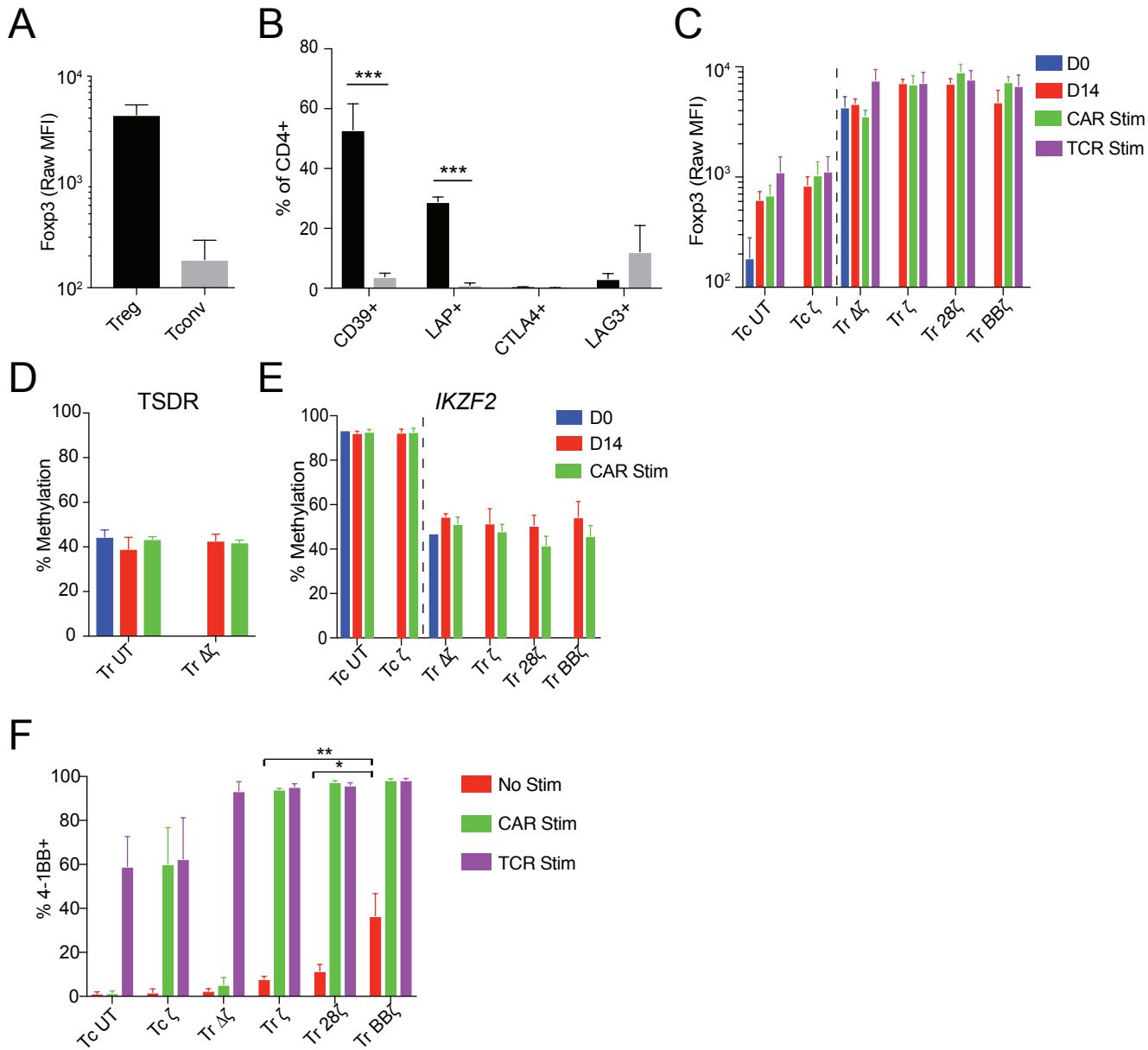


Supplemental Materials**Supplemental Table 1.****Supplemental Figures and Legends.****Supplemental Methods.****Supplemental Table 1: Treg expansion data after bead stimulation**

Sample	Mean no. of population doublings	Standard deviation
Tr BB ζ	5.26	0.76
Tr 28 ζ	5.05	0.70
Tr ζ	5.24	0.43
Tr $\Delta\zeta$	4.59	0.52

Number of Treg population doublings after 14 days in culture (7-day bead expansion followed by 7 days of rest) from starting numbers of $2e5$ - $4e5$ sorted Tregs/group before transduction (n=5 human donors).

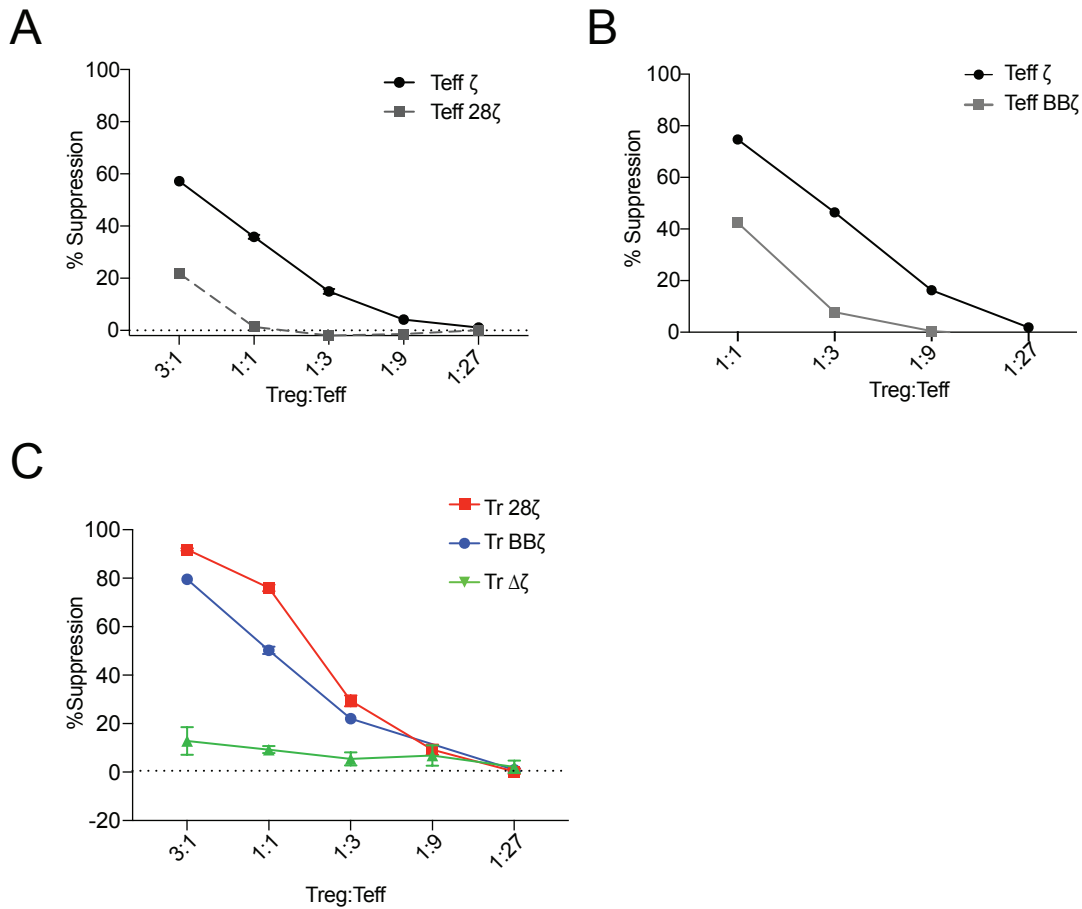
Supplemental Figure 1



Supplemental Figure 1. Treg phenotype and Foxp3 stability

Flow cytometry of Tregs (Tr) and Tconv (Tc) cells gated on CD4⁺ cells. A) Intracellular staining of Foxp3, displayed as mean fluorescence intensity (MFI) and B) surface expression of markers LAP, LAG3, CD39 and CTLA4 (n>3 human donors) on T cells after fluorescence activated cell sorting (FACS) and before expansion. C) Foxp3 intracellular staining (MFI) after FACS (Day 0), after bead expansion and rest (Day 14) and on day 23, after 9 days in culture with irradiated anti CD3 K562 (TCR Stim) or irradiated CD19 K562 stimulation (CAR Stim) (n=6 human donors). Methylation status of the D) TSDR and E) IKZF2 (Helios) promoter from mCherry⁺ cells D0, D14 and after CAR stimulation (9 days after the addition of CD19-K562 cells) using direct bisulfite modification and pyrosequencing (n=3 human donors). F) Percentage of cells expressing 4-1BB (CD137) surface expression of live CD4⁺ CAR⁺ gated cells (n=3 human donors). Blue bars for D0 represent untransduced (UT) Treg (Tr) and UT T conv (Tc) directly after FACS. All data represent mean +/- SEM, *P < 0.05; **P < 0.01; ***P < 0.001 by paired t test between Tr and Tc (B) and by paired t tests across no stim conditions (F).

Supplemental Figure 2

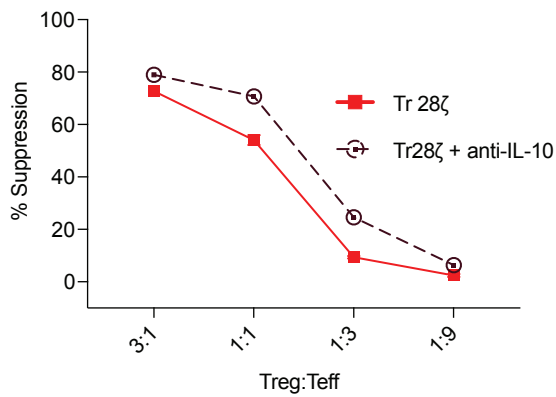


Supplemental Figure 2. Suppression of proliferation by CAR-Tregs.

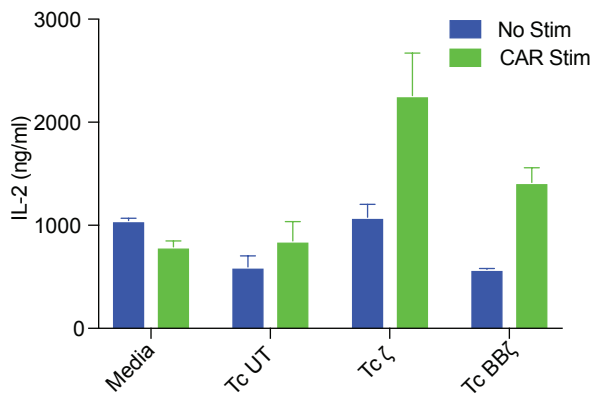
Suppression by 28 CAR Tregs in mixed lymphocyte reaction (MLR) measuring the CFSE dilution of CD19 first- generation versus A) second-generation CD19-28 CAR Teff or B) second-generation CD19-BB CAR-Teff using irradiated Nalm6 as target cells. Data are representative of 2 independent donors. C) MLR of CAR-Tregs incubated with CD19- bulk (CD4+ CD8+) Teff cells with irradiated Nalm6 cells as targets. Suppression is assessed by the CFSE dilution of CD8+ CAR+ gated T cells (CD3+ CFSE+ CD8+ mCherry+). Mean +/- SEM plotted; Tr – Treg.

Supplemental Figure 3

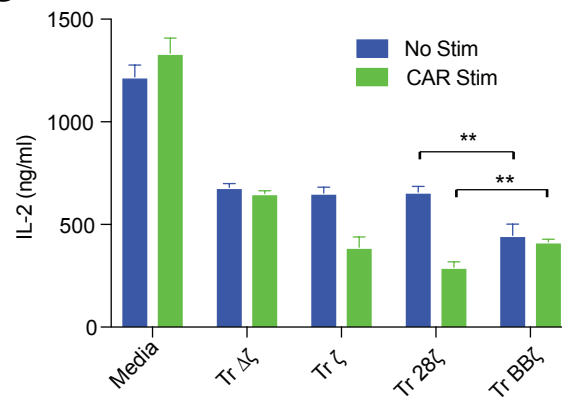
A



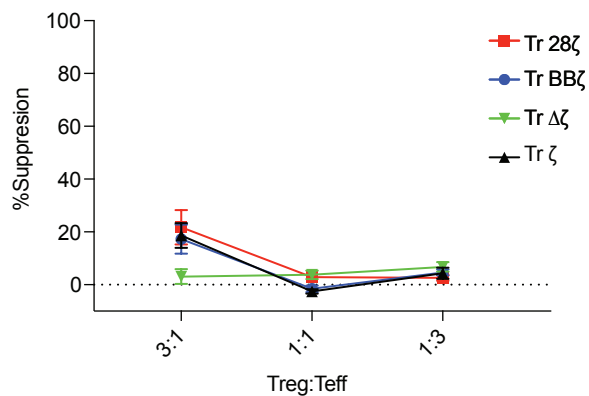
B



C



D

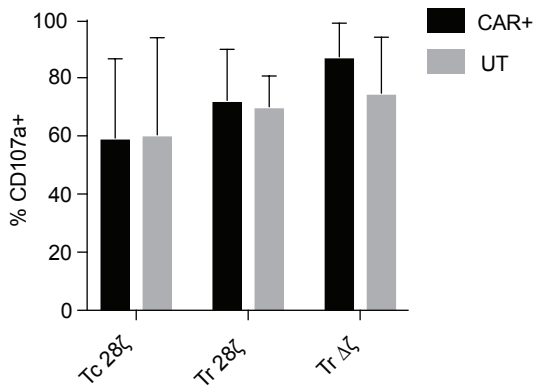


Supplemental Figure 3. CAR-Tregs differentially consume IL-2 in vitro.

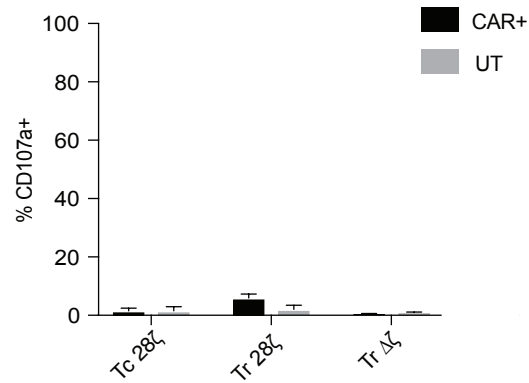
A) MLR with or without IL 10 blocking antibody at different ratios of CD19-28 CAR Tregs to CD19- CAR-Teff cells with irradiated Nalm6 cells as targets. IL 2 cytokine levels after B) Tconv and C) Treg cells expressing different CAR constructs incubated with the same initial concentrations of IL 2 (50 IU/ml) for 40 hours with or without the addition of irradiated Nalm6 cells (CAR Stim). *P < 0.05; **P < 0.01 by paired ratio t test of combined data from 3 human donors. D) MLR with indicated ratios of CAR-Tregs to CD19- Teff cells and irradiated Nalm6 cells as targets with exogenous rhIL-2 added to the media (50 IU/ml). All data are representative of 3 human donors in technical triplicates, mean +/- SEM of triplicates plotted. Tr – Treg, Tc – Tconv.

Supplemental Figure 4

A



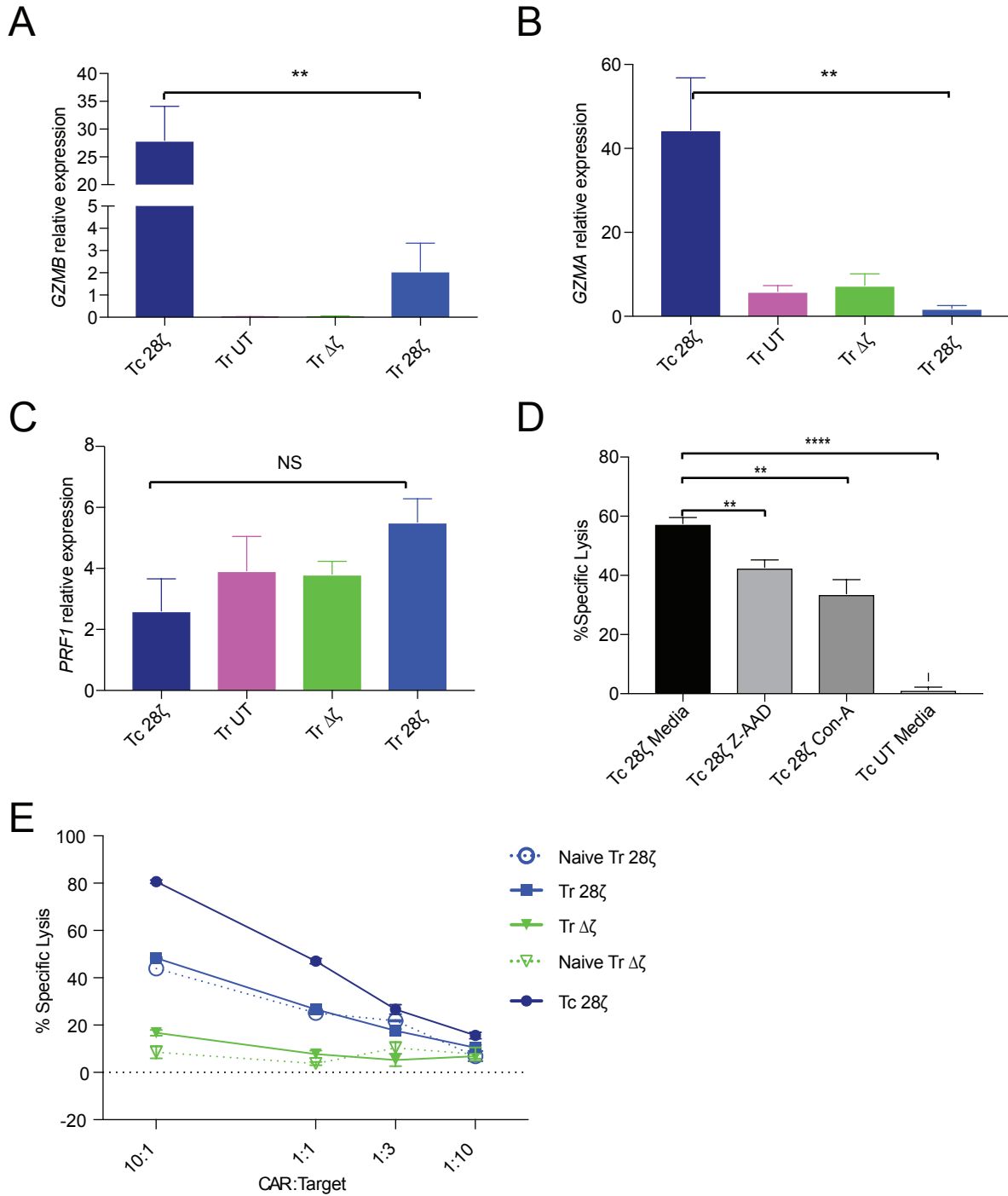
B



Supplemental Figure 4. Target specific cytotoxicity by CAR-Tregs

Degranulation assay of T cells calculated as the percentage CD107+ cells of CAR+ (CD3+mCherry+) or UT (CD3+mCherry-) T cells per well over A) 2 hours with PMA/ionomycin stimulation or B) 6 hours with no stimulation. (n=3 human donors) Tc – Tconv cells; Tr – Treg; UT – untransduced.

Supplemental Figure 5



Supplemental Figure 5. Target specific lysis of by CAR Tregs is Perforin/ Granzyme dependent

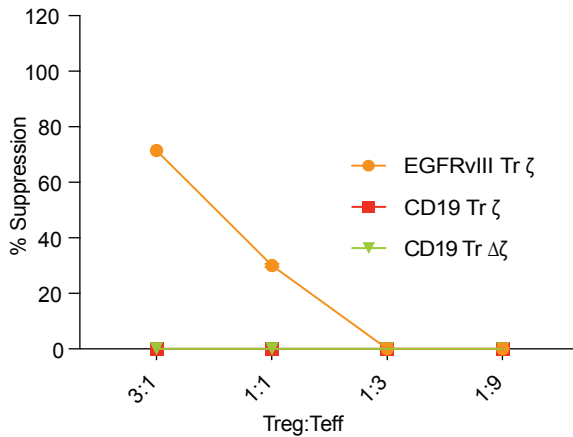
Expression of A) GZMB, B) GZMA and C) PRF1 from CD19 CAR-Tconv and CD19 CAR-Treg cells stimulated with irradiated Nalm6 for 24 hours and sorted by CD4+ mCherry+. Gene expression from cDNA was measured by digital droplet PCR and expressed as a relative ratio to internal control gene TBP (n = 4 normal donors, Mean SEM plotted). D) Luciferase based killing assays of CBG-GFP+ Nalm6 cells with CD19-28 Tconv plus granzyme/ perforin inhibitors CMA and Z AAD CMK (1:3 Tcell-to-target ratio, 16 hour incubation time). UT Tconv cells shown as the negative control for antigen-specific cytotoxicity. E) Luciferase based cytotoxicity assays using Tregs sorted on CD45RA+ (naïve) Treg cells with Nalm6 CBG GFP target cells. Data are representative of 3 normal donors in technical triplicate. Mean +/- SEM of triplicates plotted. **P < 0.01; ****P < 0.0001 by paired ratio t test comparing Tc 28 to Tr 28 (A-C) or by unpaired t test comparing (D). Tc – Tconv cells, Tr – Treg, UT – untransduced.

Supplemental Figure 6

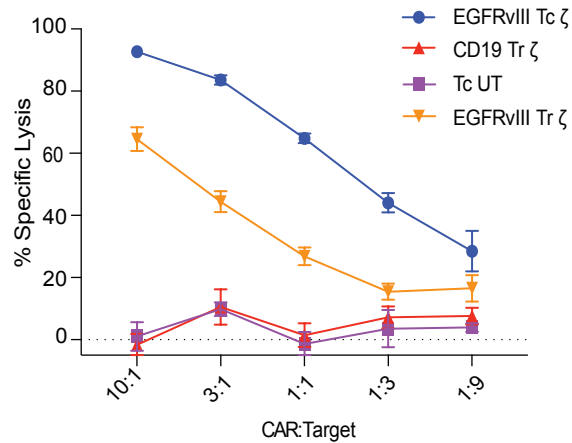
A



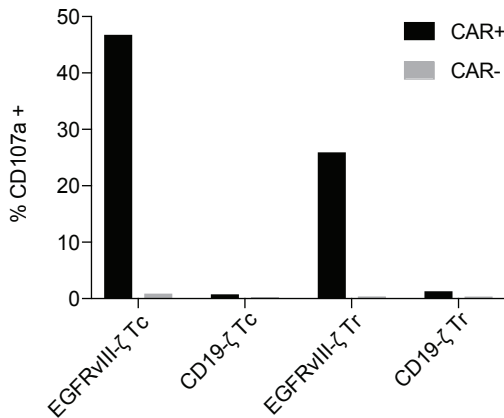
B



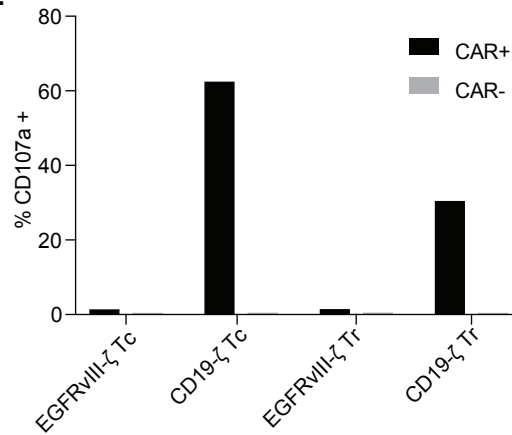
C



D



E



Supplemental Figure 6. Target specific cytotoxicity by CAR Tregs is independent of the affinity of the scFv.

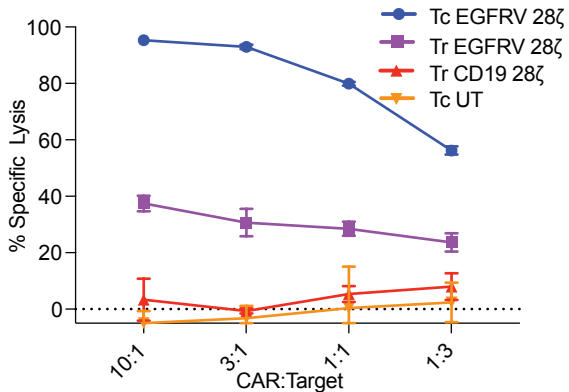
A) Vector map of low-affinity EGFRvIII CAR constructs. LTR, long terminal repeat; L, leader sequence; scFv, single-chain variable fragment; TM, hinge and transmembrane domain. B) MLR of CFSE labeled EGFRvIII CAR-Treg with different EGFRvIII CAR-Treg ratios stimulated with U87 EGFRvIII cells. After 4 days, Treg suppression was calculated by comparing the % of mCherry+ CFSElow cells in a Treg condition to the % CFSElow mCherry+ Treg cells with no Tregs present. C) Luciferase based killing assay using U87 EGFRvIII CBG GFP cells incubated with first generation CAR Tregs and Tconv cells with scFvs against CD19 or EGFRvIII at varying ratios for 16 hours. Mean SEM of technical triplicates plotted for B, C. Degranulation assay of CAR T cells in media with CD107a antibody and Befeladin A for 6 hour stimulation with D) U87 EGFRvIII target cells or E) U87 CD19 target cells. Cells gated on CD3+ mCherry+ (CAR+) or CD3+ mCherry- (UT) within a sample. Representative donor. Tc – Tconv cells, Tr – Treg All data (B-E) are representative of 3 human donors.

Supplemental Figure 7

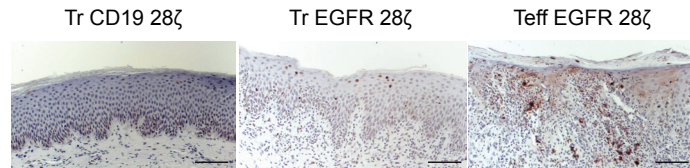
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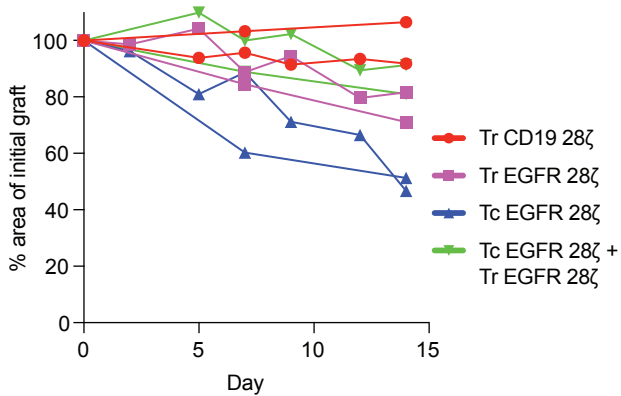
B



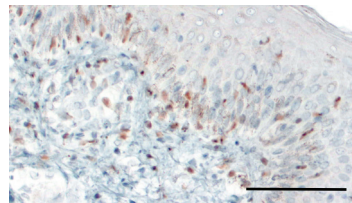
C



D



E



Supplemental Figure 7. In vivo model of Treg suppression using skin xenografts

A) Vector map of EGFR-28 CAR construct. LTR, long terminal repeat; L, leader sequence; scFv, single-chain variable fragment; TM, hinge and transmembrane domain. B) Luciferase based killing assay using U87 CBG GFP cells incubated with EGFR-28 CAR-Tregs and Tconv cells at indicated ratios for 16 hours. Data are representative of n=3 human donors in technical triplicate. C) TUNEL stained graft sections 2 weeks after Teff or Treg administration (10x objective lens, representative image of donor 1A, n=3 skin donor/T cell donor pairs). D) Size of graft measured as a percentage of the size prior to CAR T cell injection (Donor 1/A and 2/B) . E) Immunohistochemistry of nuclear Foxp3 in skin xenograft of EGFR-28 CAR-Treg treated mouse (20x objective lens). Scale bar – 100 m in all photomicrographs.

Supplemental Methods

Generation and passaging of artificial antigen presenting cell lines

Cell lines were lentivirally transduced to express the click beetle green luciferase and green fluorescent protein (GFP) under control of the EF-1 α promoter followed by single cell sorting to grow a clonal population. Lentiviral particles, produced to express EGFRvIII, human CD19 or membrane-bound OKT3 scFv under the control of the EF-1 α , were used to generate U87-CD19, U87-EGFRvIII K562-CD19 and K562-OKT3 by transduction and single cell sorting for positive populations by FACs. U87 and HEK293T cells were passaged using 0.25% and 0.05% Trypsin-EDTA (Thermo Fisher Scientific) respectively. Target cells were irradiated with 10 000 rads and frozen in FBS with 10% DMSO to be thawed prior to stimulation of CAR T cells. Cell lines were tested for mycoplasma contamination every 3 months.

Intracellular staining

Blue, Aqua or Violet LIVE/DEAD fixable dyes (Thermo Fisher) were used to stain dead cells in PBS for 20 min at 4°C before surface staining and fixation. Cells were fixed and permeabilized for 45 min and then washed in permeabilization buffer and blocked with rat serum 2 μ l/100ul for 15 min and room temperature (RT). Fixed cells were stained for 30 min with 2 μ l of Foxp3 antibody/100 μ l at 4°C. Fluorescence was measured on a BD Fortessa x-20 and data were analyzed using FlowJo (Tree Star).

Cytokine detection by Luminex

For cytokine release assays, Tregs or Tconv cells were stimulated in a 96-well round-bottom plate with 100 000 CAR T-cells/well combined with irradiated K562-OKT3 or K562-CD19 target cells at a CAR T cell-to-target ratio of 2:1 in a total volume of 200 μ l. Supernatants were harvested after 24 hours and frozen at -80°C . 50 μ l of supernatant/ sample was analyzed for cytokine levels using FLEXMAP 3D $\text{\textcircled{R}}$ platform from Lumina Instrumentation (Thermo Fisher Scientific) according to manufacturer's instructions with a panel of the following cytokines: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL12p70, IL-13, IL-18, IFN- γ , GM-CSF, TNF- α and IL-10. Plates were read using xPONENT Software 4.1. All samples were measured in technical triplicates and with $N \geq 3$ normal donors. Technical triplicates were averaged before graphing with Prism (Graphpad software).

Violet labeling

Cells were washed 2 times in PBS and labeled with 1 ml of cell trace violet dye at a concentration of 1 μ M in PBS for 10 min at room temperature. Labeling was stopped by incubating cells for 1 minute with 1ml of FBS and for a further 10 minutes, after the addition of 10ml of R10. Tregs were then washed three times before resuspending in media for assays.

IL-2 sink assay

Tregs or Tconv cells were plated at 1e5 CAR T cells/well with technical triplicates in a 96-well round-bottomed plate and stimulated with 1e5 irradiated Nalm6 or no

stimulation. We also included a media only condition where no T cells were added. All cells were in OpTmizer with a final starting concentration of 50 IU/ml IL-2 in 200 μ l. Cells were incubated for 40 hours at 37°C after which, supernatants were frozen at -80°C. Cytokine levels were measured from 50 μ l of supernatant via luminex.

Degranulation assay

Degranulation of Tregs and Tconv cells was calculated by incubating 3e5 T cells in a 24-well plate with live, Nalm6 at a 1:1 T cell-to-target ratio for 6 hours at 37°C in media with CD107a (14 μ l/ml) and brefeldin A (BD golgiplug 1 μ l/ml). PMA ionomycin (cell stimulation cocktail, eBioscience) was used at 1X concentration in culture for 2 hours. Cells were stained with live/dead fixable stain according to manufacturer's instructions (Invitrogen) followed by surface staining for CD3 and CD4. To determine the Foxp3 expression levels of degranulating cells, we fixed and permeabilized cells before staining with Foxp3. Both unfixed, surface only stain as well as fixed and permeabilized cells were analyzed by flow cytometry.

H&E, immunohistochemistry and TUNEL staining

Tissue was collected and fixed in 10% formalin for 24 h followed by standard paraffin embedding. 5 μ m paraffin-embedded tissue sections on glass slides were baked at 60°C for 30 minutes, followed by deparaffinization in xylene and rehydration in graded alcohol into water. After washing with TBS/Tween 20, antigen retrieval was performed by boiling the slides in 10mM Sodium Citrate buffer pH=6.0 for 30 minutes. Endogenous peroxidase activity was quenched with Dual Endogenous Enzyme Block (DAKO) for 5

minutes. After washing, tissue sections were incubated with 1:100 dilution of mCherry rabbit polyclonal antibody (Abcam, ab183628) or 1:400 dilution of CD3 rabbit polyclonal antibody (Dako A0452) in 1% TBS/BSA inside a humidified chamber 1 hour at room temperature. After washing, slides were incubated with HRP labelled anti-Rabbit Polymer (Dako) 30 minutes at RT. After washing the DAB+ reagent (DAKO) was added with monitoring for 5-10 minutes. After washing counterstain was done using Harris type Hematoxylin. Slides were briefly dehydrated and then mounted with Histomount solution (Life Technology, 008030).

For TUNEL stain, slides were baked and deparaffinization and rehydrated as above. After washing with TBS/Tween 20 proteinase K (DAKO) was added to slides at a dilution of 1:10 in Tris buffer. TUNEL staining was performed using the S7100 | ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to manufactures instructions. DAB substrate and DAB Enhancer (DAKO) were used for detection followed by hematoxylin counterstain. Slides were hydrated and mounted as for IHC.

Quantification of T cells and TUNEL + cells was performed with a light microscope counting positively stained cells per high power field (hpf) with either 200x to 400x magnification. 8-10 sections were randomly chosen in the area described in the figure legend.

Automation RNA in situ hybridization (ISH) assay

Automated RNA-ISH assay was performed using the RNAscope 2.5 LS Reagent Kit-Brown from Advanced Cell Diagnostics (ACD) (Catalogue No.322100) on the BondRx platform. 5-µm sections of FFPE tissue were mounted on Surgipath X-tra glass slides, baked for 1 hour at 60°C, and placed on the BOND RX for processing. On the BOND RX, the staining protocol used was the ACD ISH DAB Protocol. The RNA unmasking conditions for the tissue consisted of a 15-minute incubation at 95°C in Bond Epitope Retrieval Solution 2 (Leica Biosystems) followed by 15-minute incubation with Proteinase K which was provided in the kit. Probe hybridization was done for 2 hours with RNAscope probes which were provided by ACD. The probes used for this study were, 2.5 LS *TGFB1* Probe (Catalogue No. 443488); 2.5 LS *PRF1* Probe (Catalogue No. 550288); 2.5 LS *GZMB* Probe (Catalogue No. 550328) and 2.5 LS *IL10* Probe (Catalogue No. 550348). The RNA-ISH assay uses highly specific, branched DNA technology in which signal amplification is implemented to detect target mRNAs within the FFPE tissue section via a series of sequential hybridization steps in which the probe binds to the target mRNA. Subsequent binding of the preamplifier, amplifier and alkaline phosphatase-labelled probe molecules creates a signal amplification structure which can then be visualized with the 3,3'-Diaminobenzidine (DAB) as a chromogen to form a brown dot which can then be visualized using a standard bright field microscope.

ddPCR

CD19 CAR T cells were transduced, expanded and rested for 7 days. 1e6 cells were stimulated with 1e6 Nalm6 cells over 24 hours and then stained with CD4 antibody. 5e5 cells were collected by FACs and resuspended in 350µl of lysis buffer with 1%

2-mercaptoethanol. RNA was extracted and purified using Aurum Total RNA Mini Kit (Bio-Rad) and cDNA was generated using iScript Reverse Transcription supermix (Bio-Rad). Digital Droplet PCR was performed using ddPCR supermix with no dUTPs (Bio-Rad) with a QX200 Droplet Digital PCR (ddPCR™) System (Bio-Rad) platform for quantification. Droplet generation, PCR and detection of positive droplets were performed according to manufacturer's instructions (Instruction Manual, QX200™ Droplet Generator – Bio-Rad).

The PCR cycling protocol was according the manufacturer's instructions with a 57°C melting temperature. Human TBP was used as the reference gene in each reaction, (HEX fluorophore : TBP PrimePCR™ ddPCR™ Expression Probe Assay: **Unique Assay ID:** dHsaCPE5058363 (Bio-Rad)). The following FAM fluorophore primer probes were used:

GZMB: PrimeTime Std® qPCR Assay unique assay ID Hs.PT.58.26439821.g (IDT)

GZMA: PrimePCR™ PCR Primers unique assay ID **dHsaCPE5047756 (BioRad)**

PFR1: PrimePCR™ PCR Primers unique assay ID **dHsaCPE5030232 (BioRad)**