Supplementary Material

Supplementary Methods

Antibodies employed for FACS analysis

CD3 PB (Biolegend, cloneHIT3a), CD45 BV510 (Biolegend, clone HI30), CD271 PE-Cy7 (BD, clone CD40-1457), CD271 PE (BD, clone C40-1457), CD4 FITC (Biolegend, clone SK3), anti-mouse CD45 PerCP (Biolegend, clone 30-f11), CD14 APC (Biolegend, clone M5E2), CD19 APC/Cy7 (Biolegend, clone HIB19), CD56 PE-Cy7 (Biolegend, clone HCD56), HLA-DR APC/Cy7 (Biolegend, clone L243), CD45RA FITC (Biolegend, clone HI100), CD62L APC (Biolegend, clone DREG-56), CD8 PerCP (BD, clone SK1), CD107a FITC (Biolegend, clone H4A3), Ki-67 Pacific Blue (Biolegend clone KI-67), CD69 APC (Biolegend, clone FN50), CD25 APC/Cy7 (Biolegend, clone BC96), CD163 FITC (Biolegend, clone GHI/61), CD54 PE (Biolegend, clone HA58), CD80 PE-Cy7 (Biolegend, clone 2D10) and CD86 APC (Biolegend, clone IT2.2).

Supplementary Figures

Supplemental Figure 1. Transduction levels and killing ability of CD4 and CD8 CAR-T cells

Supplemental Figure 2. Tripartite *in vitro* co-cultures with monocyte-like THP-1 cells

Supplemental Figure 3. Antitumor activity, T-cell expansion and survival rates related to severe CRS in tumor-bearing HuSGM3 mice treated with CD4 and CD8 CAR-T cell products

Supplemental Figure 4. Antitumor activity and survival rates related to severe CRS in tumor-bearing HuSGM3 mice treated with differentially co-stimulated CD4:CD8 1:1 CAR-T cell products

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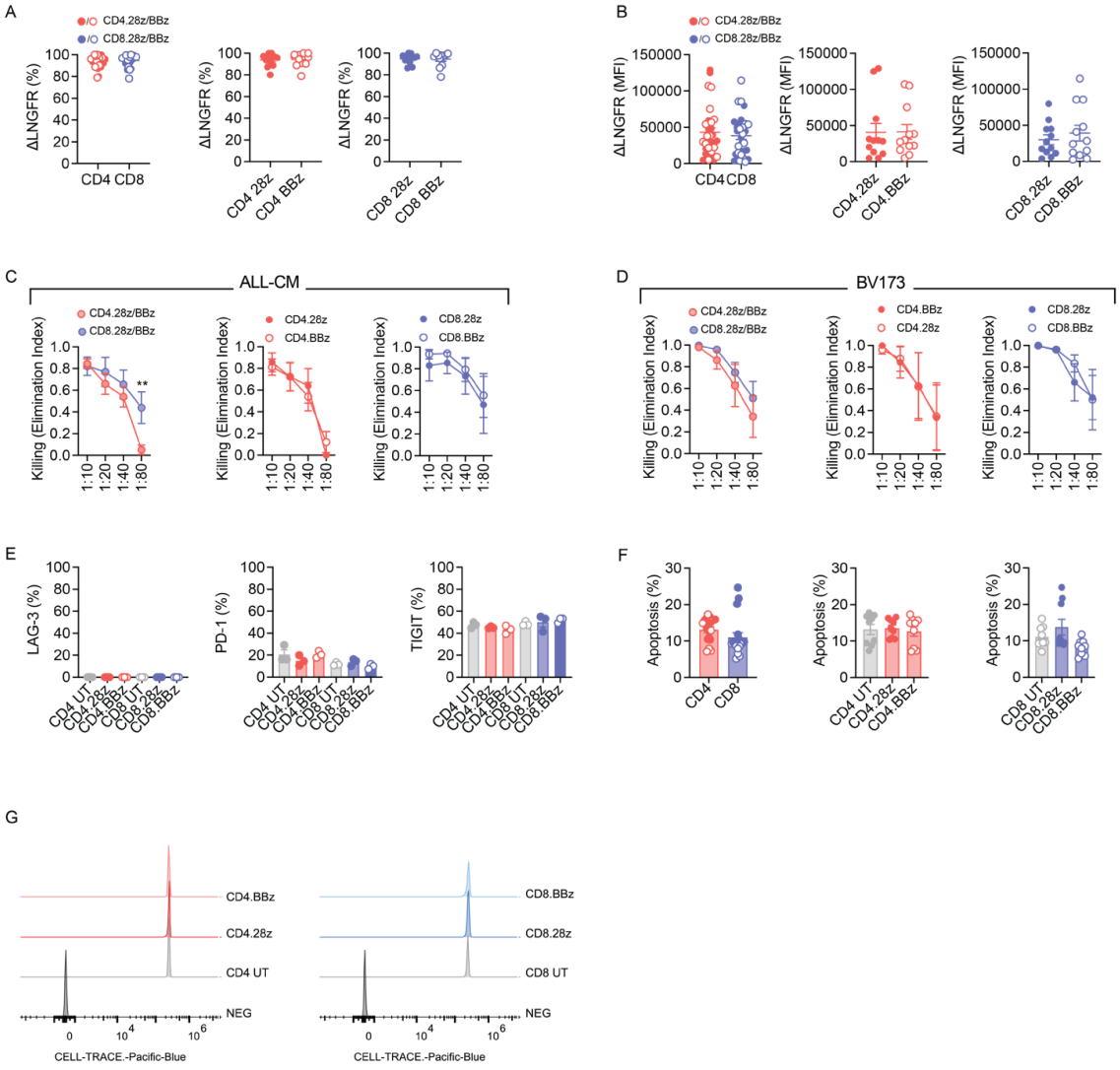

Supplemental Figure 1. Transduction levels, killing ability and tonic signaling of CD4 and CD8 CAR-T cells. ΔLNGFR marker expression reported as (**A**) percentage and (**B**) MFI at the end of manufacturing (n=12). **C**) Killing activity expressed as elimination index (see Methods) and measured by co-culturing CD4.28z (n=3), CD4.BBz (n=3), CD8.28z (n=3) and CD8.BBz (n=3) with CD19+ ALL-CM cells for 4 days at different effector/target (E:T) ratios. **D**) Killing activity measured by co-culturing CD4.28z (n=3), CD4.BBz (n=3), CD8.28z (n=3) and CD8.BBz (n=3) with CD19+ BV173 cells for 4 days at different effector/target (E:T) ratios. **E**) LAG-3, PD-1 and TIGIT exhaustion markers expression at the end of manufacturing (n=3). **F**) Apoptosis analysis at the end of manufacturing (n=3). **G**) Antigen-independent proliferation expressed as Cell-Trace

dilution (n=3). Data are represented as mean ± SEM or mean ± SEM together with overlapping scattered values. By paired t test or 2-way ANOVA.

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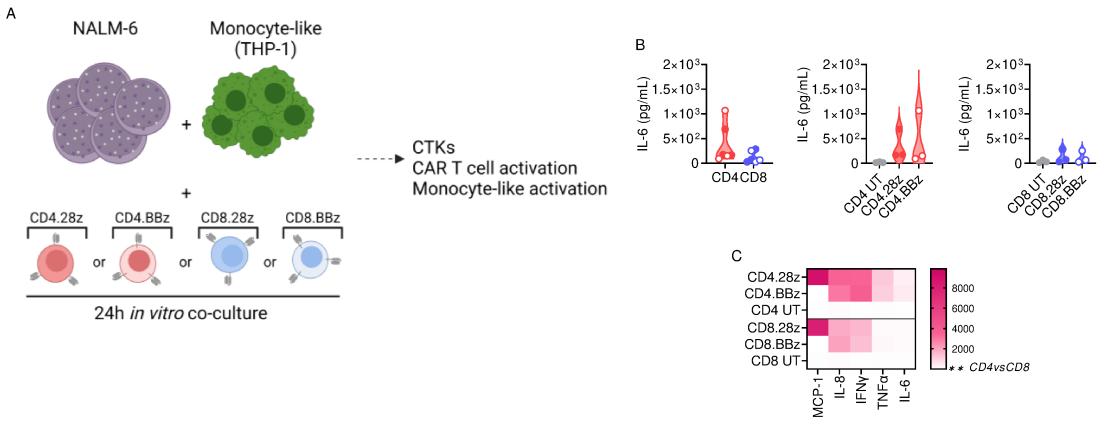
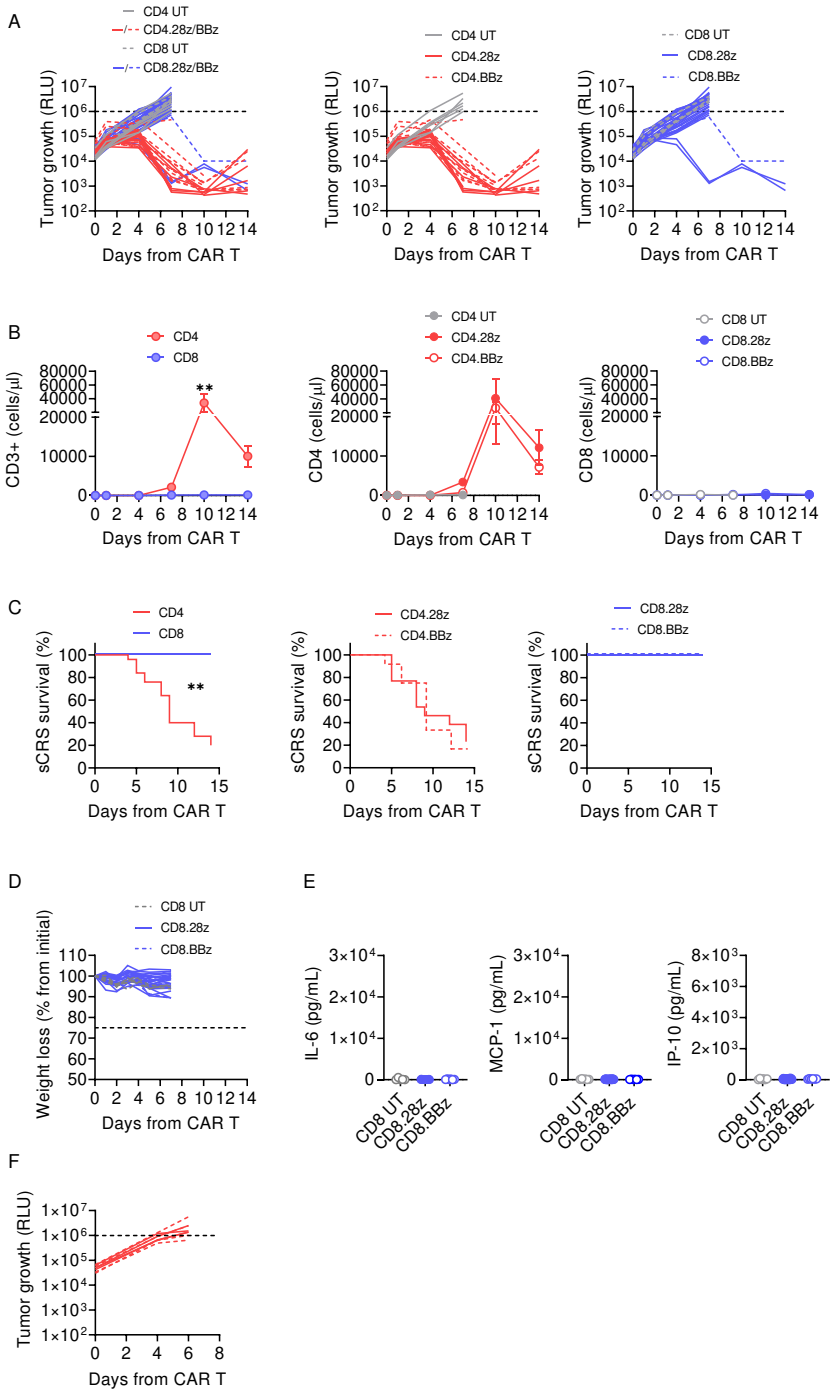
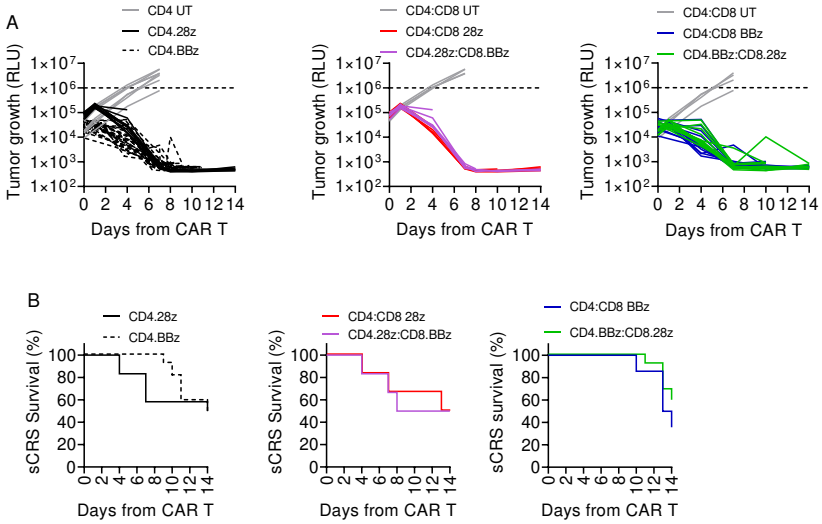


Supplemental Figure 2. Tripartite *in vitro* co-cultures with monocyte-like THP-1 cells. A) Schematic representation of tripartite co-cultures consisting of NALM-6 leukemia cells, CD4 or CD8 CAR-T cells and the monocyte-like THP-1 cell line. **B)** IL-6 production and (**C)** heatmap visualization of cytokine release 24 hours after plating (n=3). Data are represented as mean ± SEM together with overlapping scattered values. **P < 0.01, by paired t test or 2-way ANOVA.



Supplemental Figure 3. Antitumor activity, T-cell expansion and survival rates related to severe CRS in tumor-bearing HuSGM3 mice treated with CD4 and CD8 CAR-T cell products. A-C) Additional data from the experiment reported in Figure 3A-F. **A)**

LUCIA+/NGFR+/NALM-6 bioluminescence signal measured at different time points after treatment with CD4 UT (n=6), CD4.28z (n=13), CD4.BBz (n=12), CD8 UT (n=6), CD8.28z (n=24) and CD8.BBz (n=7) and expressed as relative light units (RLU). **B)** CAR-T cell expansion peak monitored in the peripheral blood of mice receiving CD4 UT (n=6), CD4.28z (n=13), CD4.BBz (n=12), CD8 UT (n=6), CD8.28z (n=2) and CD8.BBz (n=1). **C)** sCRS-related Kaplan-Meier survival analysis of mice treated with CD4.28z (n=13), CD4.BBz (n=12), CD8.28z (n=24) and CD8.BBz (n=7). **D)** Weight loss and **E)** IL-6, MCP-1 and IP-10 serum levels in mice who did not achieve tumor control after treatment with CD8 UT (n=6), CD8.28z (n=22) and CD8.BBz (n=5). **F)** Additional data from the experiment reported in Figure 3G and H. LUCIA+/NGFR+/NALM-6 bioluminescence signal measured at different time points after treatment with CD4.28z (n=4) and CD4.BBz (n=4). Data are represented as mean ± SEM together with individual and overlapping scattered values. **P < 0.01 by 2-way ANOVA, unpaired t test and Mantel-Cox 2-sided log-rank test were performed.



Supplemental Figure 4. Antitumor activity and survival rates related to severe CRS in tumor-bearing HuSGM3 mice treated with differentially co-stimulated CD4:CD8 1:1 CAR-T cell products. Additional data from the experiment reported in Figure 5. **A)** LUCIA+/NGFR+/NALM-6 bioluminescence signal measured at different time points after treatment with CD4:CD8 UT (n=8), CD4:CD8 28z (n= 6), CD4.28z:CD8.BBz (n=6), CD4:CD8 BBz (n= 14) and CD4.BBz:CD8.28z (n=13). **B)** sCRS-related Kaplan-Meier survival analysis of mice. Data are represented as mean ± SEM together with individual scattered values by 2-way ANOVA, unpaired t test, Mantel-Cox 2-sided log-rank test.