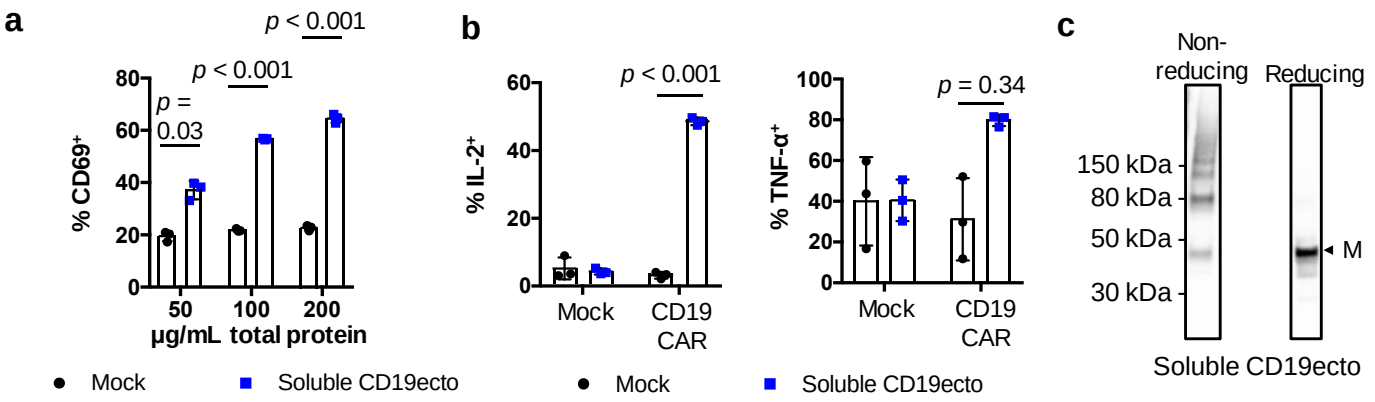
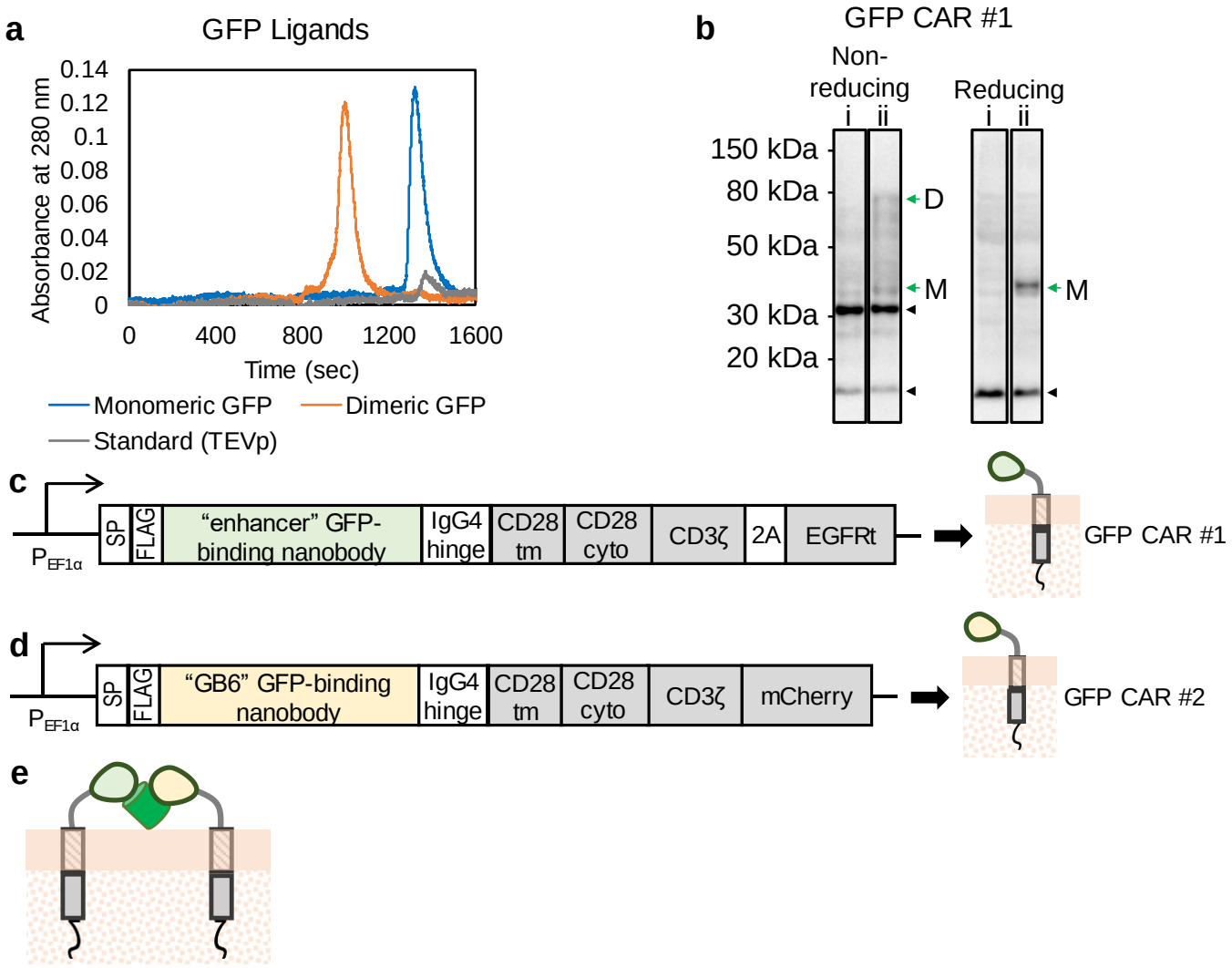
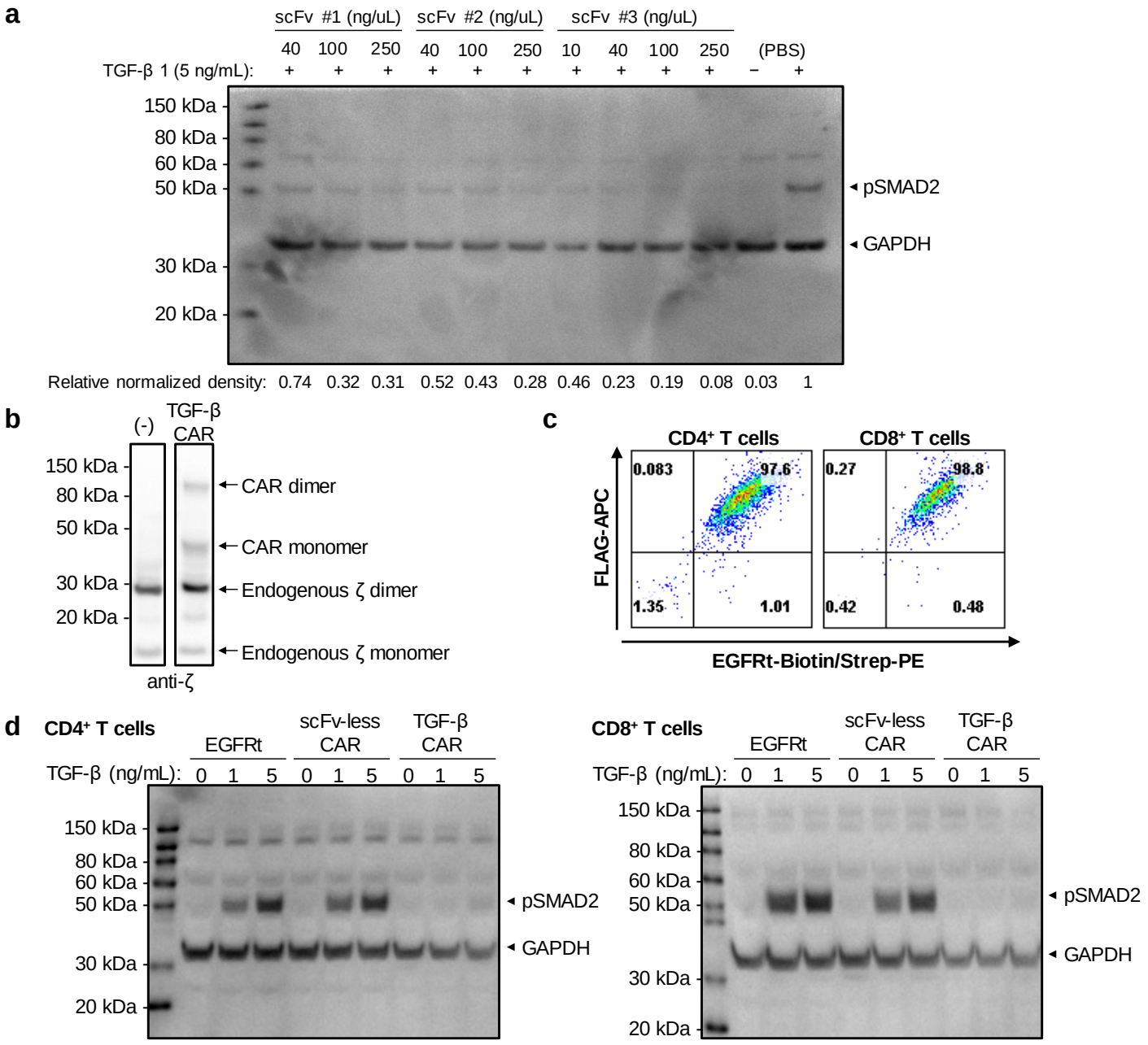


Supplementary Figure 1: CD19 CAR can be activated by soluble ligand. HEK293T cells were transfected with an empty plasmid (mock) or a plasmid encoding CD19 ectodomain (CD19ecto) tagged with a secretion signal. (**a**) CD69 expression was assessed in a CD19 CAR Jurkat cell line exposed to varying levels mock or soluble CD19ecto-containing concentrated supernatant. (**b**) Interleukin (IL)-2 or tumor necrosis factor alpha (TNF-α) production were assessed by intracellular cytokine staining of primary human $CD4^+$ mock-transduced (i.e., CAR-less) T cells or CD19 CAR-T cells exposed to mock or soluble CD19ecto-containing concentrated supernatant at 200 µg/mL total protein. (**c**) Non-reducing and reducing western blots of soluble CD19ecto-containing supernatant, representative of n = 3 independent experiments. In all graphs, data points from n = 3 biologically independent cell cultures are shown with means ± 1 standard deviation (SD). Statistical comparisons performed with the two-tailed Student's *t* test and the Sidak correction for multiple comparisons.

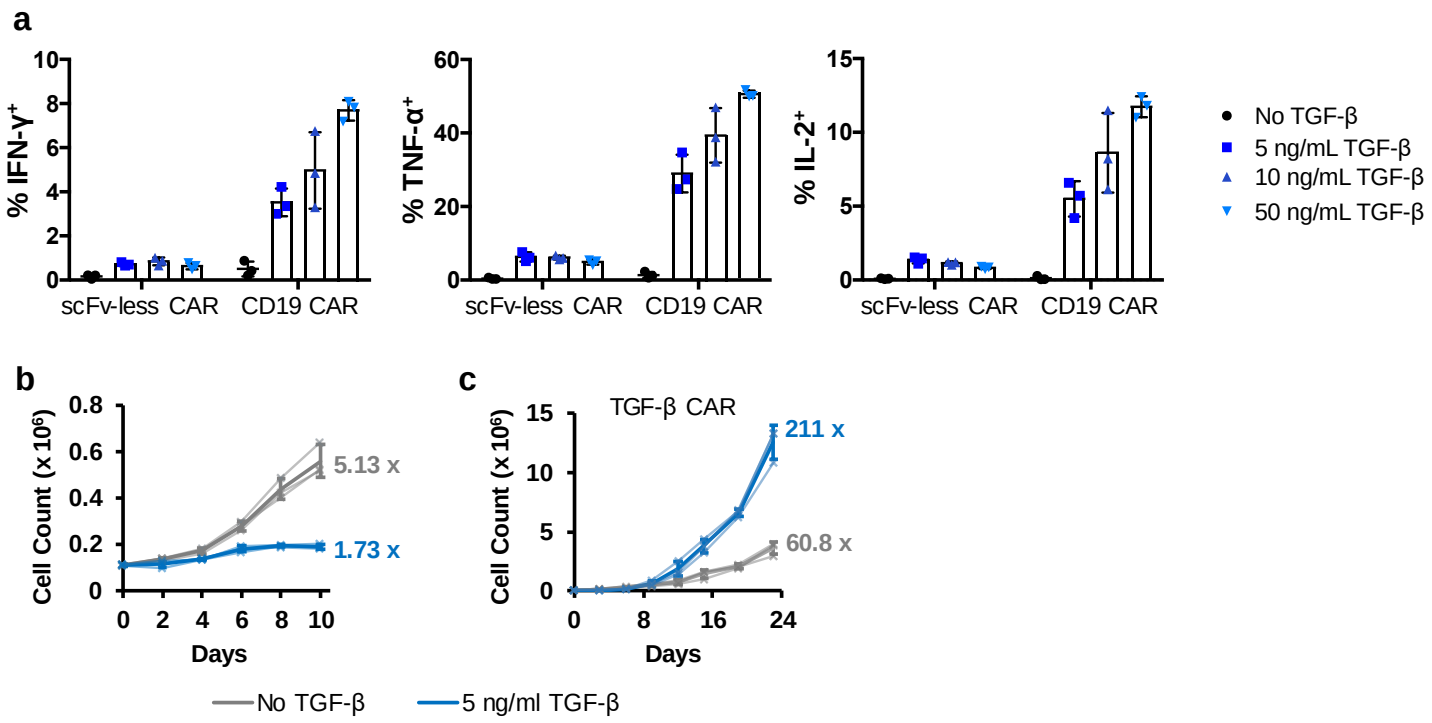


Supplementary Figure 2: Components of the GFP CAR model system. (**a**) Size-exclusion chromatography was performed once to show the two ligands run at distinct sizes. The monomeric sfGFP runs similarly to tobacco etch virus protease (TEVp), which is of similar molecular weight to sfGFP. (**b**) Reducing and non-reducing western blots of (**i**) parental Jurkat cells and (**ii**) GFP CAR #1-expressing Jurkat cells, representative of n = 3 independent experiments. Green arrows point at bands corresponding to monomeric (M) and dimeric (D) CARs. Black arrowheads indicate the molecular weights for endogenous dimeric and monomeric CD3ζ chains. (**c**) GFP CAR #1 contains the "enhancer" GFP-binding nanobody linked to the CD28 and CD3ζ endodomains. SP, signal peptide; FLAG, DYKDDDDK epitope; V_H, heavy-chain variable domain; V_L, light-chain variable domain; tm, transmembrane; cyto, cytosolic; 2A, 2A "self-cleaving" peptide; EGFRt, truncated epidermal growth factor receptor. (**d**) GFP CAR #2 contains the "GB6" GFP-binding nanobody linked to the CD28 and CD3ζ endodomains. (**e**) The GFP-binding nanobodies in GFP CAR #1 and GFP CAR #2 are chosen so that they can concurrently bind monomeric EGFP.

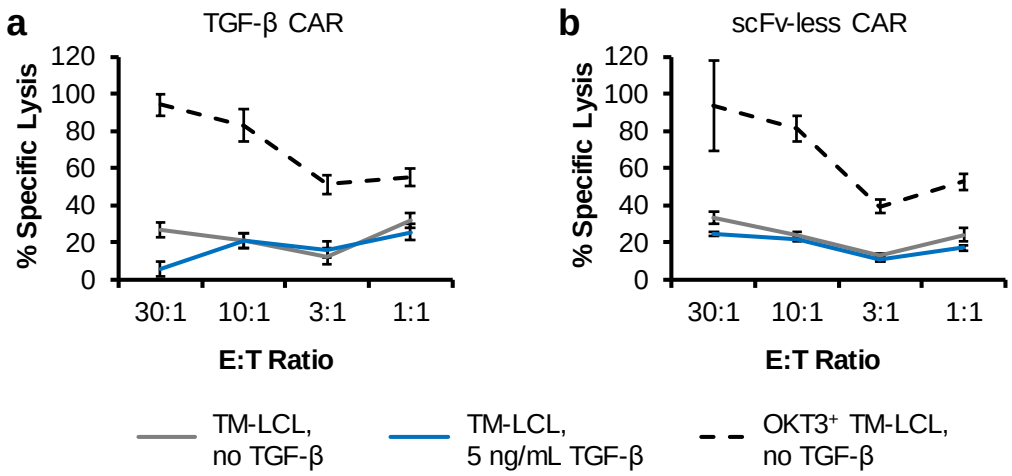


Supplementary Figure 3: Generation of a TGF-β–binding CAR that efficiently presents to the cell surface and inhibits native TGF-β signaling. (**a**) Three TGF-β–binding single-chain variable fragments (scFvs) were generated and confirmed to reduce TGF-β–mediated SMAD2 phosphorylation in HepG2 cells. Inhibition occurs in a dose-dependent manner, with scFv #3 showing the highest potency of inhibition. The intensity of the phosphorylated SMAD2 (pSMAD2) band was normalized to that of the GAPDH band, and the intensity ratio of each sample was subsequently normalized to that of the right-most lane (i.e., sample with 5 ng/mL TGF-β and no scFv). (**b**) Non-reducing Western blot for the CD3ζ endodomain performed on unmodified and TGF-β CAR–expressing CD4+ T cells. (**c**) Cross-staining for the EGFRt transduction marker and the N-terminal FLAG

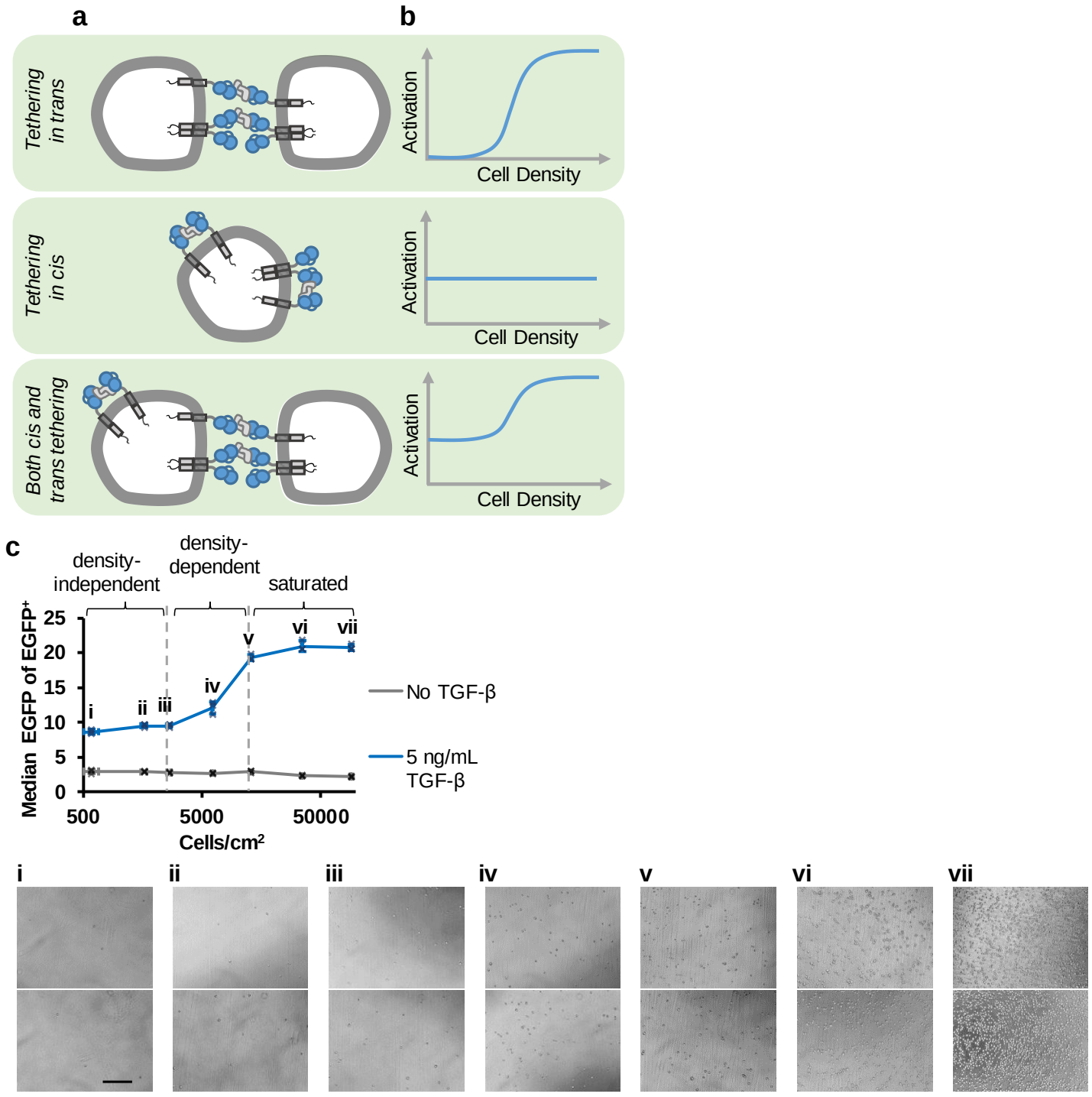
epitope on the TGF-β CAR shows that the CAR is efficiently expressed on the surface of primary human $CD4^+$ and $CD8^+$ T cells. (**d**) TGF-β was added to $CD4^+$ and $CD8^+$ T cells expressing no CAR (EGFRt only), an scFv-less CAR, or the TGF-β CAR. A western blot for phosphorylated SMAD2 (pSMAD2) shows that the TGF-β CAR inhibits native TGF-β signaling (cropped blots shown in Fig. 2b). All figure panels are representative of at least three independent experiments.



Supplementary Figure 4: The TGF-β CAR converts soluble TGF-β into a stimulatory molecule for CD8⁺ T cells. (**a**) CD8⁺ TGF-β CAR-T cells were exposed to varying concentrations of TGF-β and assayed for interferon gamma (IFN-γ), TNF-α, and IL-2 production by intracellular cytokine staining. (**b**-**c**) Primary human CD8⁺ T cells were cultured in the presence of irradiated feeder cells plus IL-2, IL-15, and either no TGF-β or 5 ng/mL TGF-β supplemented every two days. (**b**) Expansion of unmodified CD8⁺ T cells. (**c**) Expansion of CD8⁺ T cells transduced to express the TGF-β CAR. In all graphs, data points from n = 3 biologically independent cell cultures are shown with means ± 1 SD.

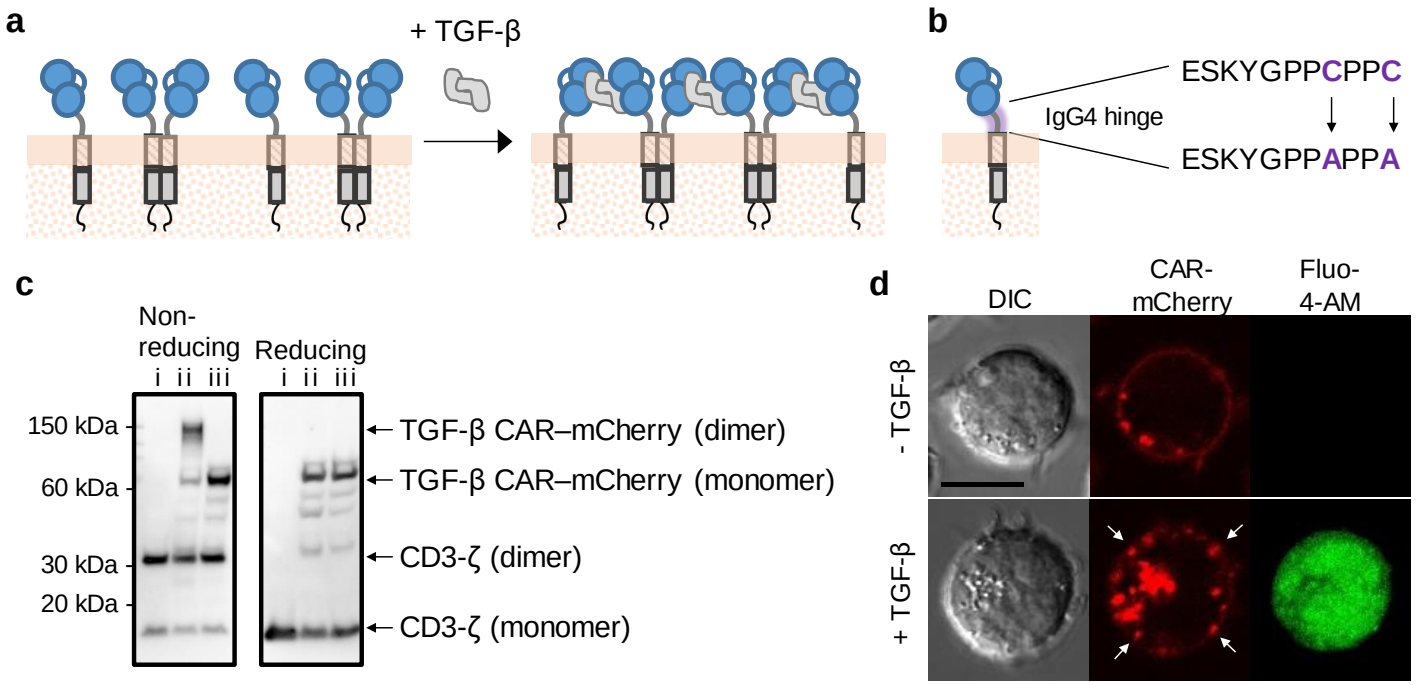


Supplementary Figure 5: TGF-β CAR-T cells do not kill antigen-negative bystander cells. $CD8^+$ T cells expressing (**a**) the TGF-β CAR or (**b**) an scFv-less CAR were co-incubated with off-target TM-LCLs at various effector-to-target (E:T) ratios in the presence or absence of TGF-β for 4 hours. Co-incubation with an on-target $OKT3^+$ TM-LCL line served as a positive control for target-cell lysis. The % specific lysis is calculated from the results of three independent cell cultures with means ± 1 SD. Data and calculations are available in the Supplementary Data Set.

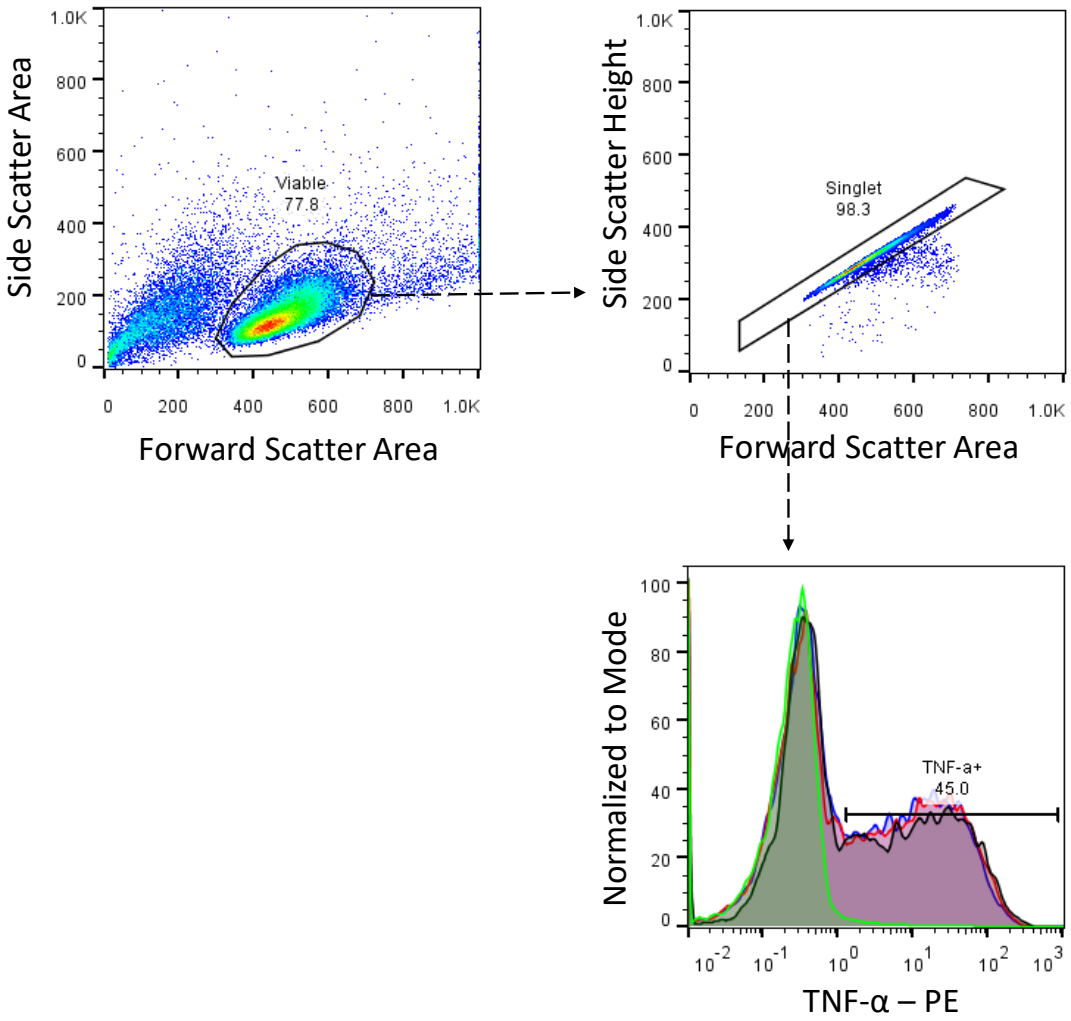


Supplementary Figure 6: Relationship between cell density and cell activation informs a working model of TGF-β–mediated CAR dimerization. (**a**) TGF-β, a homodimer, may tether TGF-β–binding CARs on adjacent cells (in *trans*) or on the same cell (in *cis*). (**b**) The mode of TGF-β CAR activation should affect how activation responds to varying cell densities. Activation by tethering in *trans* requires cell-cell encounters, which increase with increasing cell density until saturation is reached. If activation requires *cis* tethering, then the probability of activation should be independent of cell density. If *trans* and *cis* tethering can both activate the TGF-β CAR,

then a combination of both behaviors should occur. (**c**) Bright-field snapshots of Jurkat cells used in the variable cell-density experiment from Figure 4e. Data points from n = 3 biologically independent cell cultures are shown with means ± 1 SD. The microscopy experiment was performed once to visually verify cell density. Scale bar denotes 100 μm.



Supplementary Figure 7: Monomeric TGF-β CARs remain capable of CAR signaling and microcluster formation. (**a**) Schematic of TGF-β bridging dimeric CAR molecules to form a "polymer" of TGF-β CARs. (**b**) A monomeric CAR was constructed by replacing cysteines with alanines in the IgG4 hinge. (**c**) Non-reducing western blot for the CD3-ζ endodomain was performed once on the lysate of (**i**) parental Jurkat, (**ii**) TGF-β CAR Jurkat, and (**iii**) C→A mutant TGF-β CAR Jurkat cells. The C→A mutation eliminated CAR dimerization. (**d**) Jurkats expressing mCherry-tagged TGF-β CARs containing the C→A mutation were loaded with Fluo-4-AM and imaged 15 min after the addition of TGF-β. White arrows point at examples of peripheral clusters. Images are representative of multiple cells across two independent experiments. Scale bar denotes 10 μm.



Supplementary Figure 8: Example of gating strategy for flow cytometry data. Cell populations were initially viewed with the Forward Scatter Area/Side Scatter Area axes and gated for viable cells. Viable cells were viewed under Forward Scatter Area/Forward Scatter Height axes gate for single cells. Single cells were then assessed for staining by PE-conjugated anti-TNF-α antibody. The green curve corresponds to the negative control, a “true negative” sample that was not stimulated to produce TNF-α but processed alongside the other samples, undergoing the same fluorophore-conjugated antibody staining and buffer washes. This negative control was used to draw the TNF-α+ gate shown.

Supplementary Data Set: Lack of bystander cell lysis by TGF-β CAR-T cells

TGF-β CAR-T cells do not kill antigen-negative bystander cells. $CD8^+$ T cells expressing the TGF-β CAR or an scFv-less CAR were co-incubated with off-target TM-LCLs at various effector-to-target (E:T) ratios in the presence or absence of TGF-β for 4 hours. Co-incubation with an on-target $OKT3^+$ TM-LCL line served as a positive control for target-cell lysis. The % specific lysis is calculated from the results of three independent cell cultures with means ± 1 SD. Formulas used in % specific lysis calculation are specified in the Online Methods and in the Excel spreadsheet.