CHIMERIC ANTIGEN RECEPTOR-T CELLS WITH CYTOKINE NEUTRALIZING CAPACITY

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Supplementary Material

(Supplementary Methods; Figures S1-S8)

SUPPLEMENTARY METHODS

Retroviral transduction, surface marker immunophenotyping, cytokine measurements

For T cell transduction, T cells were incubated with retroviral supernatant in the presence of RetroNectin (Takara, Kusatsu, Japan) at 37°C. Retroviral supernatant was replaced with freshly harvested supernatant every 12 hours thereafter for the next three days. Transduced T cells were subsequently harvested and cultured in RPMI-1640 supplemented with 10% FBS, and 200 IU/mL IL-2.

Surface markers were detected with the following antibodies: anti-CD69-PE, anti-CD62L-APC, anti-CD45RA-APC-H7, anti-CCR7-PE, anti-CD7-PE, anti-CD25-PE-Cy7, anti-CD3-APC, anti-CD8-PE, and anti-CD4-PE-Cy7 (all from BD Biosciences), and anti-PD1-APC, anti-TIM3-PE, and anti-LAG3-PE-Cy7 (BioLegend, San Diego, CA).

For IFNγ production, T cells were cultured with target cells at a 1:1 ratio. After 1 hour, GolgiPlug (BD Biosciences) was added and cultures continued for another 5 hours. After cell membrane permeabilization with 8E (a permeabilization reagent developed in our laboratory), cells were labelled with anti-human IFNγ-PE (BD Biosciences), and analyzed by flow cytometry. In some experiments, supernatant from CAR-T/OP-1 co-culture was harvested after 48 hours and added to T cells mixed at 1:1 with either undifferentiated or differentiated THP-1 cells. Cytokine levels were measured after a further 72 hour of culture with the BD Cytometric Bead Array Human Inflammatory Cytokines Kit.

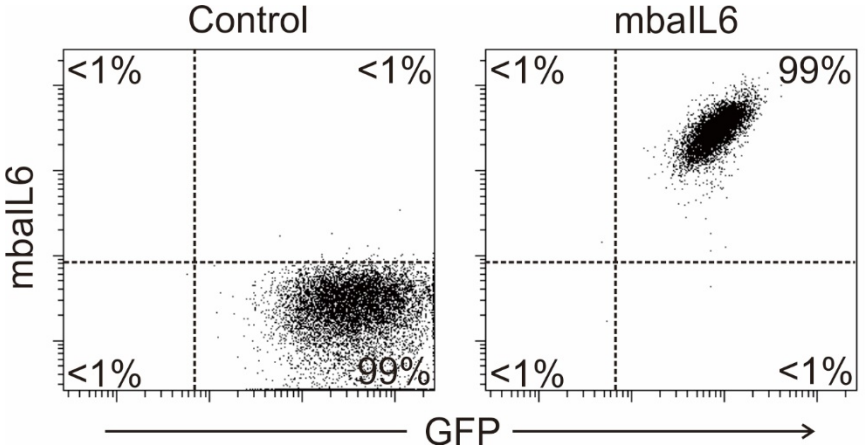

Figure S1. Stability of mbaIL6 expression in Jurkat cells. Expression of mbaIL6 was assessed 30 months and more than 100 passages after initial transduction. Flow cytometry dot plots illustrate GFP fluorescence, and mbaIL6 expression after staining with biotin-conjugated goat anti-human F(ab′)2 antibody and streptavidin-APC (Jackson ImmunoResearch) in Jurkat cells transduced with either GFP alone ("Control") or GFP plus mbaIL6.

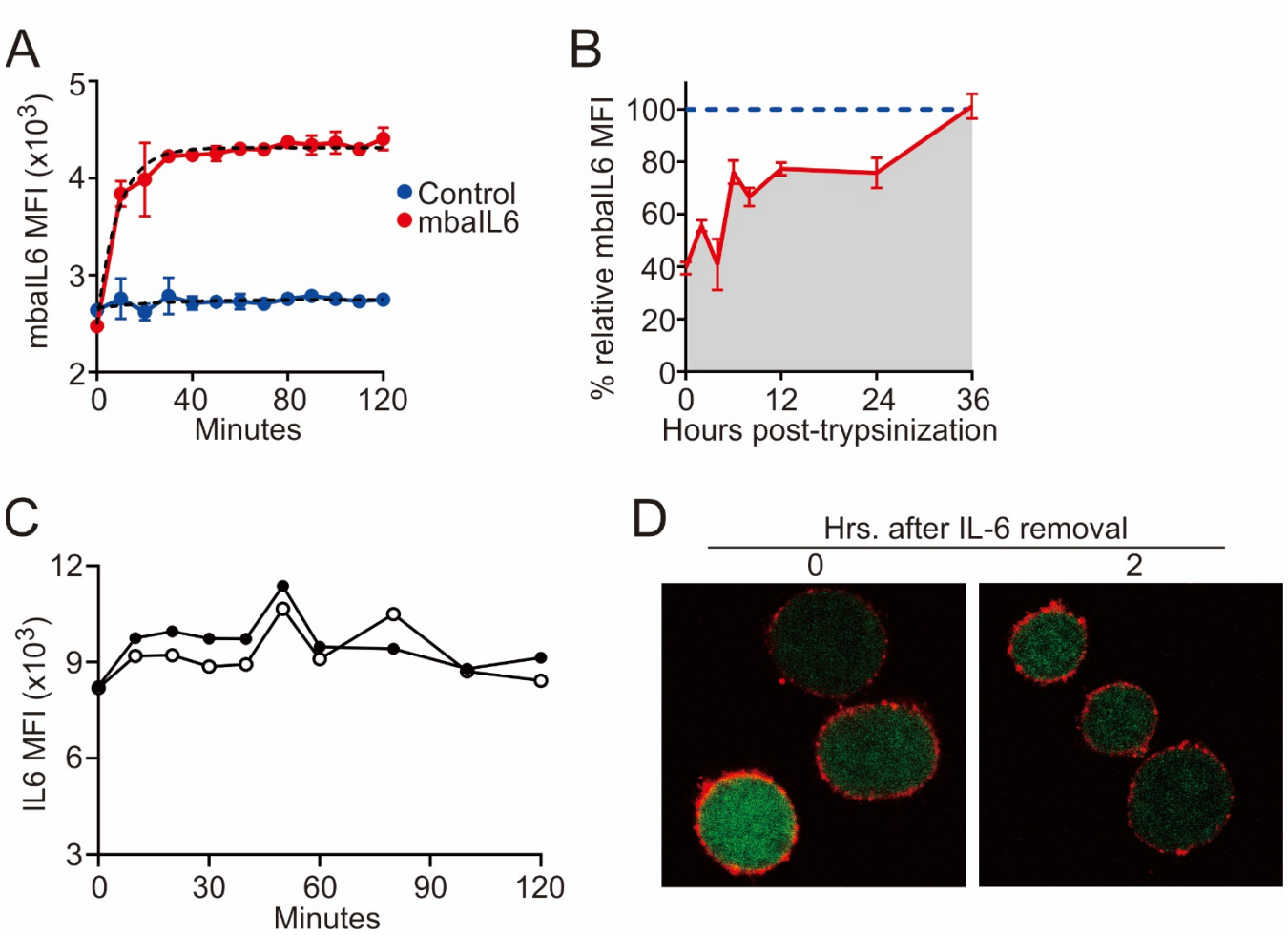

Figure S2. Functionality of the mbaIL6 receptor. **A.** Jurkat cells (0.1 x 10^6/mL) were cultured with 10 ng/mL IL-6. IL-6 levels were measured by ELISA. Mean ± SD of 3 experiments for each time point is shown. Assuming negligible dissociation, 90% binding of mbaIL6 was reached after 20.3 minutes, 99% binding after 40.5 minutes, and 99.9% binding after 60.8 minutes. **B.** Jurkat cells were treated with trypsin (HyClone; 0.25 g/mL) for 30 minutes, washed and stained with biotin-conjugated goat anti-human F(ab')2 followed by streptavidin-APC (Jackson ImmunoResearch) at different time points. Non-trypsinized cells cultured in parallel were used

as a control (dashed blue line). Mean ± SD of 3 parallel cultures are shown. **C.** Jurkat cells expressing mbaIL6 (1 x 10^6/mL) were cultured with IL-6 (5 ng/mL) for 2 hours; after washing, cells were cultured for another 2 hours and periodically stained with anti-IL-6-PE (BD Biosciences). Data are from 2 independent experiments. Flow cytometric dot plots at 0 and 2 hours are shown in Fig. 2H. **D**. Confocal image analysis (60x objective lens; Olympus) of IL-6 bound to the surface of Jurkat cells from one of the experiments shown in **C**.

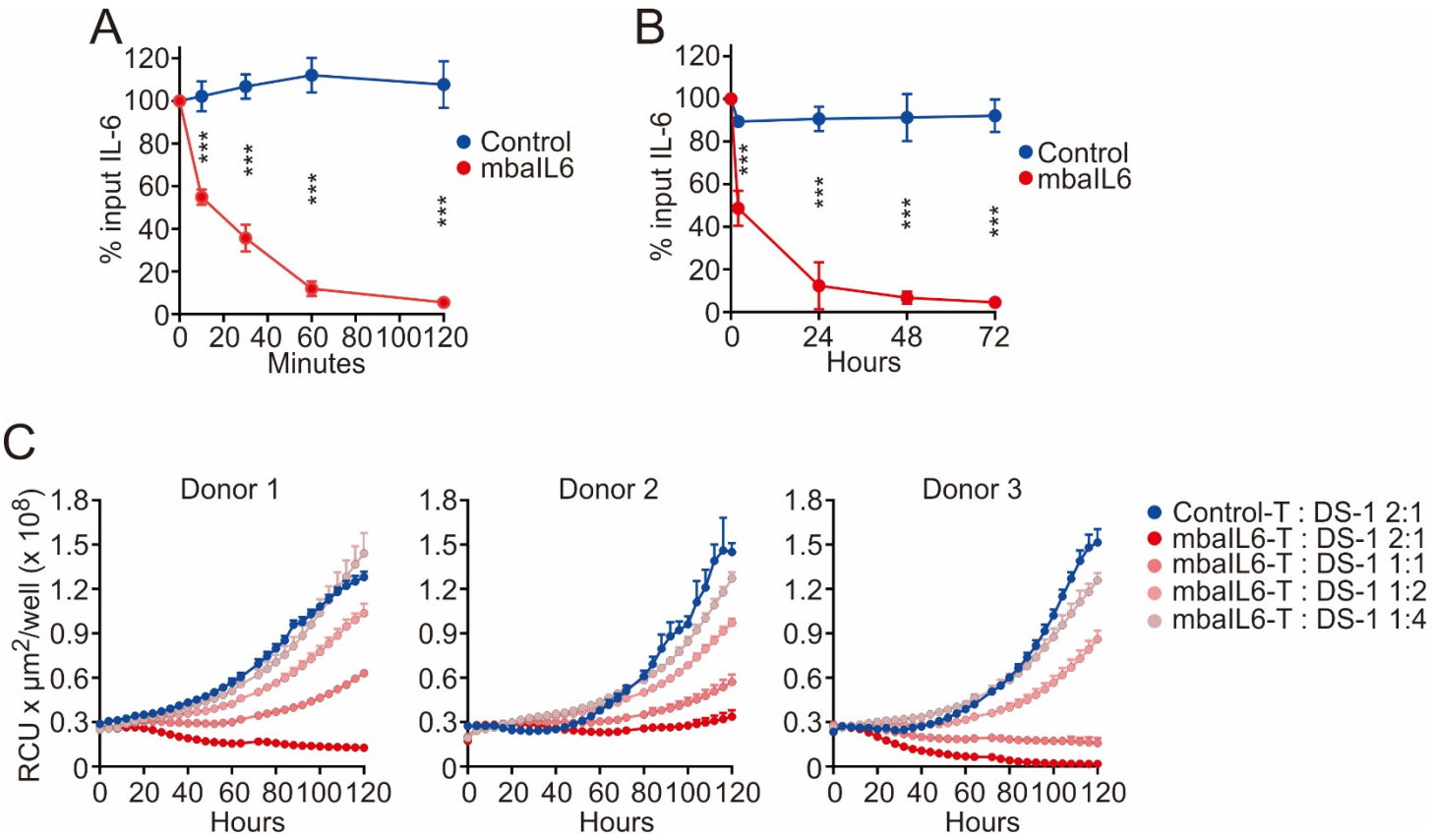

Figure S3. Functionality of mbaIL6 in T lymphocytes. **A.** Peripheral blood T lymphocytes ($2x10^6$/mL) transduced with either GFP alone ("Control") or GFP plus mbaIL6 were cultured for 2 hours with 1 ng/mL human IL-6; IL-6 in the supernatant was measured by ELISA. Mean (±SD; n=3) is shown. ***, P <0.001. **B.** Cultures were set up as in **A**, with $0.1x10^6$ T lymphocytes/mL. IL-6 levels were measured after the indicated time. Mean (±SD; n=3) is shown. ***, P <0.001. **C.** DS-1-mCherry cells were co-cultured with Control or mbaIL6-transduced T cells from 3 donors at the indicated ratios, with IL-6 (0.5 ng/mL). DS-1 proliferation was quantitated using the IncuCyte System (Essen); shown are mean (± SD) of red calibrated unit (RCU) x μm^2/well in triplicate measurements.

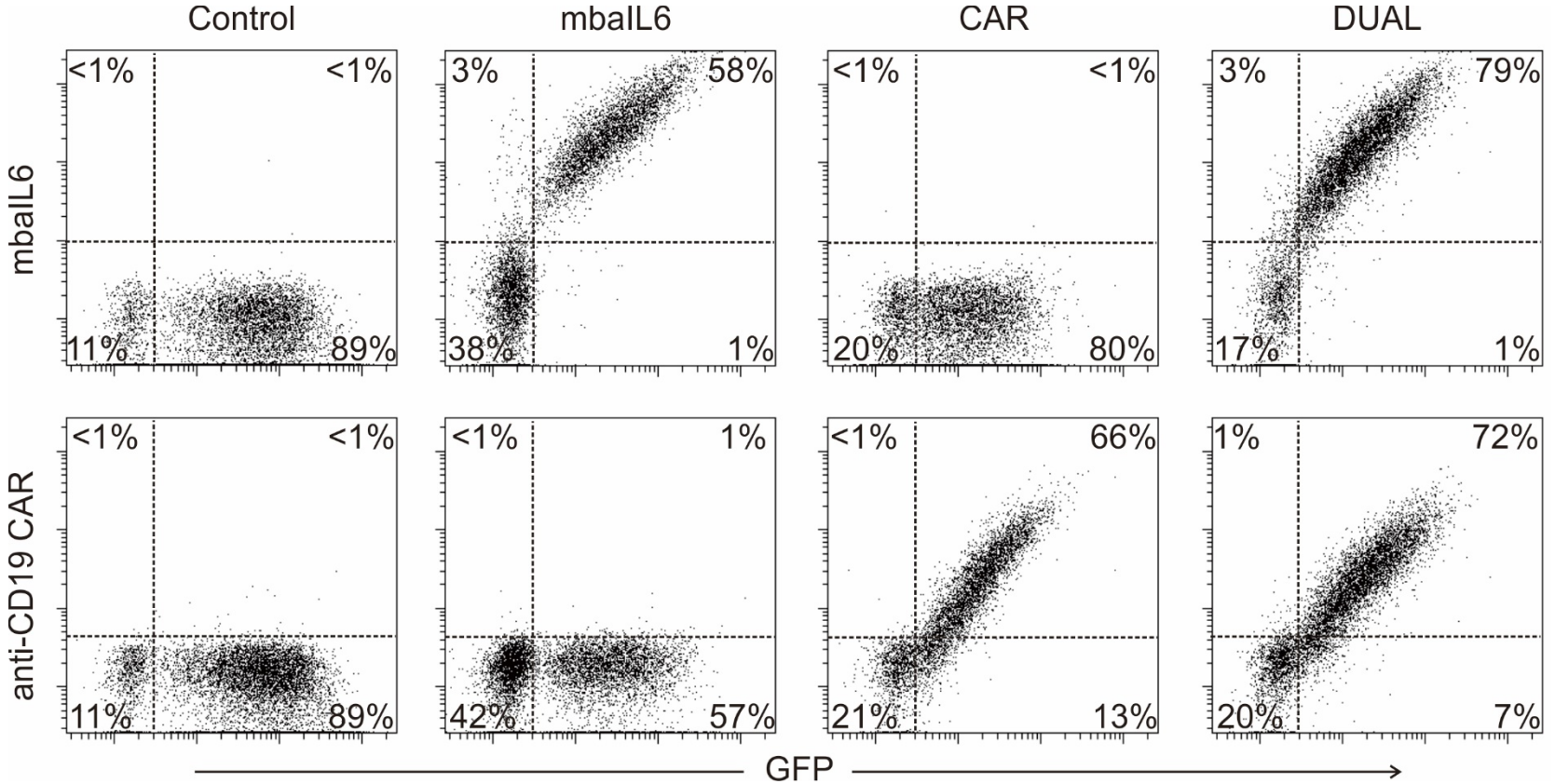

Figure S4. Flow cytometric analysis of peripheral blood T-cells transduced with either GFP alone (Control), GFP plus anti-CD19-CAR, mbaIL6, or both. Dot plots illustrate mbaIL6 expression after staining with biotin-conjugated goat anti-human F(ab′)2 antibody and streptavidin-APC (Jackson ImmunoResearch; top row), and anti-CD19 CAR expression after staining with CD19-myc, followed by R-phycoerythrin (PE)-conjugated anti-myc (Cell Signaling Technology; bottom row). Expression of both receptors in the same plot is shown in Fig. 4B.

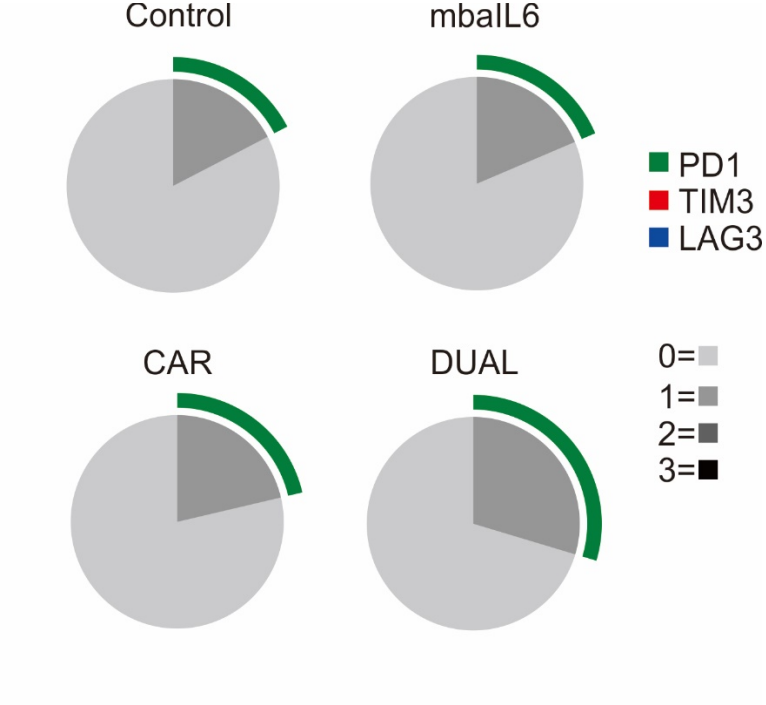

Figure S5. PD1, TIM3, and LAG3 expression in unstimulated T cells was plotted with Python 3 using Matplotlib package (https://matplotlib.org/). The profile of the same cells stimulated with CD19+ target cells is shown in Fig. 5F.

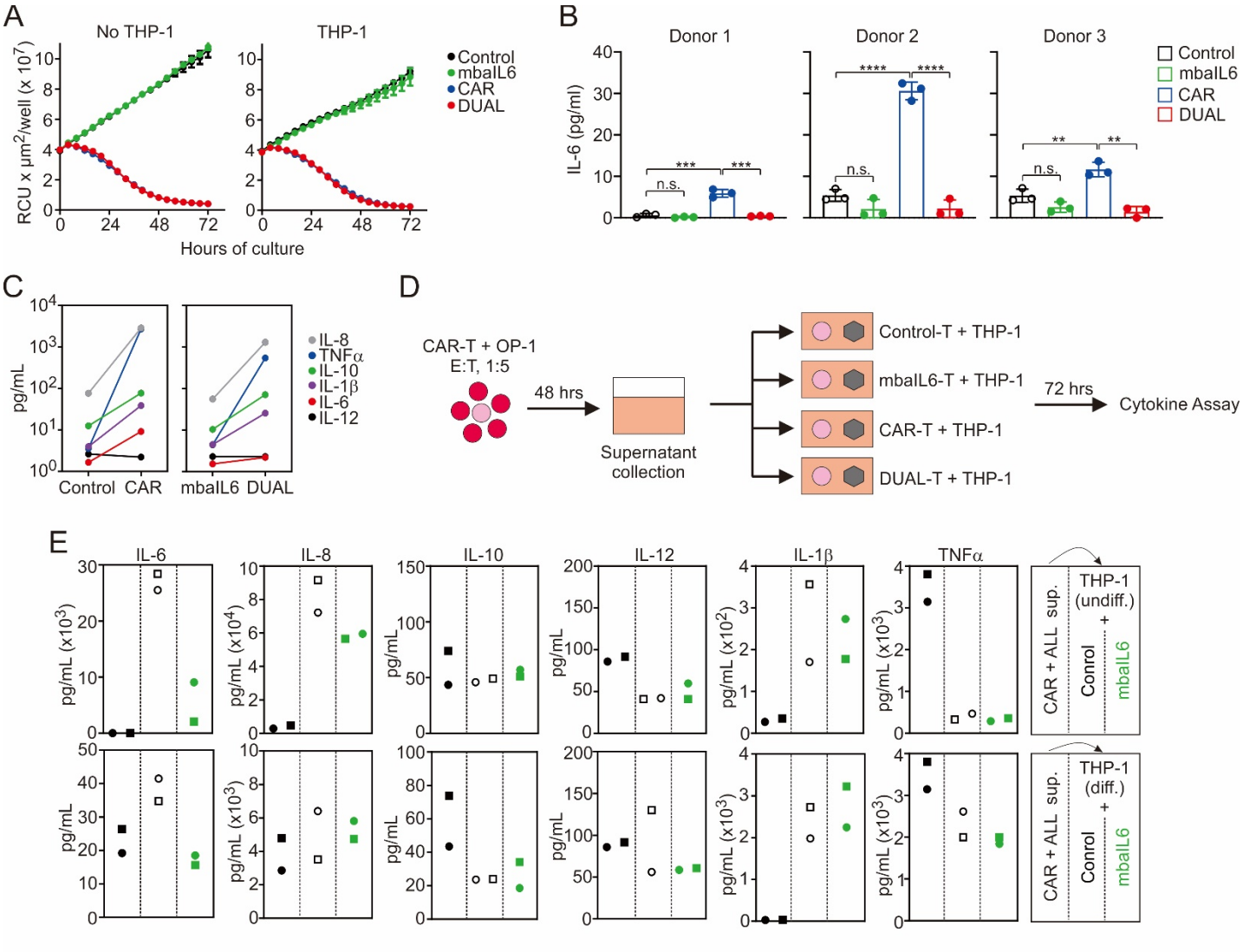

Figure S6. Capacity of T cells expressing mbaIL6 and anti-CD19-CAR to exert cytotoxicity and quench IL-6 in the presence of monocytic cells. **A.** Cytotoxicity of T cells against OP-1-mCherry cells, with or without differentiated (PMA-treated) THP-1 cells (1:5:1 T-cell:OP-1:THP-1 ratio). OP-1 cell numbers were quantitated using the IncuCyte System (Essen); results with T cells from 3

donors are expressed as mean (± SD) of red calibrated unit (RCU) x μm^2/well. **B.** IL-6 levels measured by ELISA in the supernatant of 48-hour cultures with T cells from each of the donors shown in **A**. n.s., not significant; P>0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. **C**. T cells from 1 donor were cultured with OP-1 at a 1:5 ratio. Levels of cytokines in supernatant obtained after 48 hours were measured using the BD Cytometric Bead Array Human Inflammatory Cytokine Kit. **D.** Experimental design for the data shown in **E** and in Fig. 6C. **E.** Supernatant from CAR-T/OP-1 co-culture was harvested after 48 hours and added to T cells mixed at 1:1 with either undifferentiated or differentiated THP-1 cells. Cytokine levels were measured after a further 72 hours of culture. Results with T cells transduced with CAR or mbaIL6-CAR GFP are shown in Fig. 6C.

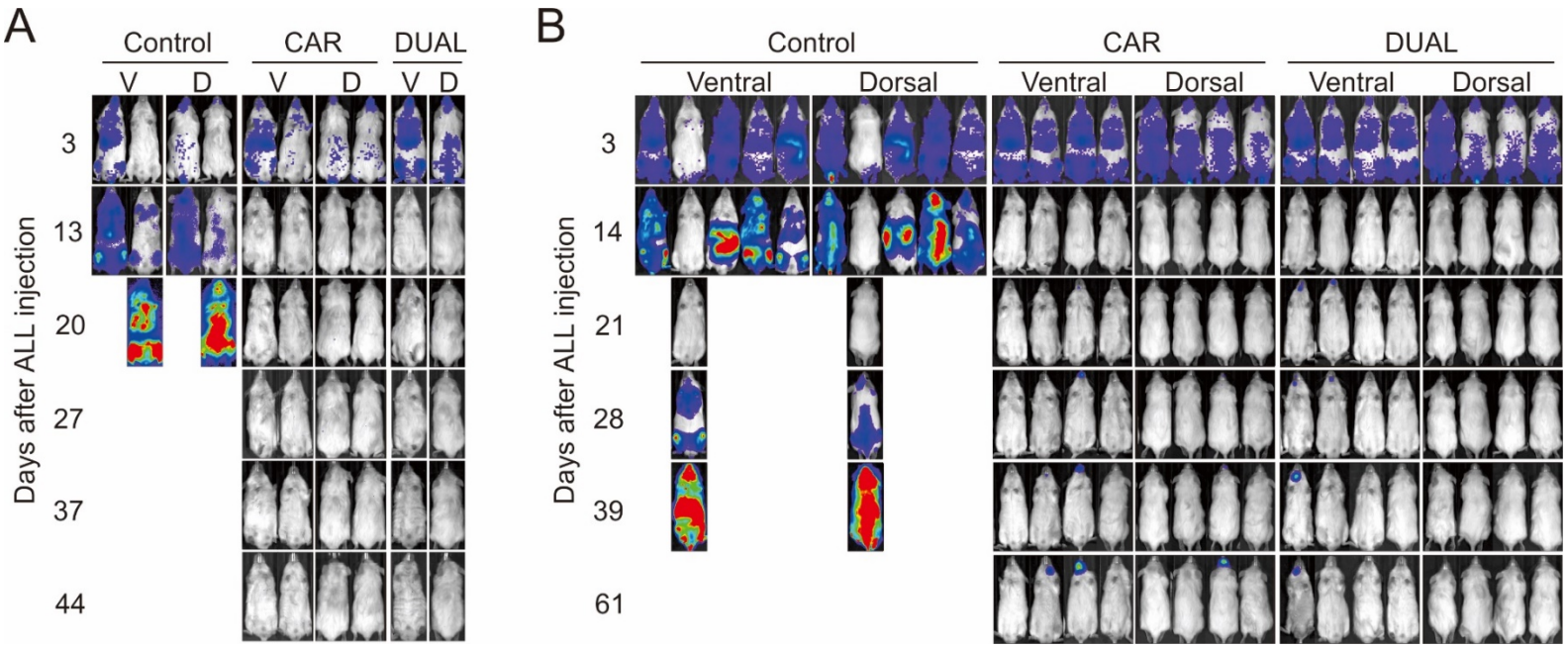

Figure S7. NOD/scid-IL2RGnull mice were injected i.v. with 0.5 x 10^6 (**A**) or 1 x 10^6 (**B**) Nalm-6-luciferase cells; 20 x 10^6 T-cells were injected i.v. 3 days after Nalm-6 engraftment. All mice received 20000 IU IL-2 i.p. every 2 days. Ventral and dorsal image of mice collected by Xenogen IVIS-200 system (Caliper Life Sciences) after D-luciferin injection are shown (day 3 images were collected with enhanced sensitivity to visualize Nalm-6 engraftment). Mice were euthanized when the total bioluminescence signal reached 1 x 10^{10} photons per second.

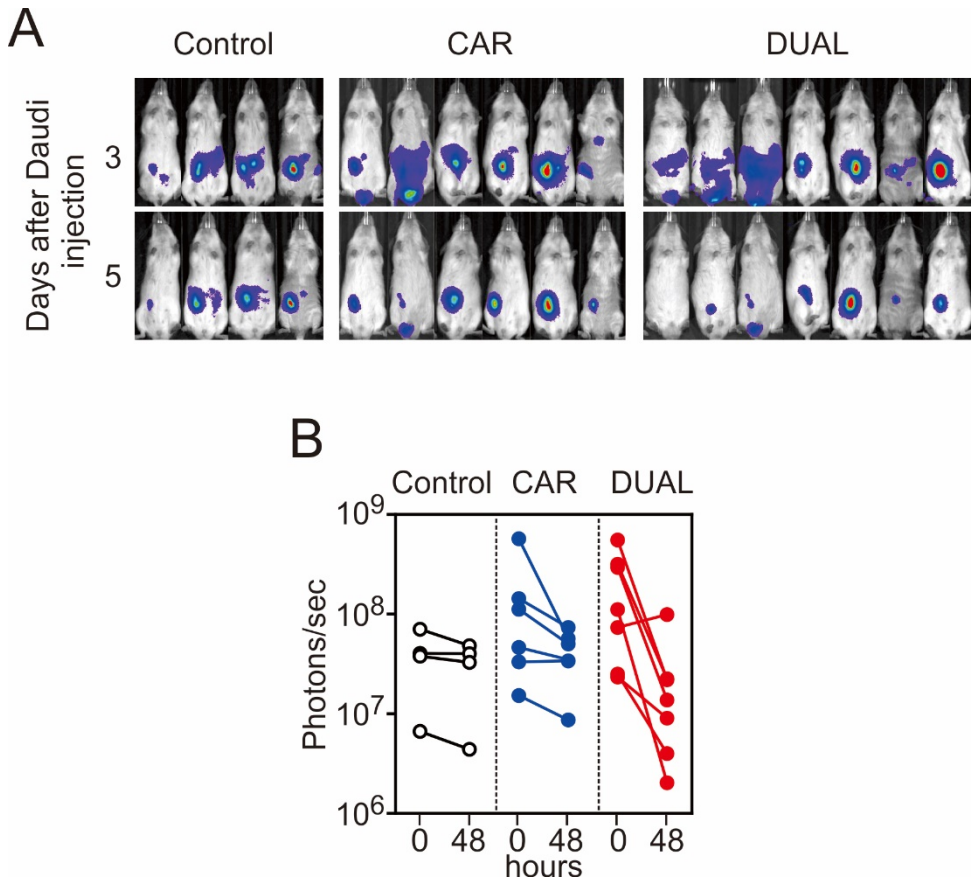

Figure S8. A. NOD/scid-IL2RGnull mice were injected i.p. with 20 x 10^6 Daudi-luciferase cells; 20 x 10^6 of both T-cells and THP-1 cells were injected i.p. 3 days after Daudi engraftment. Mice were euthanized 48 hours after T-cell and THP-1 injection. Shown are ventral images by Xenogen IVIS-200 system (Caliper Life Sciences) after D-luciferin injection. **B.** Changes in tumor cell volume before T-cell injection and 48 hours after injection. Leukemic growth in mice is expressed as photons per second. Each point on the graph represents a bioluminescence measurement of each mice.