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Supplemental information

CD3 engagement as a new strategy for allogeneic “off-the-shelf” T cell therapy

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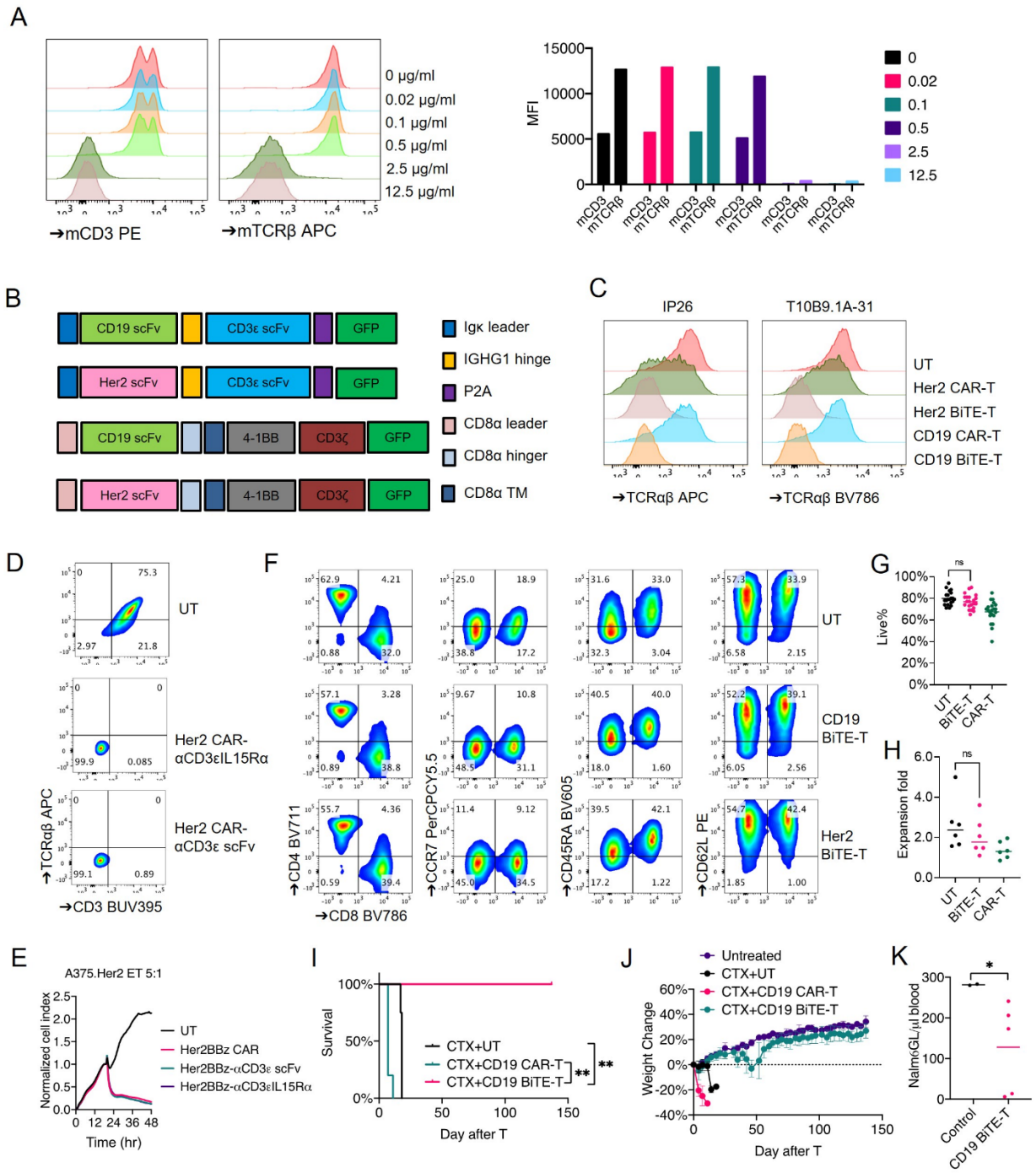

Fig. S1. CD3 engagement induced TCRαβ/CD3 downregulation and BiTE-T cells have decreased allo-reactivity. (A), Mouse TCR/CD3 expression on mouse T cells after treated with plate-coated mCD3ε antibody for 24 hr. **(B)**, Schematic diagrams of BiTE and CAR constructs used in the study. **(C)**, TCRαβ evaluation on UT, BiTE-T or CAR-T cells using two different clones of TCRαβ antibodies. **(D)**, TCRαβ/CD3 expression on CAR-T cells engineered with a membrane-bound or soluble secreted CD3ε

scFv. **(E)**, Cytotoxicity of CAR-T cells engineered with a membrance-bound or solube secreted CD3ε scFv. **(F)**, Immune phenotype of BiTE-T and CAR-T cells. Panel g-i show a GvHD risk study using chemotherapy. Mice were treated with cyclophosphamide (CTX) at 250 mg/kg and then given 10 million of transduced T or untransduced T cells (UT). Viability (**G**) and expansion (**H**) of BiTE-T cells compared to CAR-T and UT cells in *in vitro* production. **(I)**, Survival. **(J)**, Weight change. **(K)**, Long-term leukemia protection by BiTE-T cells. In the same study, survived mice were challenged with NALM6GL cells 4.5 months after BiTE-T cells. Leukemia cells in peripheral blood were evaluated 3 weeks after challenge.

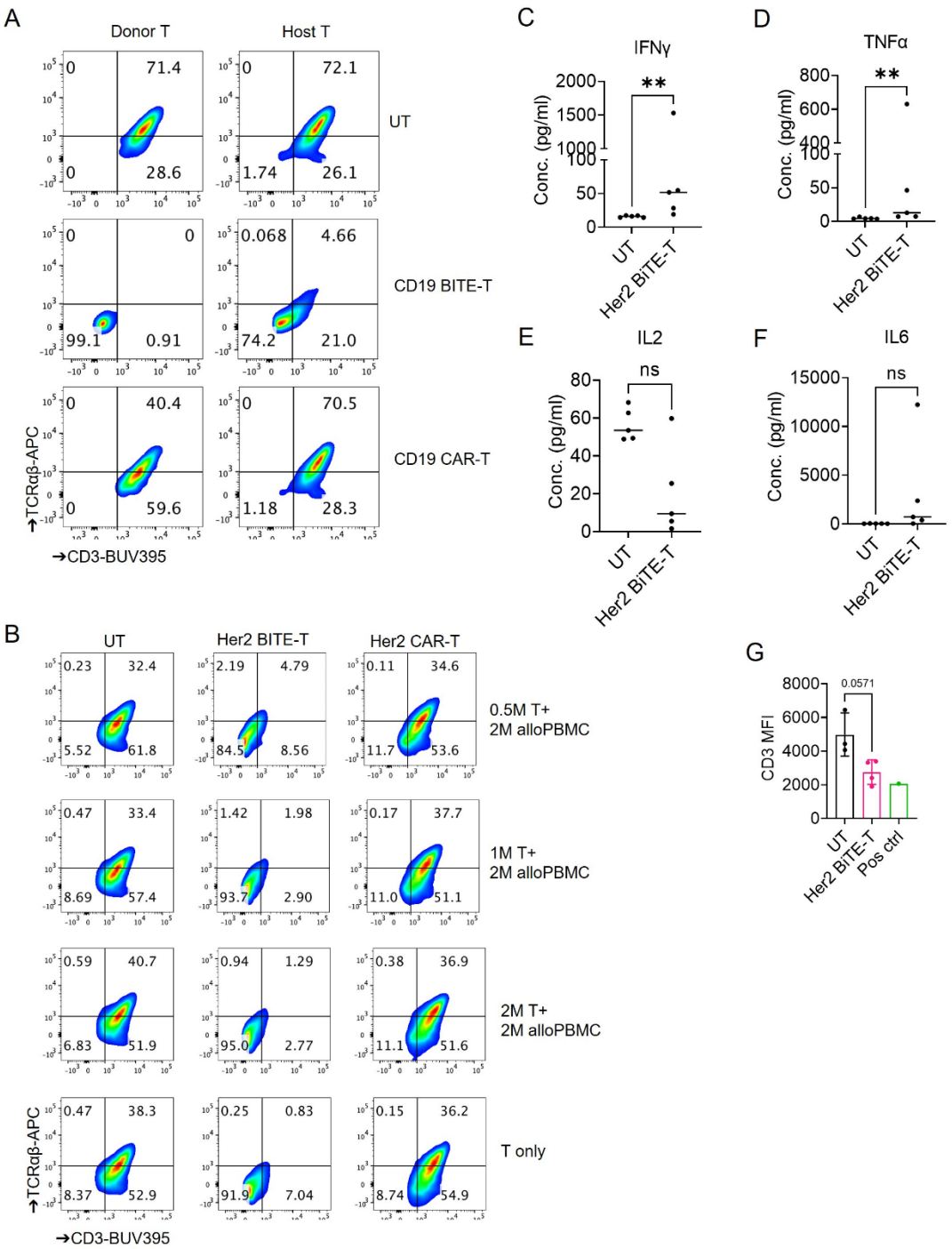

Fig. S2. BiTE-T cells decrease TCRαβ/CD3 on bystander T cells and secrete significant levels of BiTEs in circulation. (A), Representative flow plots of CD19 BiTE-T cells inducing CD3/TCRαβ downregulation on HLA-mismatched donor T cells. **(B)**, Her2 BiTE-T cells reduced CD3/TCRαβ expression on allogeneic T cells. HLA-A2+ Her2 BiTE-T cells were co-cultured with HLA-A2- PBMCs for 5 days without cytokines. Total cells were subjected to flow analysis. **C-G** are from an in vivo study

showing BiTE-T cells secrete significant levels of BiTEs in circulation. NSG mice were subcutaneously transplanted with A375.Her2 cells and subsequently treated with Her2 BiTE-T cells. Sera were isolated one week after T cell injection and added to A375.Her2 cells in the presence of untransduced T cells. Seventy-two hour later, cytokines in the supernatant were measured. **(C),** IFNγ; **(D),** TNFα; **(E),** IL2**; (F),** IL6**.** CD3 on T cells co-cultured with mice sera was measured by flow cytometry (**G**). T cells co-cultured with supernatant from BiTE-T cell culture were used as positive controls.

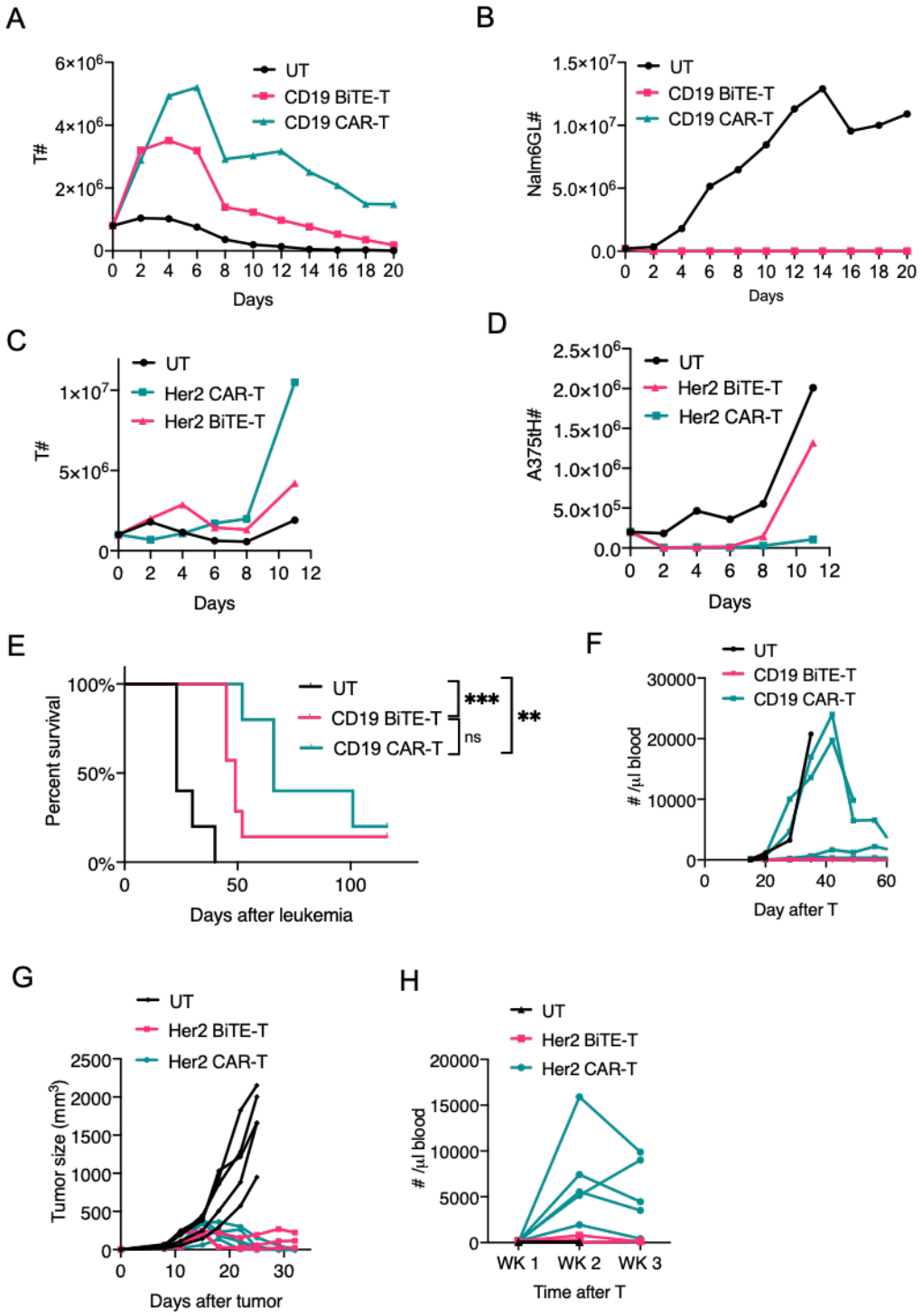

Fig. S3. BiTE-T cells are not as durable as CAR-T cells. BiTE-T cells are not as durable as CAR-T cells. **(A)**, T cell expansion in CD19 serial killing assay. **(B)**, NALM6GL cell killing in serial killing assay. **(C)**, T cell expansion in A375.Her2 serial killing assay. **(D)**, A375.Her2 cell killing in serial killing assay. Panel **E** (survival) & **F** (T cell persistence) show in vivo efficacy comparison of CD19 BiTE-T and

CAR-T cells. Study design see Figure 3i. Panel **G** (tumor growth) & **H** (T cell persistence) show in vivo efficacy comparison of Her2 BiTE-T and CAR-T cells. Study design see Figure 3D.

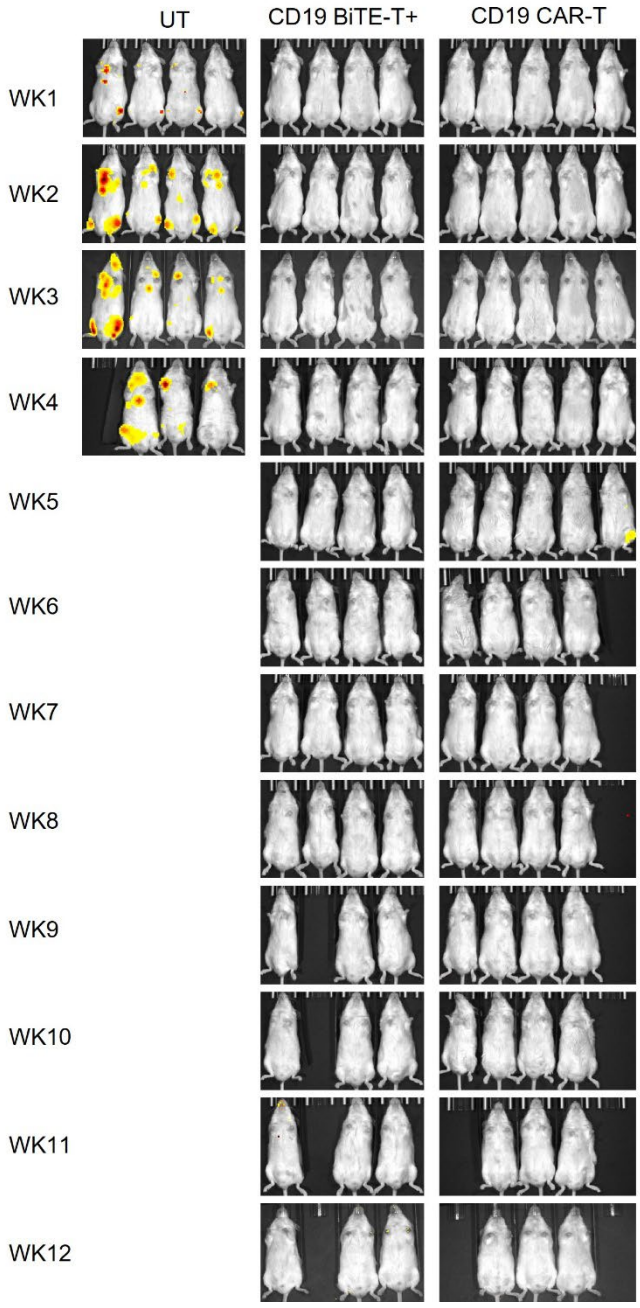

Fig. S4. Bioluminescence data of co-stimulation enhanced CD19 BiTE-T cells (BiTE-T+) compared to CD19 CAR-T cells.