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## Supplemental Information

# Generation of highly proliferative, rejuvenated cytotoxic T cell clones through pluripotency reprogramming for adoptive immunotherapy

**Yohei Kawai, Ai Kawana-Tachikawa, Shuichi Kitayama, Tatsuki Ueda, Shoji Miki, Akira Watanabe, and Shin Kaneko**

**Fig. S1**

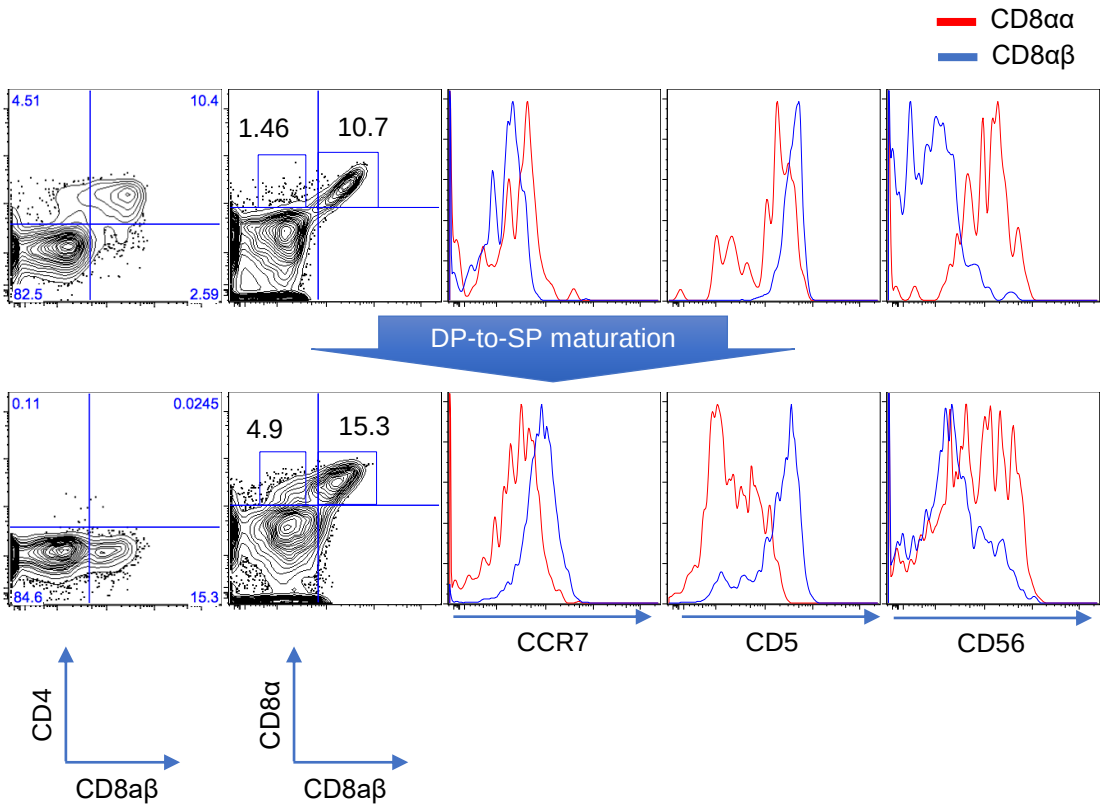


**Fig. S1. CD8αβ iPSC-CTLs express more CCR7 and CD5 but less CD56 than conventional CD8αα cells. (A)** Flow cytometric profile of CD8αβ and CD8αα iPSC-CTLs before and after modified maturation culture as described in Materials and Methods.

**Fig. S2**

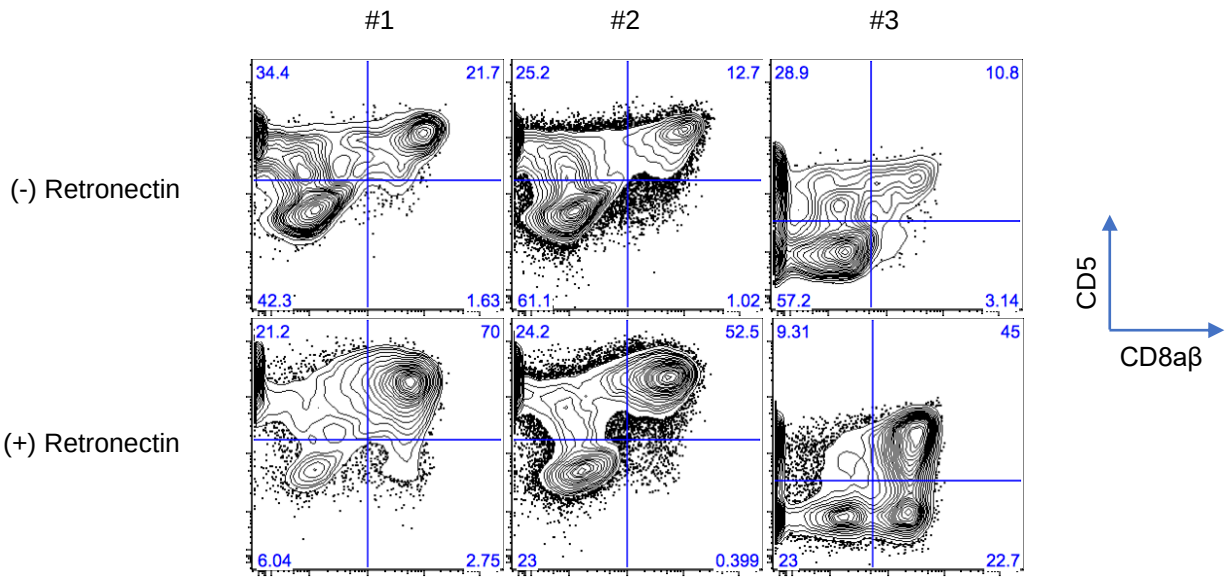


**Fig. S2. Retronection coating promotes the generation of CD8αβ$^{+}$CD5$^{+}$ iPSC-CTLs.** Flow cytometric analysis of three independent iPSC-CTLs matured with or without Retronectin-coating in the presence of 1 mg/ml CD3ε antibody and 10 ng/ml IL-7, but not IL-21.

**Fig. S3**

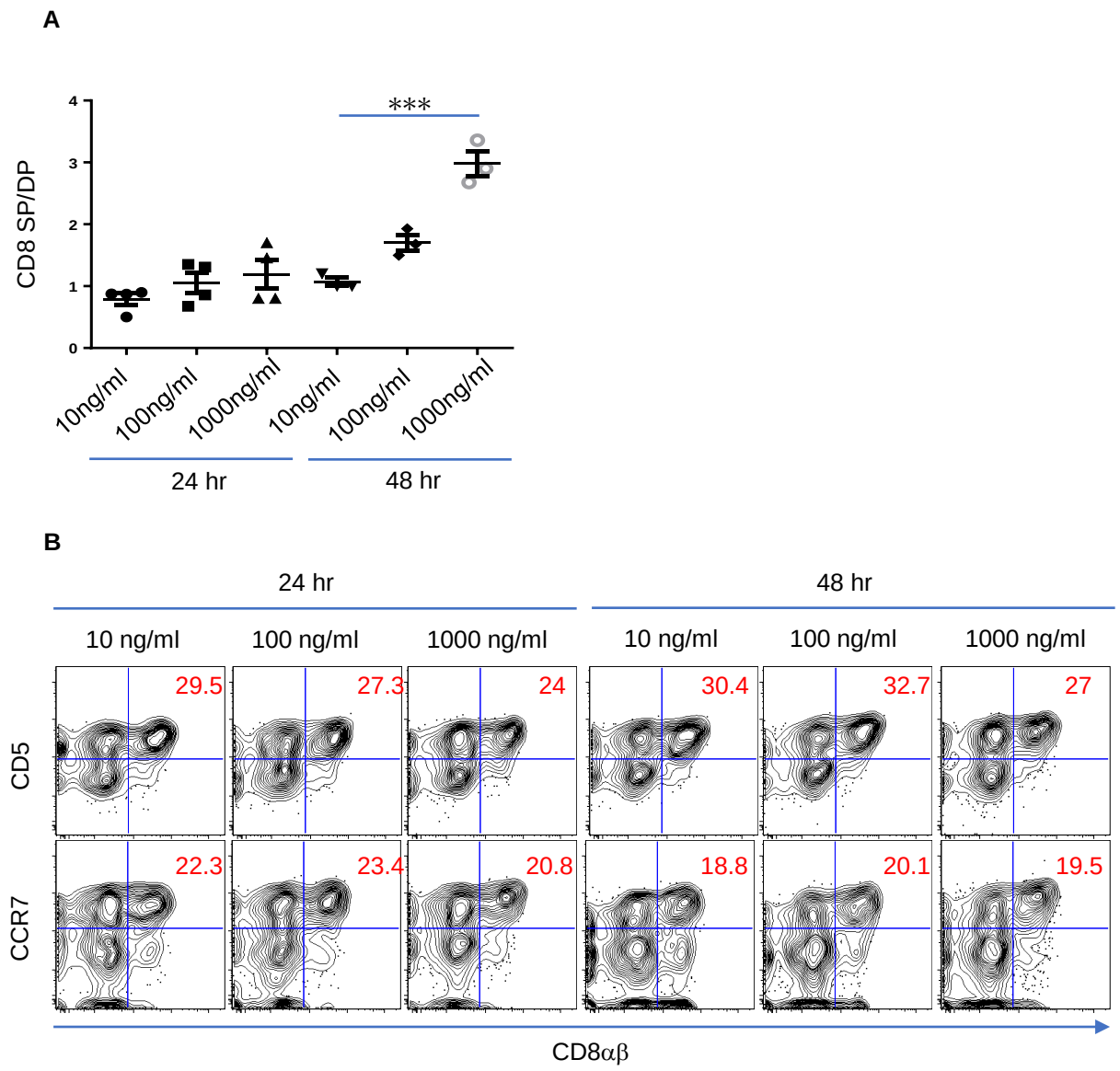


**Fig. S3. Optimization of CD3ε antibody supplement and Retronectin-coating in the DP-to-CD8SP stage.** The effect of differential duration and dose of CD3ε antibody supplement on maturation efficacy (A) and the expression of adaptive naïve associated markers (B). iPSC-derived cells on day 24 OP9/DL1 coculture were matured for the indicated period in the presence of the indicated dose of CD3ε antibody (supplemented with 10 ng/ml IL-7, but not IL-21), and the ratio of the CD8SP cell yield against the starting DP cell number was calculated as the maturation efficacy (24 hr, n=4; 48 hr, n=3; mean ± SEM; one-way ANOVA comparing mean log10 of all groups with Tukey's multiple comparisons test; ***P‹0.0005). The longer and higher the dose of the CD3ε antibody supplementation, the higher the maturation efficacy with only negligible change in the expression of adaptive naïve-associated markers. Representative of four independent experiments.

Fig. S4

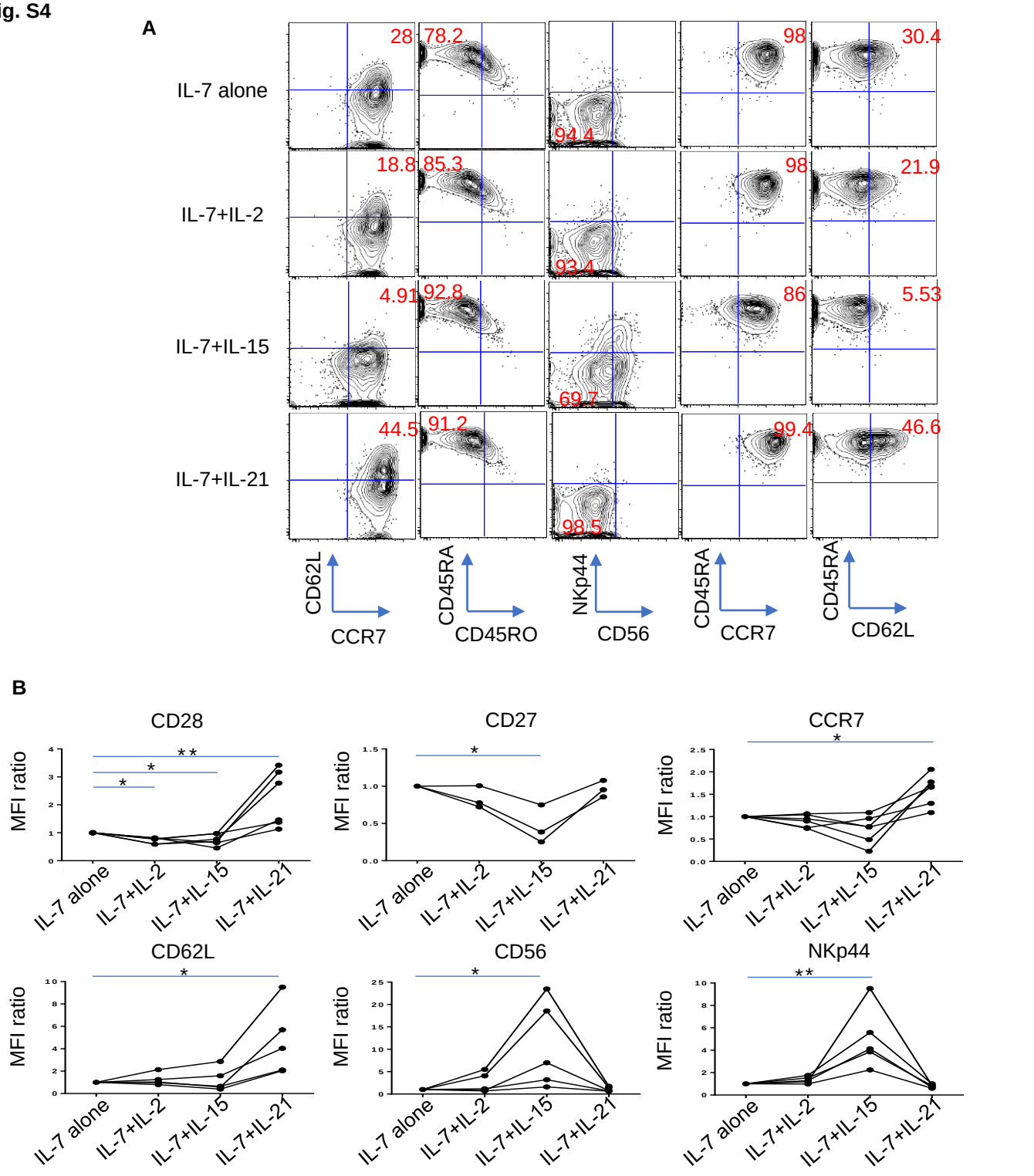


**Fig. S4. The effect of common γ-chain cytokines on the surface phenotype in the DP-to-CD8SP stage.** Representative FACS dot plot (A) and summary of the mean fluorescence intensity (MFI) ratio to control (IL-7 alone) (B) are shown for the indicated molecules on iPSC-CTLs matured in the presence of IL-2, IL-15 or IL-21. iPSC-derived cells on day 24 OP9/DL1 coculture were matured for 2 days in the presence of 1 mg/ml CD3e antibody and OP9/DL1, transferred to 5-day culture with 5 mg/ml Retronectin-coating and the indicated cytokines and then analyzed by flowcytometry. 3 (CD27), 5 (CD62L, CD56, NKp44) and 6 (CD28, CCR7) independent experiments were performed by different flow cytometers using TKT3V1-7, H254SeV3- and H2531SeV3-derived iPSC-CTLs (mean ± SEM; one-way ANOVA comparing mean log10 of all groups with Tukey's multiple comparisons test; *P‹0.05, **P‹0.005).

**Fig. S5**

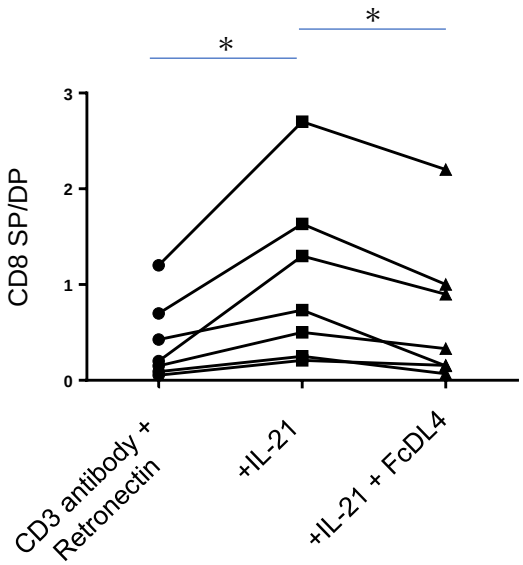


**Fig. S5. Simultaneous engagement of IL-21 and a Notch ligand hampered the yield of CD8 SP cells.** iPSC-derived cells on day 24 OP9/DL1 coculture were matured for 2 days in the presence of 1 mg/ml CD3e antibody and 5 mg/ml Retronectin-coating with or without 20 ng/ml IL-21 and 5 mg/ml Fc-DL4 fusion protein-coating. The ratio of the CD8 SP cell yield against the starting DP cell number was calculated as the maturation efficacy (n=7; mean ± SEM; one-way ANOVA comparing mean log10 of all groups with Tukey's multiple comparisons test; a = 0.05; *P‹0.05).

**Fig. S6**

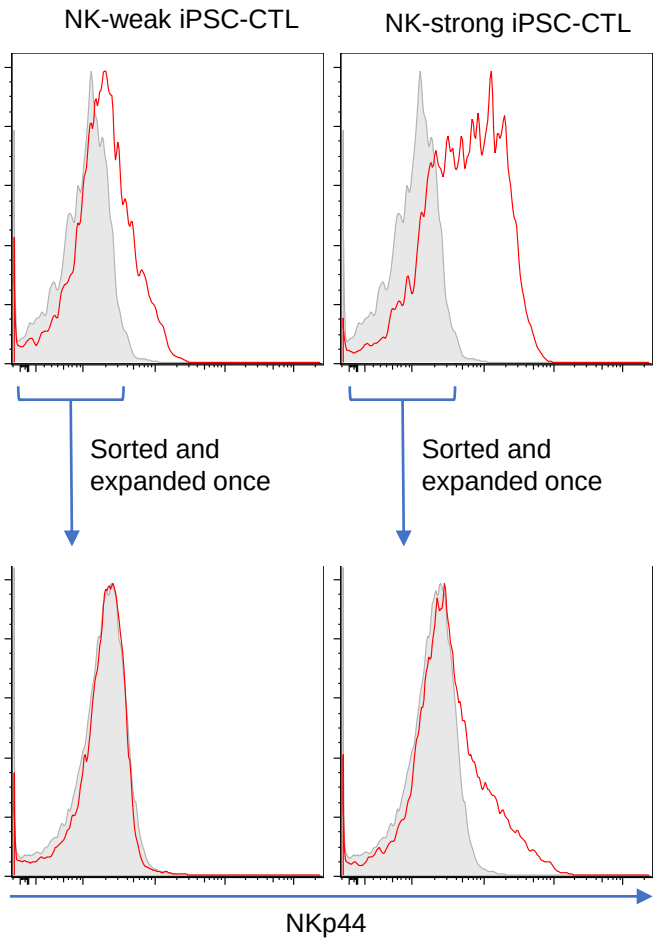


**Fig. S6. Negative sorting could not remove NKp44 expression permanently in NK-strong iPSC-CTLs.** NKp44$^{-}$ cells were sorted from NK-weak and -strong iPSC-CTLs using FACSAria II, expanded in PHA-PBMC-feeder condition and monitored again for NKp44 expression in flow cytometry. Representative of two independent experiments.

**Fig. S7**

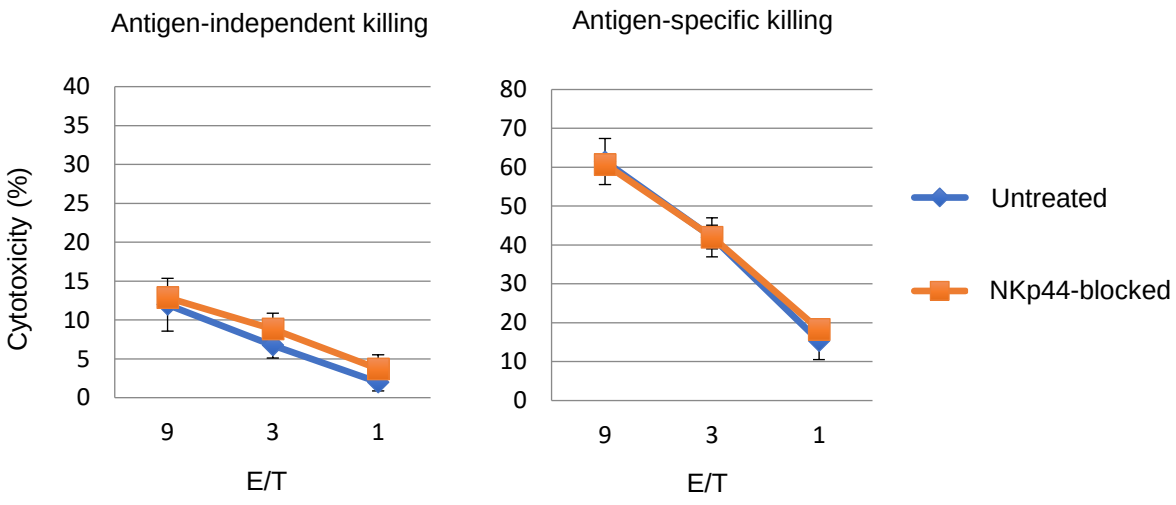


**Fig. S7. NKp44 blocking antibody did not suppress the NK activity of iPSC-CTLs.** $^{51}$Cr release assay was performed using iPSC-CTLs and 1 mM peptide-pulsed (antigen-specific) or unpulsed (antigen-independent) K562-A24 in the presence or absence of 5 mg/ml anti-NKp44 blocking antibody. Representative of two independent experiments.

**Fig. S8**

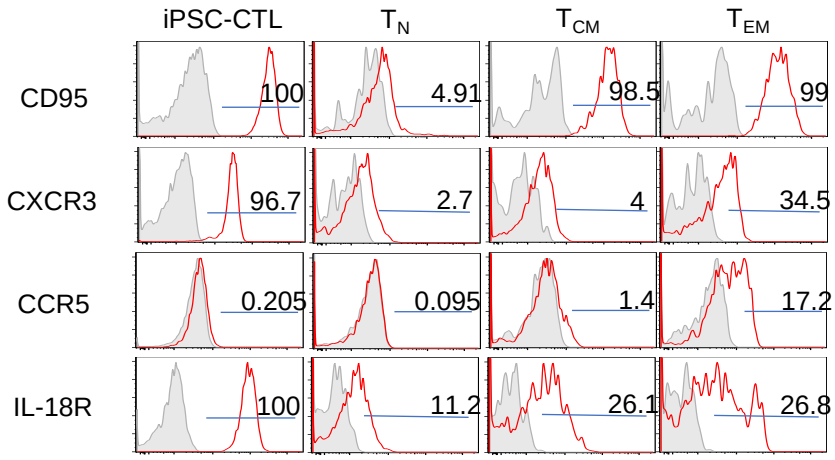


**Fig. S8. Memory-associated molecules were expressed in iPSC-CTLs.** Flow cytometric analysis of TKT3V1-7-derived iPSC-CTLs and indicated subsets of healthy donor-derived primary CTLs. Representative of two independent experiments using TKT3V1-7- and H254SeV3-derived iPSC-CTLs

**Fig. S9**

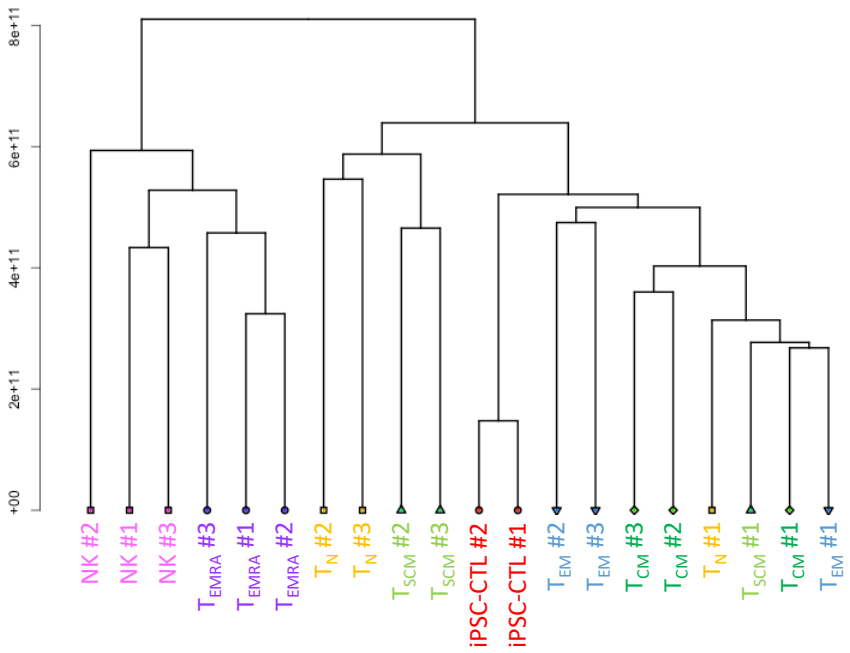


**Fig. S9. iPSC-CTLs are distinguished from fully differentiated cytotoxic effector cells such as NK cells and $T_{EMRA}$ in global gene expressions.** A dendrogram generated by global gene expression profiles related to cytotoxic T cell subsets for iPSC-CTLs and primary CD8 T cells. Messenger RNA expression in iPSC-CTLs and primary CD8 T cells was determined by RNA sequencing.

**Fig. S10**

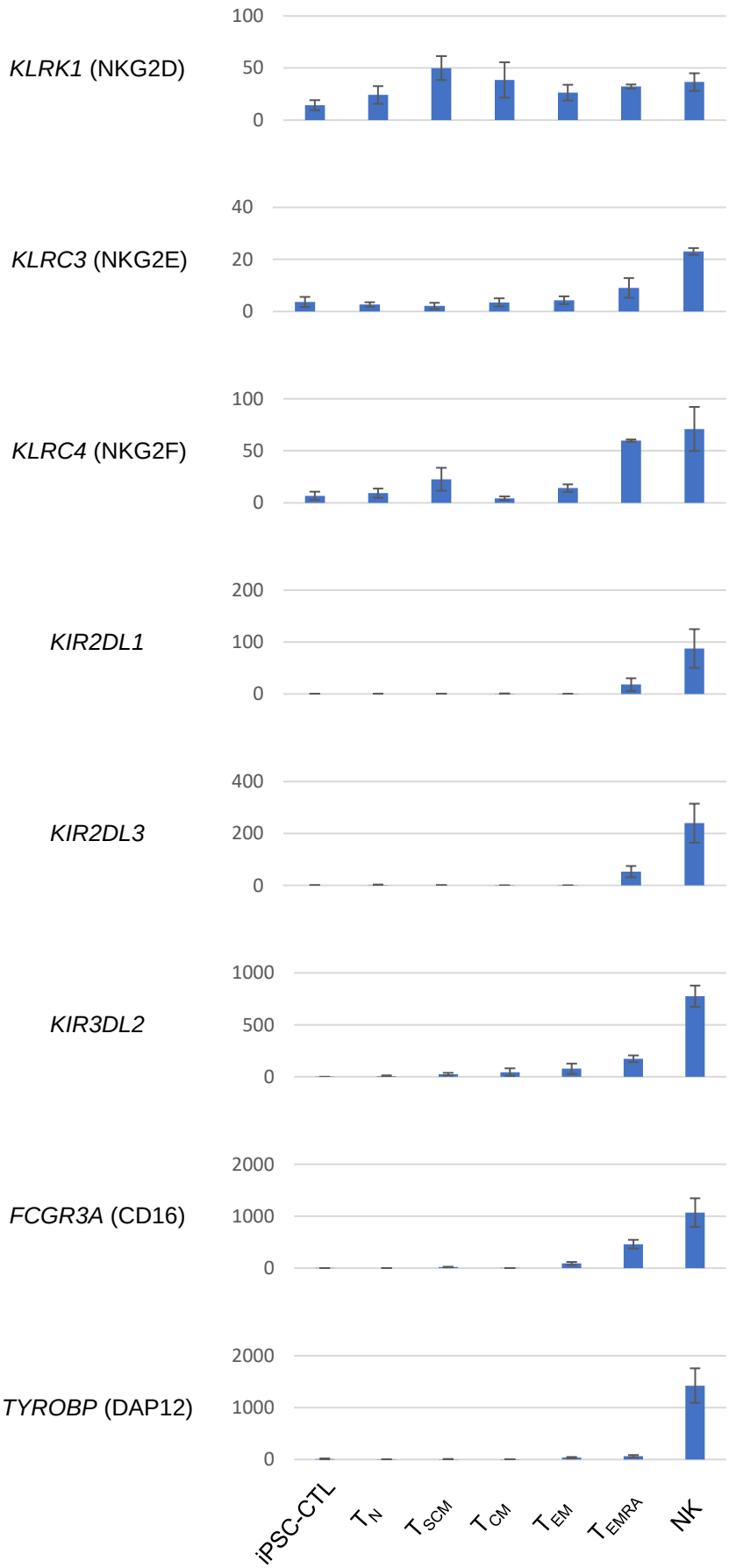


**Fig. S10. Expression of NK-associated molecules in iPSC-CTLs and indicated primary CTL subsets.** Messenger RNA expression levels (RPKM) of *KLRK1, KLRC3, KLRC4, KIR2DL1, KIR2DL3, KIR3DL2, FCGR3A* and *TYROBP* are shown for the indicated populations. TKT3V1-7 and H254SeV3 clones were subjected to RNA sequencing (iPSC-CTLs, n=2; primary CTL subsets, n=3; mean ± SEM).

**Fig. S11**

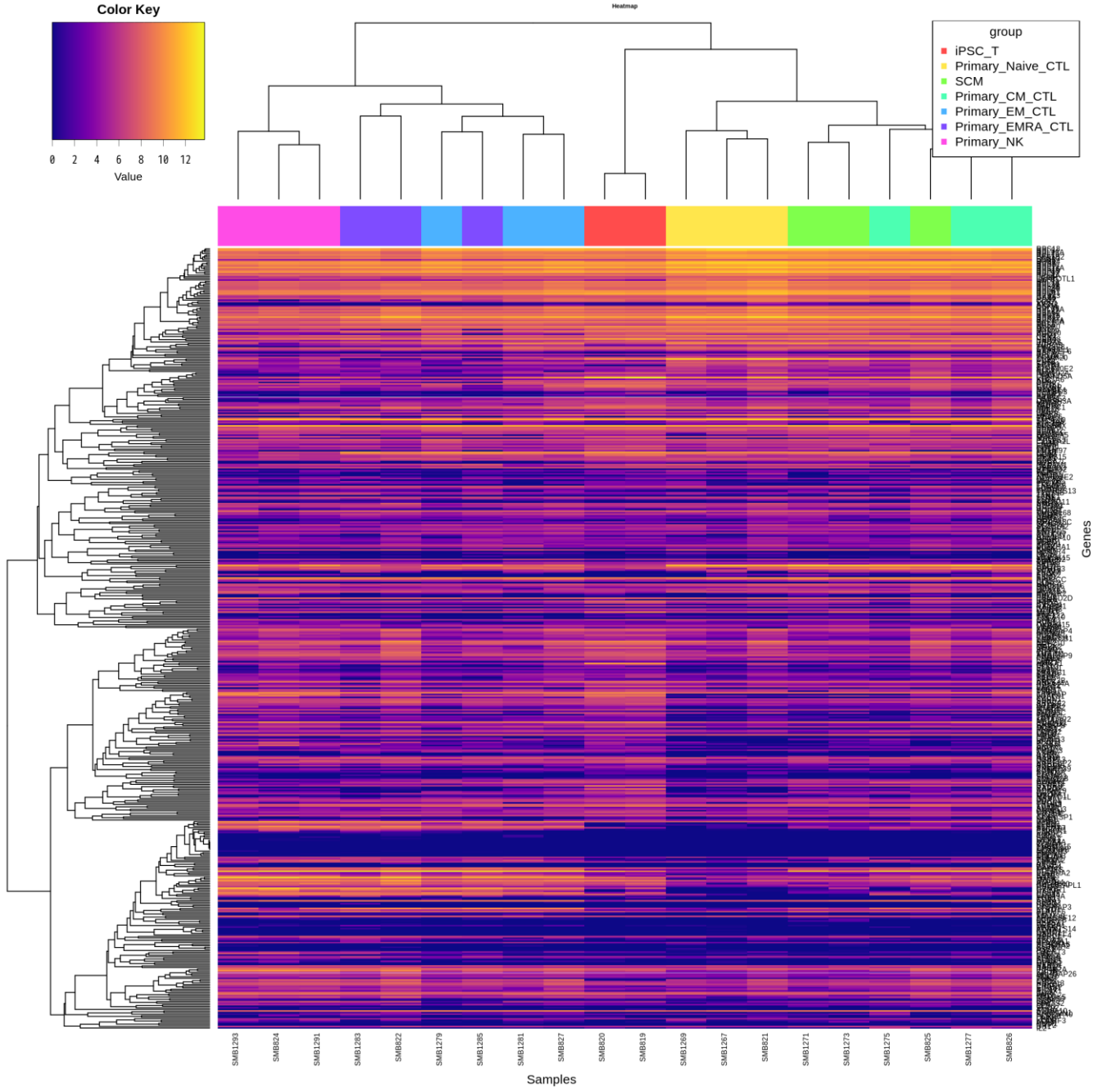


**Fig. S11. Heatmap comparing the expression of 776 T-cell associated genes in iPSC-CTLs and indicated primary CTL subsets.** The heatmap was drawn from RNA sequencing data using TKT3V1-7- and H254SeV3-derived iPSC-CTLs (iPSC-CTLs, n=2; primary CTL subsets, n=3).

**Fig. S12**

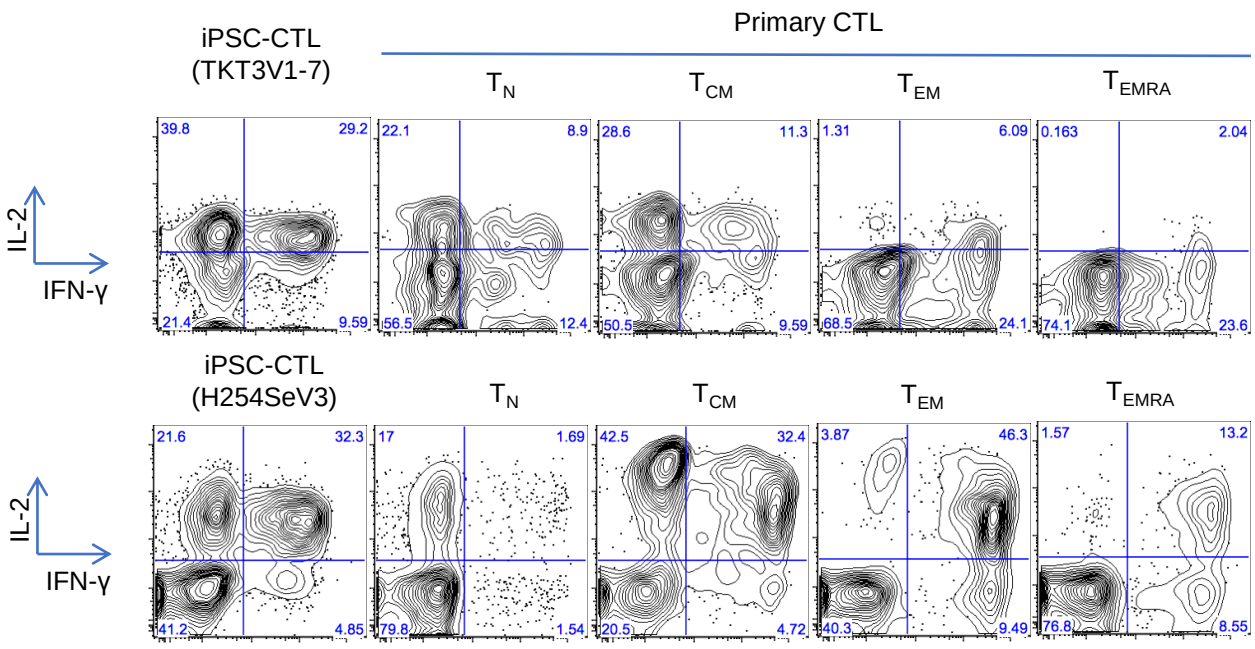


**Fig. S12. Modified iPSC-CTLs showed a $T_{CM}$-like cytokine profile.** Intracellular IL-2 and IFN-γ in TKT3V1-7- and H254SeV3-derived iPSC-CTLs were analyzed by flow cytometry after 4 hours of stimulation with 50 ng/ml PMA and 1 mg/ml Ionomycin.

**Fig. S13**

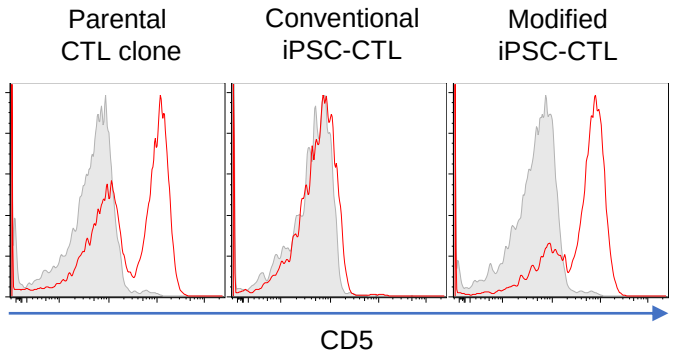


**Fig. S13. Modified iPSC-CTLs expressed CD5 over multiple expansions unlike conventional iPSC-CTLs.** Flow cytometric analysis of the parental T-cell clone and indicated iPSC-CTLs expanded four times in PHA-PBMC-feeder condition. Representative of two independent experiments.

**Fig. S14**

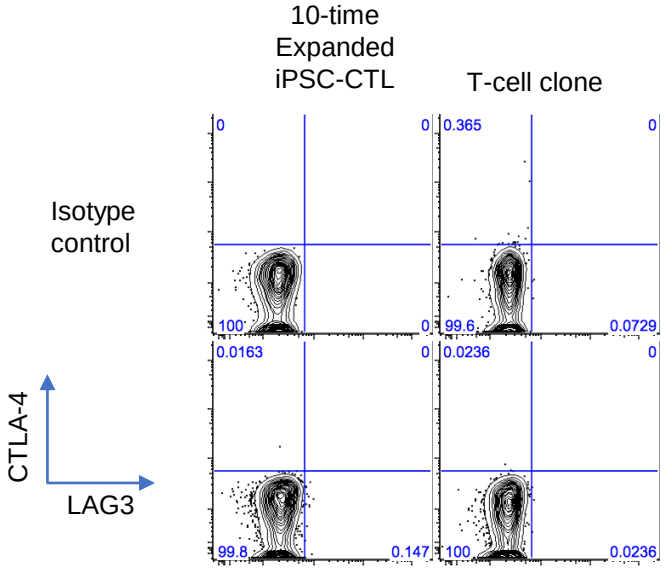


**Fig. S14. Expression of exhaustion markers in iPSC-CTLs after 10 rounds of expansions.** Expression of LAG-3 and CTLA-4 in 10-times expanded iPSC-CTLs and the parental T-cell clone in PHA-PBMC-feeder expansion culture as described in Materials and Methods.

**Fig. S15**

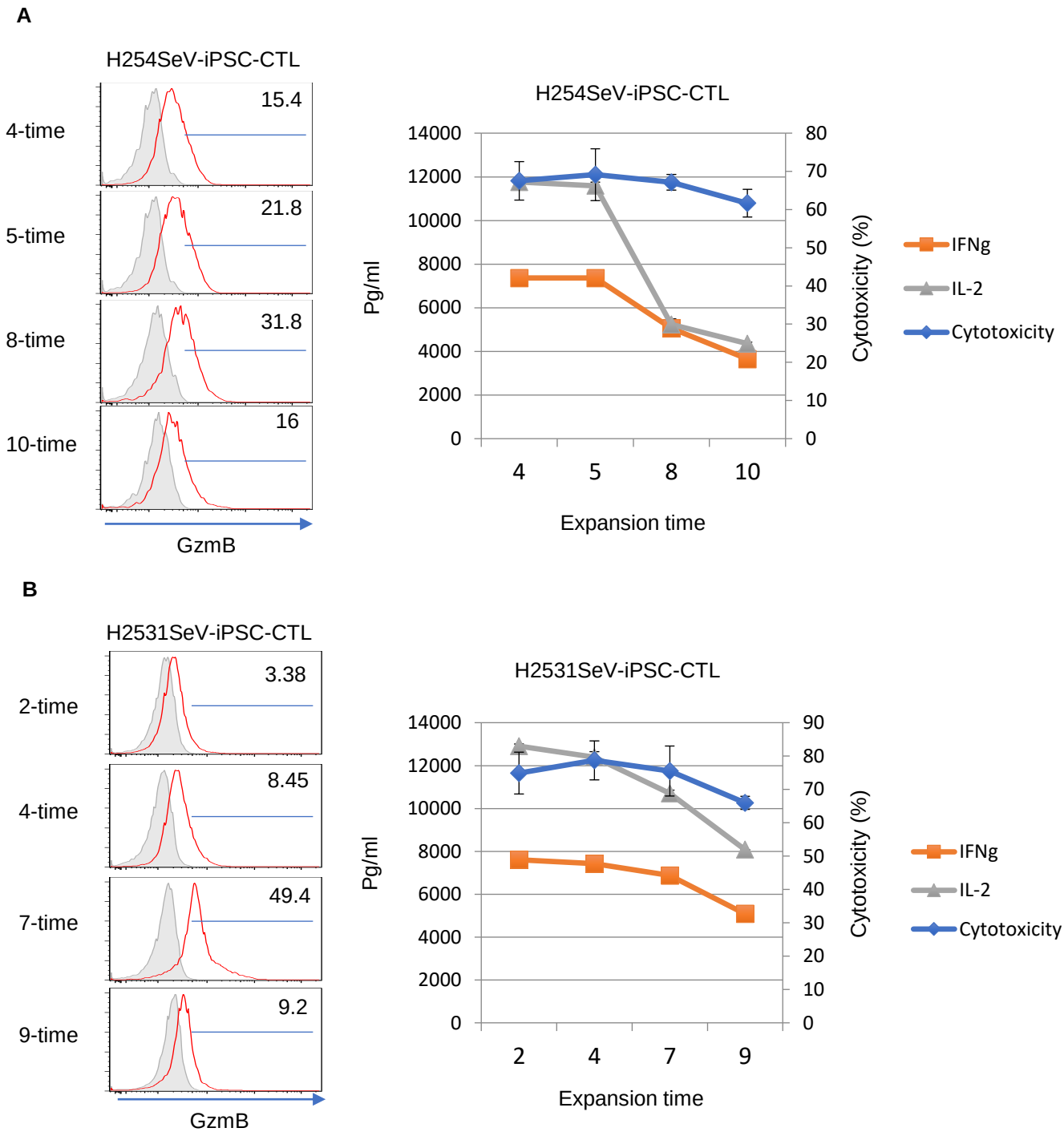


**Fig. S15. Cytokine production and killing activity of iPSC-CTLs change over multiple *in vitro* expansions.** Intracellular Granzyme B expression (left), IL-2 and IFN-γ production and cytotoxic activity (right) of H254SeV3- (A) and H2531SeV3- (B) derived iPSC-CTLs after the indicated rounds of expansions. The frozen stocks of iPSC-CTLs were made at different times, but they were thawed, rested and expanded once side by side in advance of the flow cytometry or the $^{51}$Cr release assay against 1 mM peptide-pulsed K562-A24 at an E/T=10 (mean ± SD).

**Fig. S16**

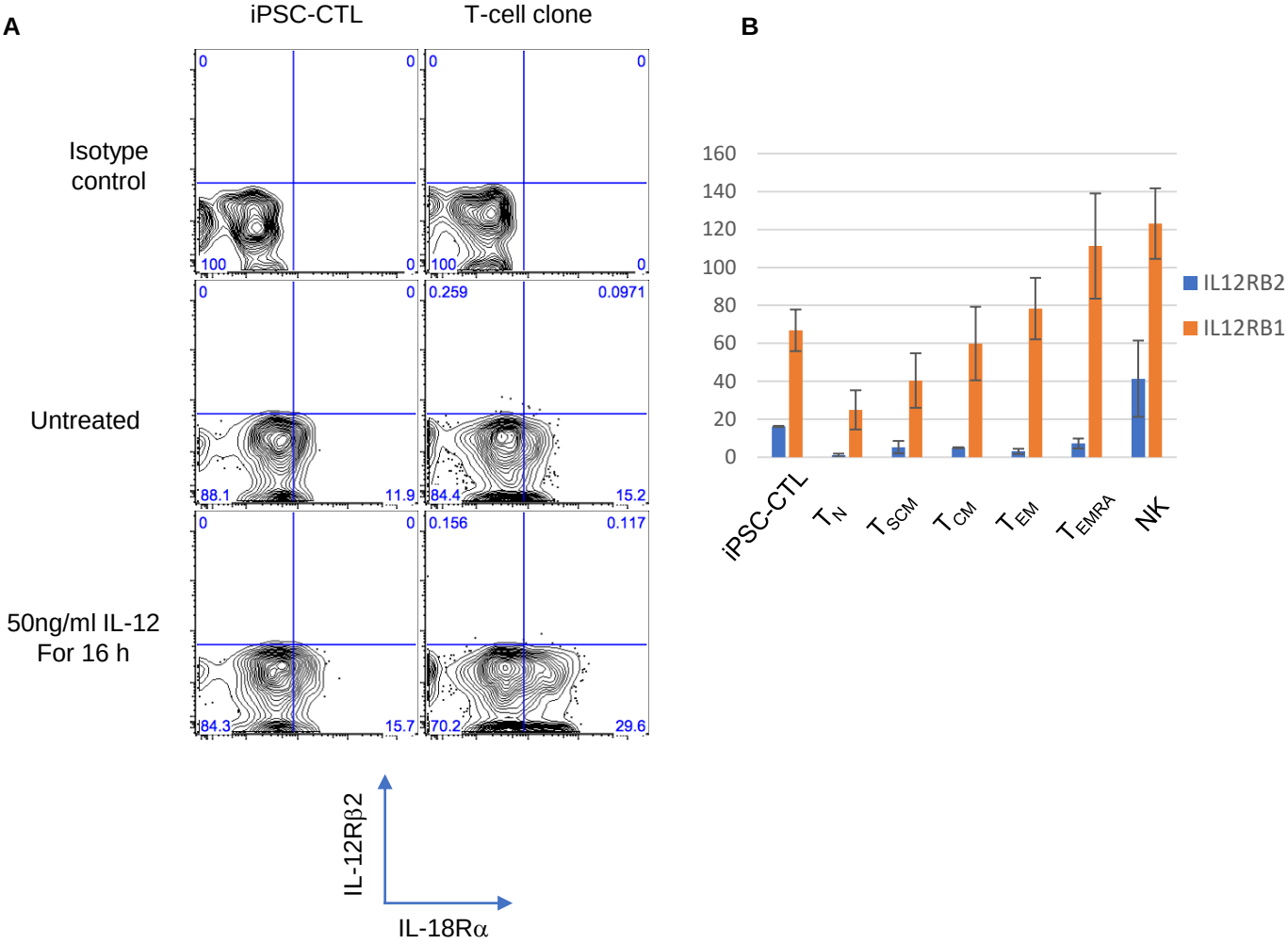


**Fig. S16. IL-12 enhances IL-18 receptor expression on iPSC-CTLs and parental T-cell clones.** (A) Flow cytometric profiles of iPSC-CTLs and T-cell clones treated with or without 50 ng/ml IL-12 for 16 h. (B) Messenger RNA expression levels (RPKM) of *IL12RB1 and IL12RB2* in iPSC-CTLs and indicated primary CTL subsets.

**Fig. S17**

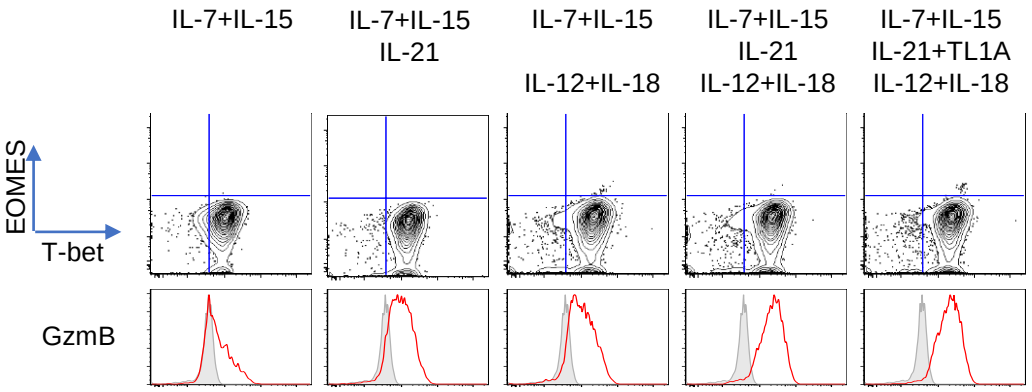


**Fig. S17. GzmB, T-bet and EOMES expression in iPSC-CTLs 2 weeks after PBMC feeder-free stimulation in the presence or absence of IL-21, IL12, IL-18 or TL1A.** The expression of GzmB was induced, but the expression of T-bet or EOMES was not 2 weeks after IL-21, IL12, IL-18 or TL1A-supplemented TCR stimulation.

**Fig. S18**

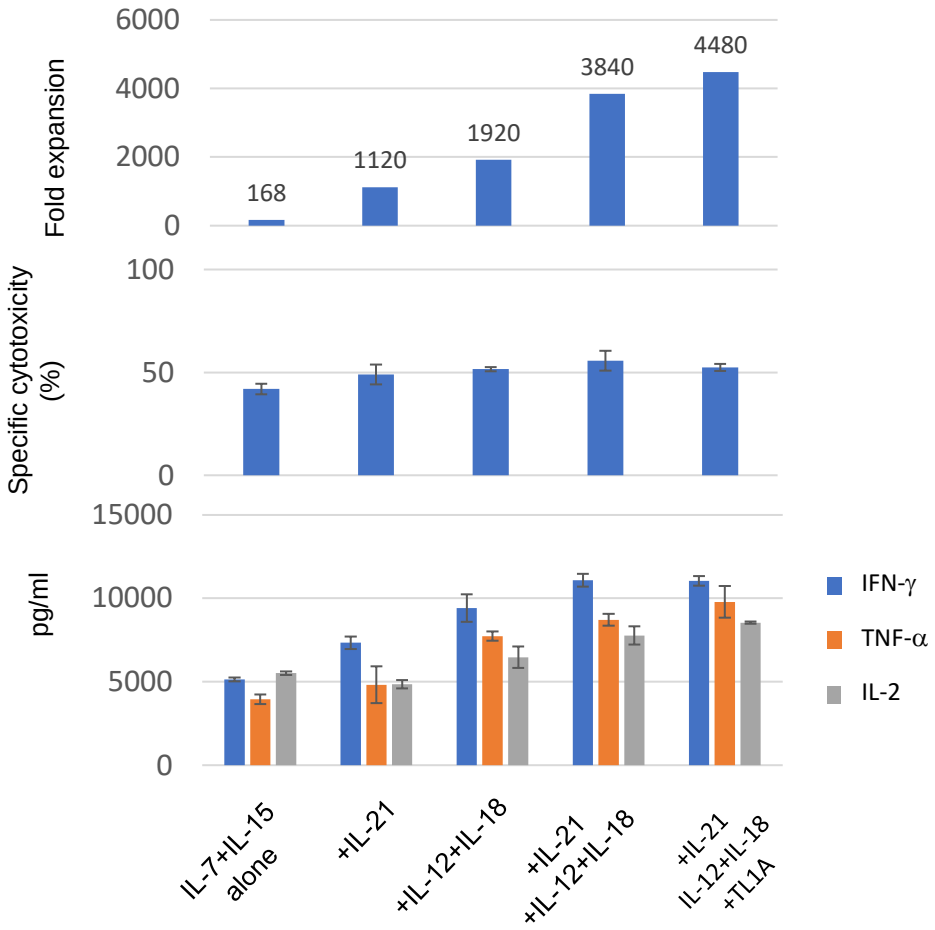


**Fig. S18. Effect of cytokines on proliferation (top), cytotoxic activity (middle) and cytokine production (bottom) in iPSC-CTLs.** iPSC-CTLs expanded in PHA-PBMC-feeder 4 times were stimulated in feeder-free condition supplemented with the indicated cytokines, enumerated to calculate the fold expansion 2 weeks later (top) and subjected to the $^{51}$Cr release assay (middle) and CBA cytokine assay (bottom) against 1 mM peptide-pulsed K562-A24 cells. Representative of three independent experiments.

**Fig. S19**

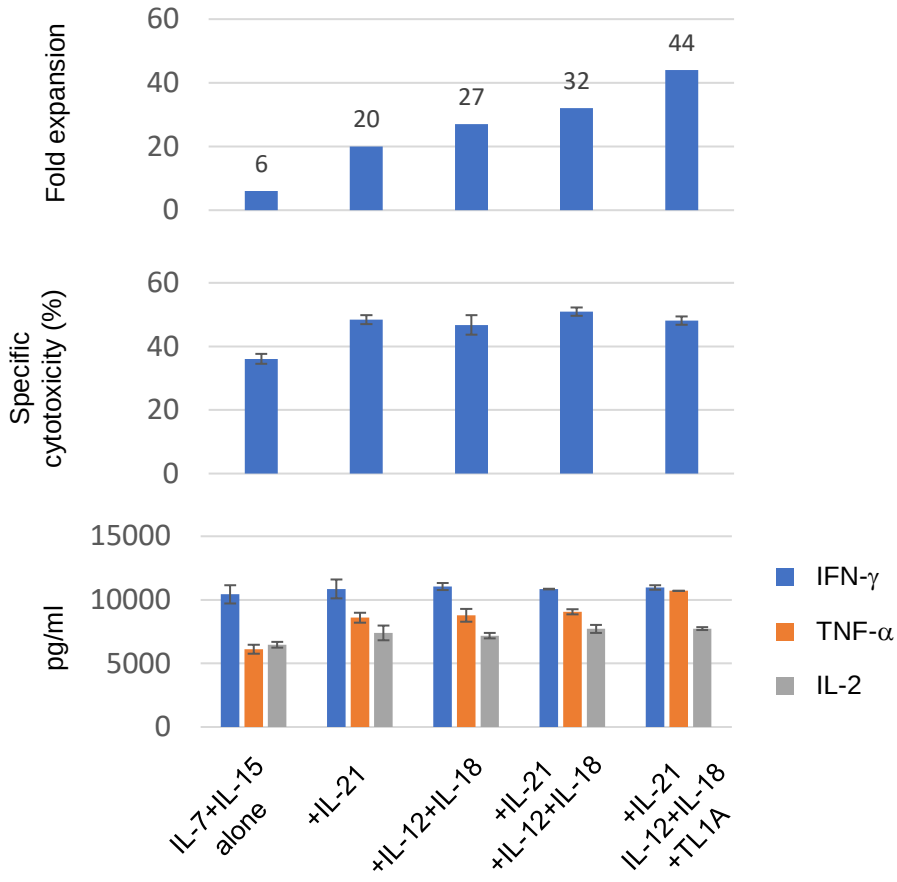


**Fig. S19. Effect of cytokines on proliferation (top), cytotoxic activity (middle) and cytokine production (bottom) in parental T-cell clones.** Parental T-cell clones were stimulated in feeder-free condition supplemented with the indicated cytokines, enumerated to calculate the fold expansion 2 weeks later (top) and subjected to the $^{51}$Cr release assay (middle) and CBA cytokine assay (bottom) against 1 mM peptide-pulsed K562-A24 cells. Representative of three independent experiments.

**Fig. S20**

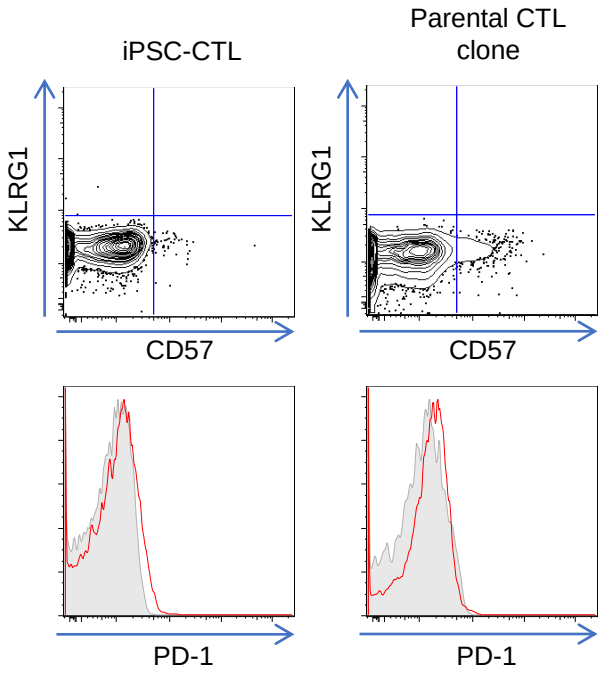


**Fig. S20. Exhaustion/senescence markers were negligibly expressed in iPSC-CTLs or the parental T-cell clone immediately before functional comparison.** Flow cytometric analysis of iPSC-CTLs and the parental T-cell clone after 2-week feeder-free expansion before the functional assay. Representative of three independent experiments.

**Fig. S21**

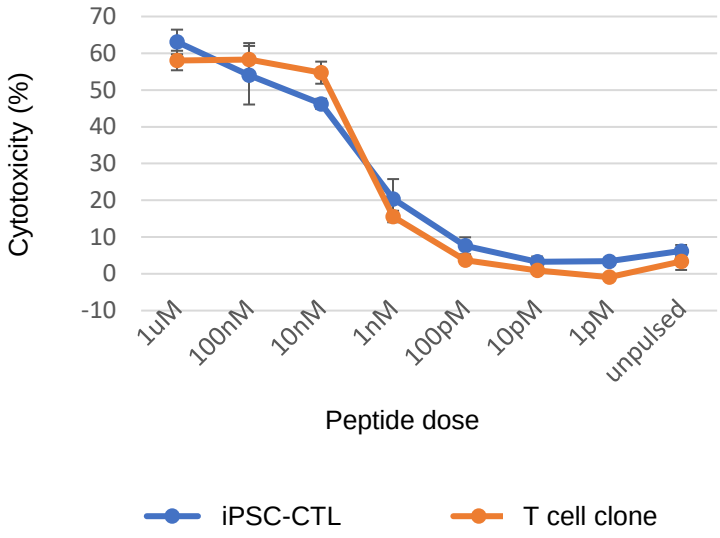


**Fig. S21. iPSC-CTLs exhibited equivalent cytotoxicity against CD4 helper GXL cells with negligible NK activity like the parental T cell clone.** $^{51}$Cr release assay was performed after 5 hours of coculture with CD4 helper GXL cells pulsed with the indicated dose of peptide at an E/T=9.

**Fig. S22**

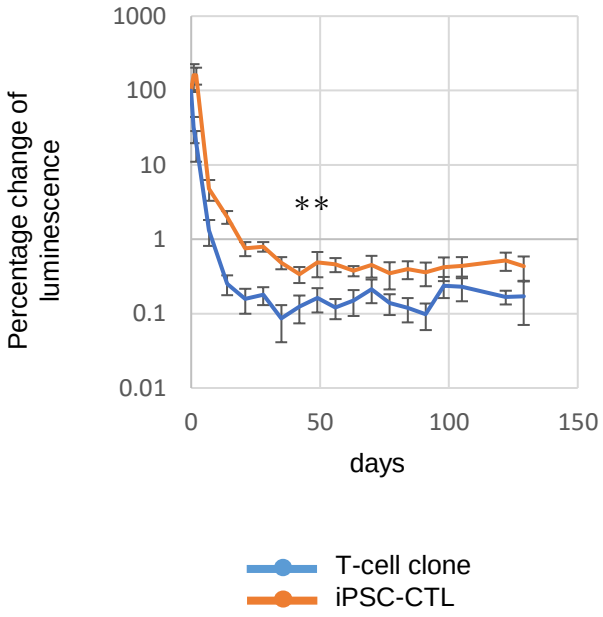


**Fig. S22. iPSC-CTLs showed superior *in vivo* persistence compared to the parental T cell clone.** Percentage change of ROI values (stomach/head) of NOD-SCID IL2Rgc$^{null}$ (NSG) mice engrafted with 2 x $10^6$ luciferase-transduced iPSC-CTLs or the parental T cell clone intraperitoneally. 100 ng/head recombinant hIL-15 was infused weekly. Representative of two independent experiments using H254SeV3-derived iPSC-CTLs (5 mice; mean ± SEM; **P‹0.005,).

**Fig. S23**

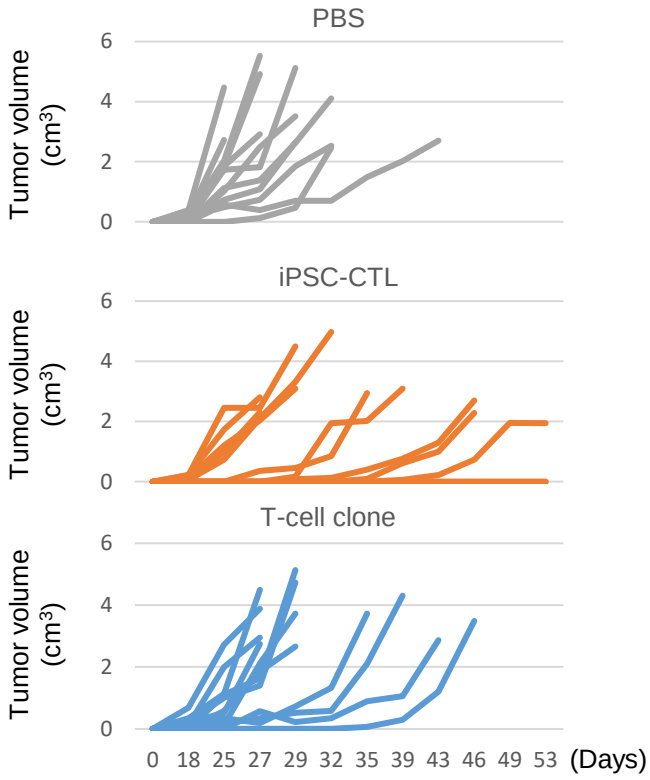


**Fig. S23. iPSC-CTLs suppressed tumor growth better than the parental T cell clone.** Kinetics of the total tumor volume in NSG mice after subcutaneous inoculation with 2 x $10^5$ cognate-Nef-peptide-expressing K562-A24-N138Rluc followed by the injection of PBS, 2 x $10^6$ iPSC-CTLs or the parental clone 4 days and 7 days later. Mice were euthanized when the tumor volume exceeded 2 $cm^3$ on either side. Shown are combined results of two independent experiments using H254SeV3-derived iPSC-CTLs (12 mice per group (5 and 7 mice for each experiment); tumor volume = (length × $width^2$)/2. [1]).

**Supplemental table.** List of 776 selected genes representing the molecular signatures of early memory cells and late memory T cells. [2]

**Supplemental movie.** iPSC-CTLs exhibited frequent serial killing while parental T-cell clone easily committed activation-induced apoptosis

**References in supplemental information**


1 Faustino-Rocha, A. *et al.* Estimation of rat mammary tumor volume using caliper and ultrasonography measurements. *Lab Anim (NY)* **42**, 217-224, doi:10.1038/laban.254 (2013).

2 Muranski, P. *et al.* Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity* **35**, 972-985, doi:10.1016/j.immuni.2011.09.019 (2011).