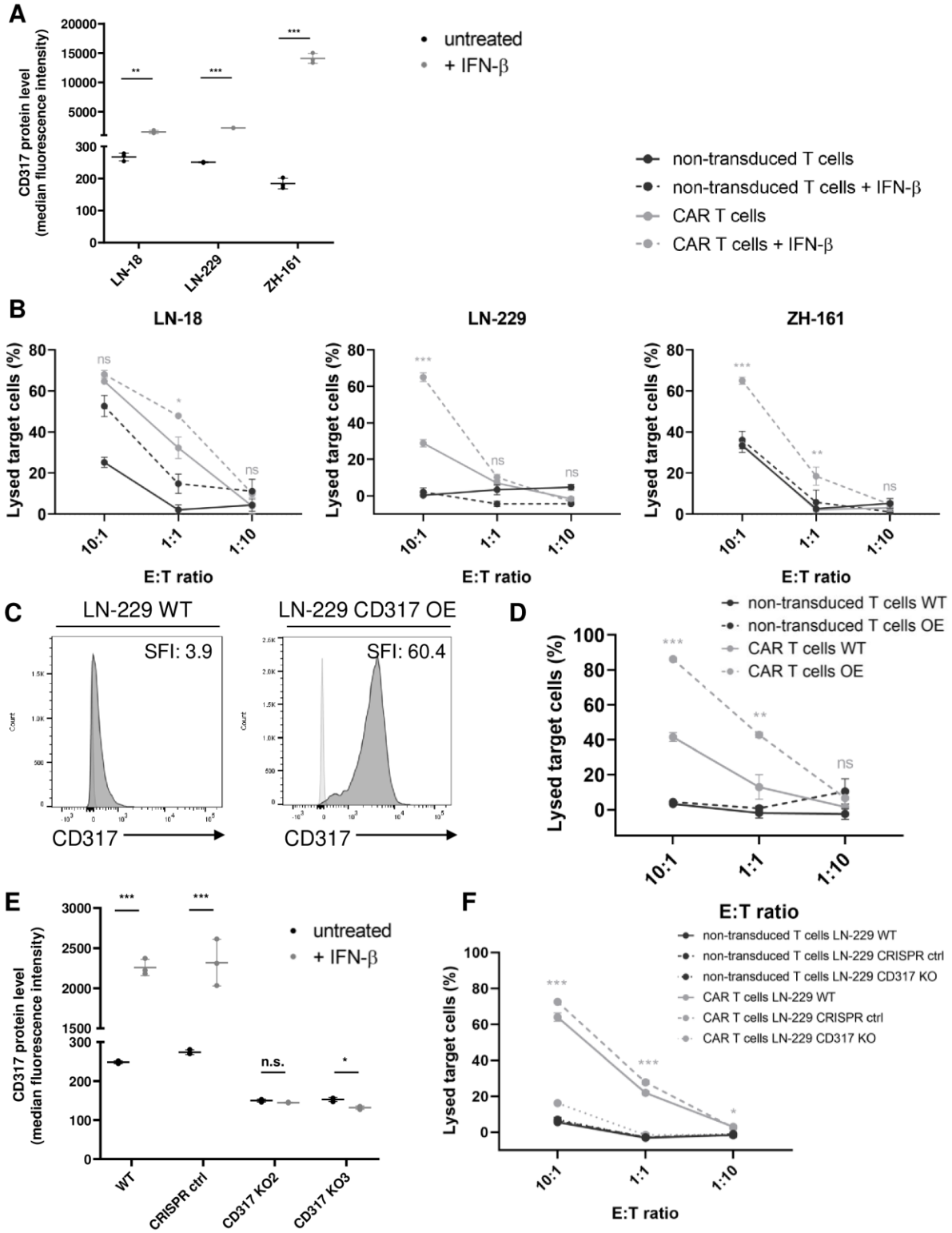

Figure 4

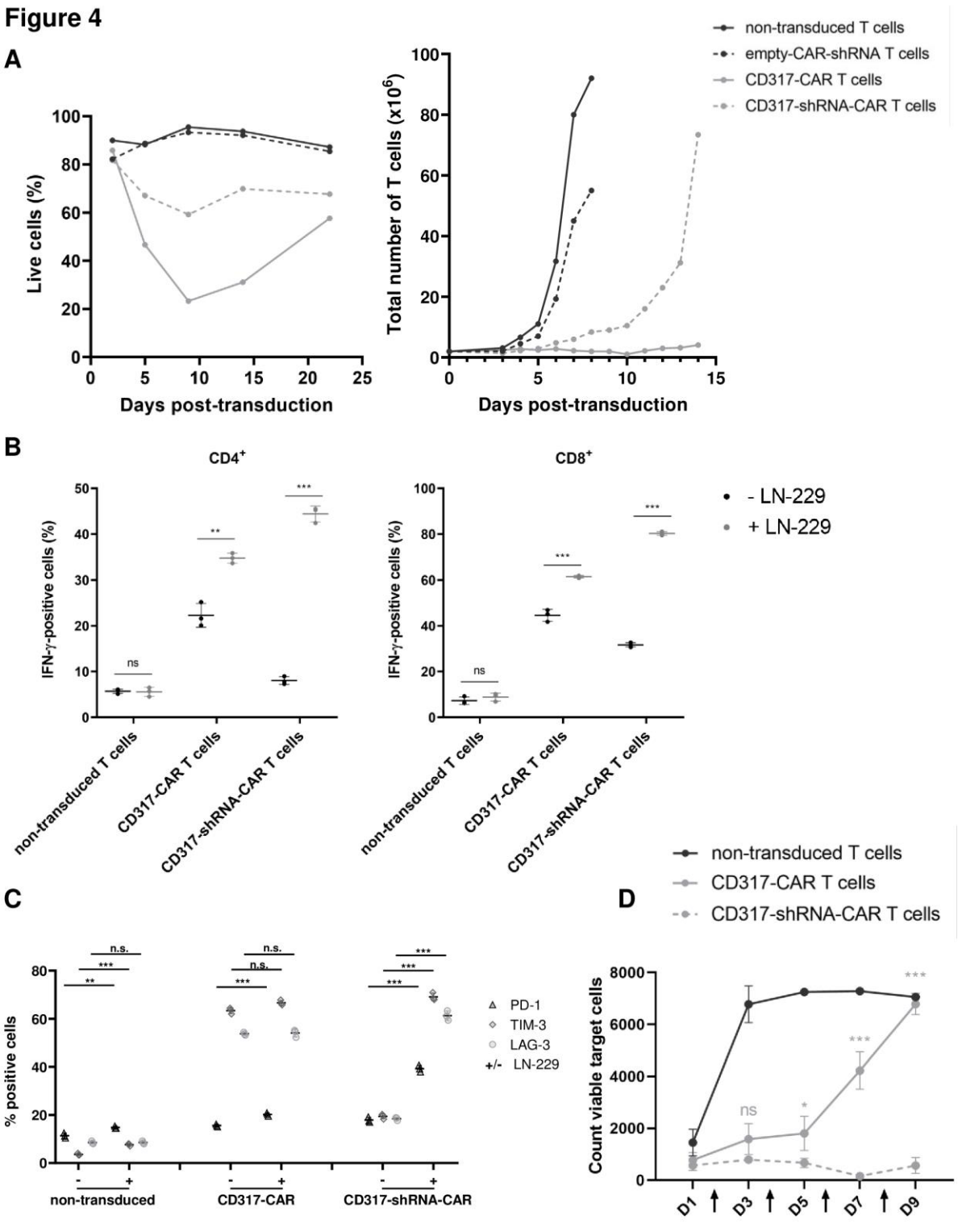

Figure 5