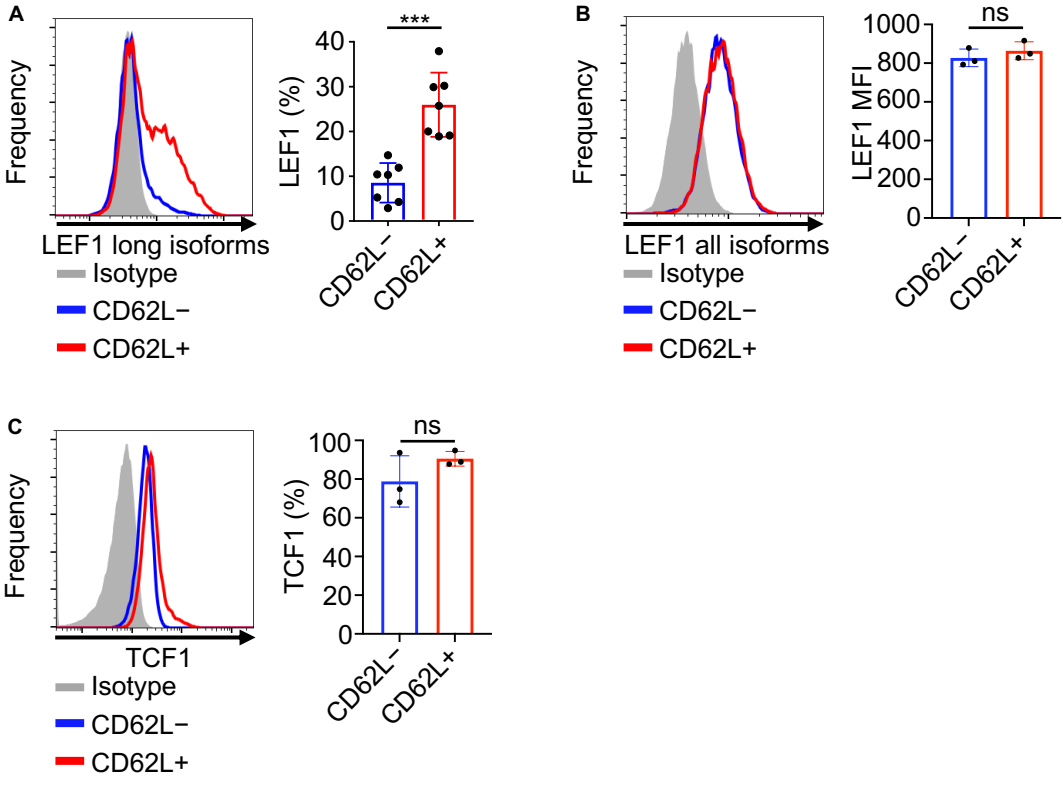
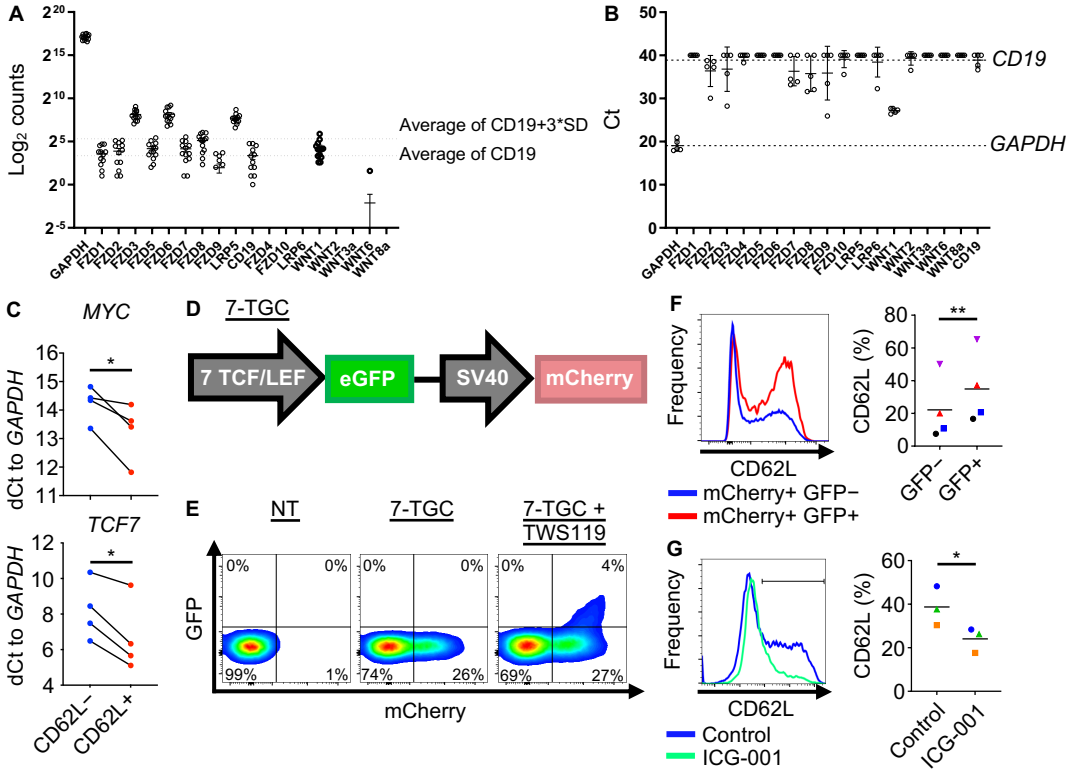
Supplementary Figure S1



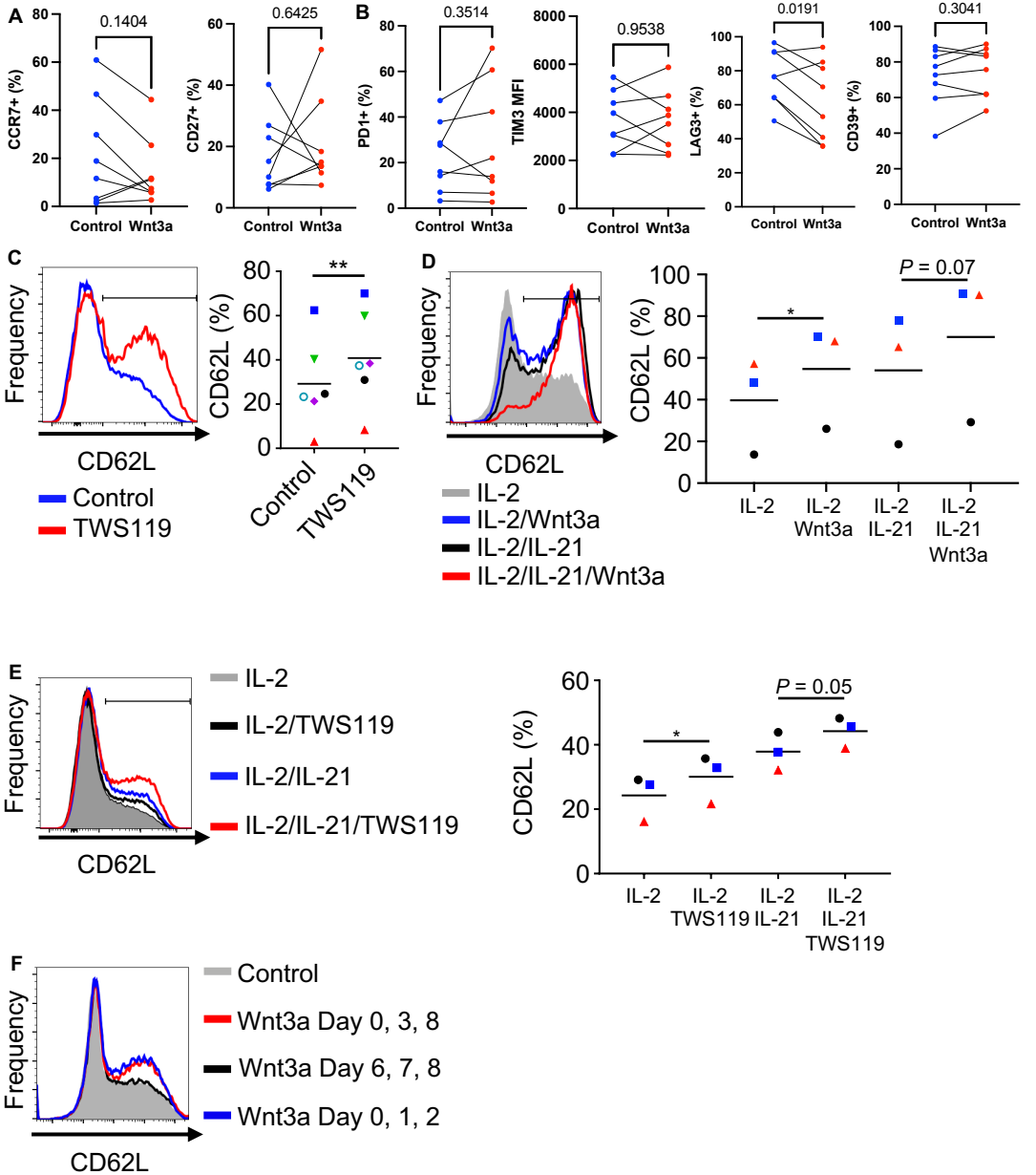
Supplementary Figure S1. CD62L+ central memory-like NKTs overexpress LEF1 long isoforms. **A-C,** Twelve days after stimulation, *ex vivo*-expanded primary NKTs were processed for intracellular flow cytometry to determine (**A**) LEF1 long isoforms (n = 7 donors), (**B**) LEF1 all isoforms (n = 3 donors), and (**C**) TCF1 (n = 3 donors) expression in CD62L+ and CD62L- gated populations. Representative donor histograms and mean ± SEM percent positive or MFI in CD62L+/- populations are shown.

Supplementary Figure S2



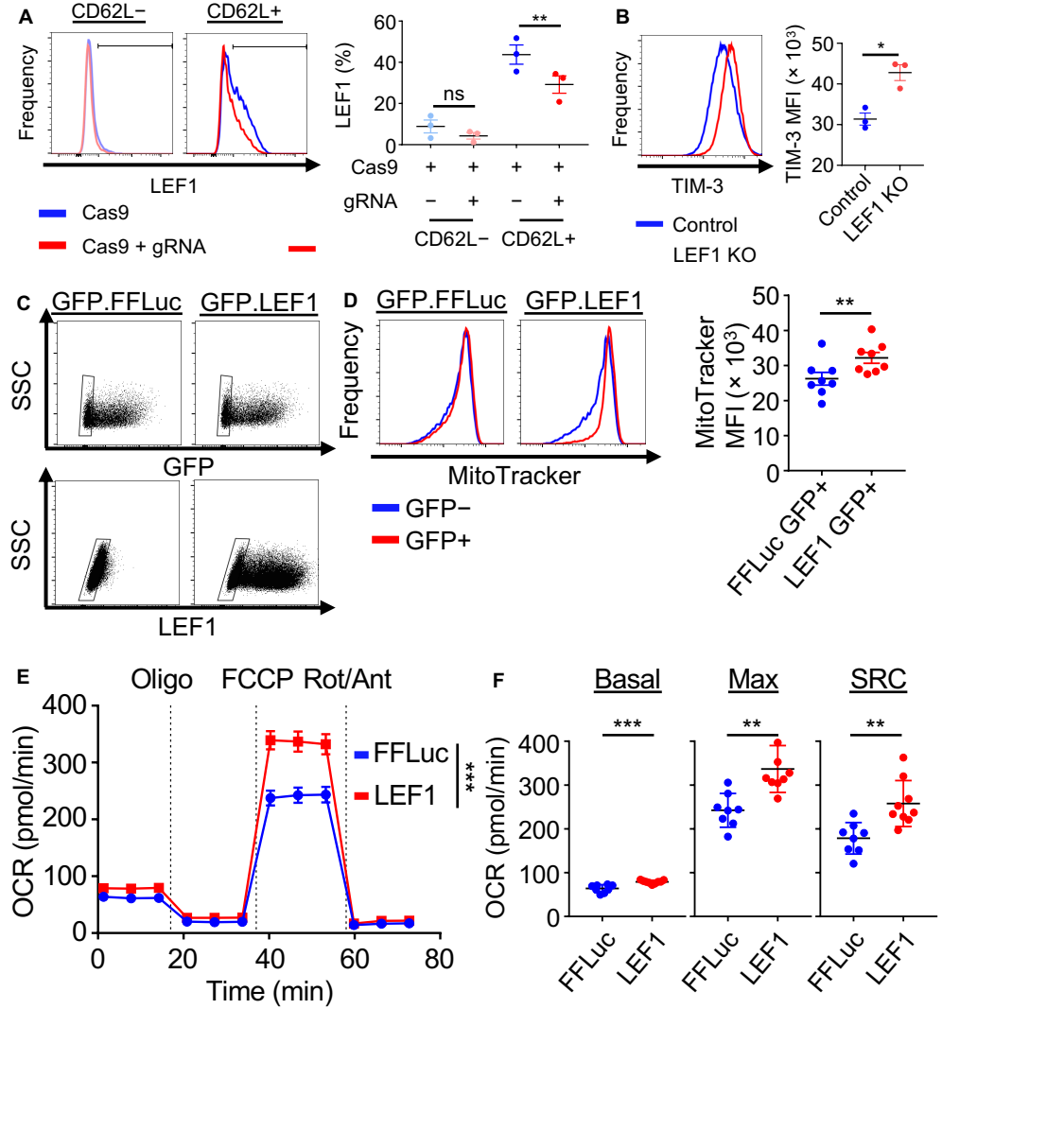
Supplementary Figure S2. Elevated Wnt/β-catenin signaling in CD62L+ supports their central memory-like phenotype. **A,** Next generation sequencing performed on RNA was isolated from expanded NKTs was used to examine expression of the Wnt-receptor, co-receptor and ligand genes. **B**, RNA was isolated from freshly isolated peripheral NKTs, and qRT-PCR was performed on isolated RNA to determine expression levels of Wnt receptor, co-receptor, and ligand mRNA. GAPDH (positive control) and CD19 (negative control) expression were used to set a range of significant Ct values. Mean ± SD are from two independent experiments with a total of four to five donors. **C**, On day 10 of *ex* vivo expansion, NKTs were magnetically sorted into CD62L+ and CD62L- subsets and expression of *MYC* and *TCF7* as Wnt target genes were determined from isolated RNA using qRT-PCR and the dCt method (n = 4 donors). **D**, Lentiviral 7-TGC construct containing eGFP as Wnt signaling reporter and mCherry as transduction marker. **E**, NKTs were transduced with 7-TGC and left untreated or treated overnight with Wnt pathway activator TWS119 (10 μM). Wnt signaling activity was assessed by flow cytometry 12 days after stimulation. Non-transduced NKTs served as negative control. **F**, Following transduction with 7-TGC, mCherry+ NKTs were gated into GFP+ and GFP- subsets. Representative donor histogram and mean CD62L+ percentage (n = 4 donors) in GFP+/- NKT populations are shown. **G**, NKTs were treated with Wnt pathway inhibitor ICG-001 (3 μM) or PBS for three days following stimulation with αGalCer-pulsed autologous PBMCs. CD62L expression was determined 12 days after stimulation. Representative donor histogram and mean CD62L+ percentage (n = 3 donors) are shown. **P* < 0.05, ***P* < 0.01 paired Student's *t* test for paired result from each donor.

Supplementary Figure S3



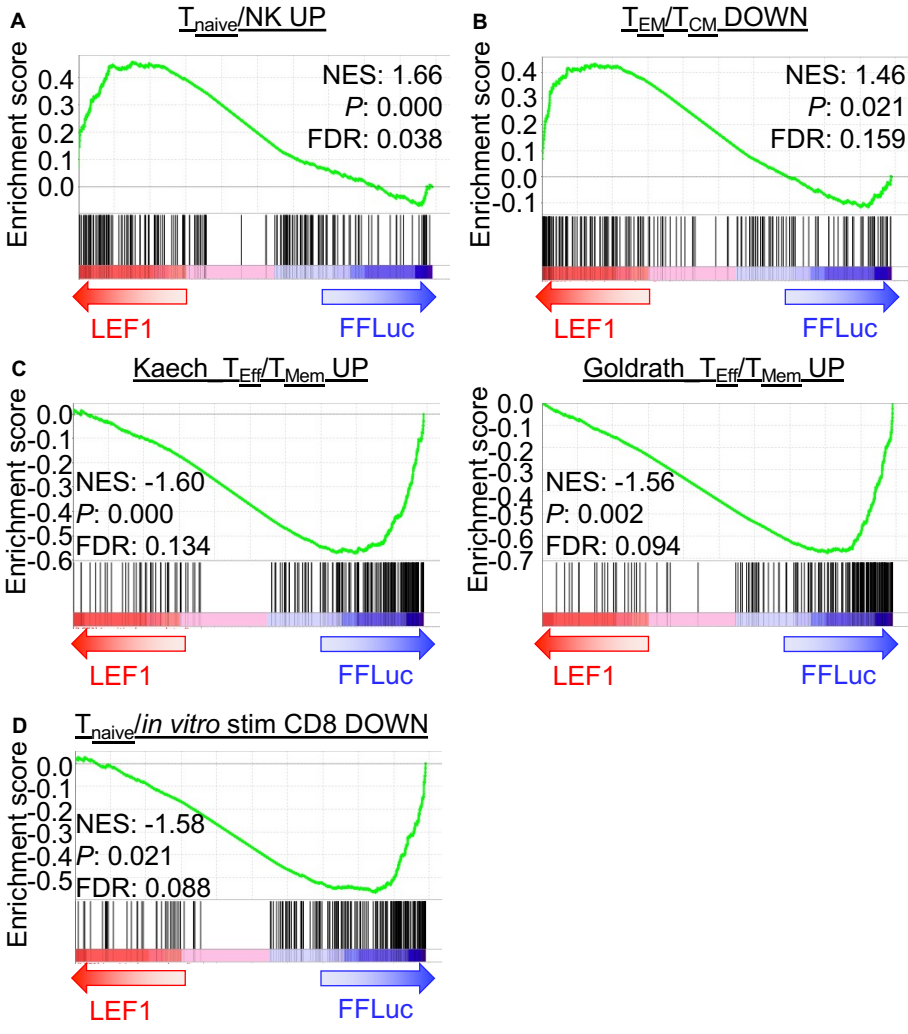
Supplementary Figure S3. Effects of Wnt signaling on NKTs. **A-B,** NKTs were treated with 500 ng/mL human recombinant Wnt3a or PBS for three days following stimulation. Surface expression of (**A**) central memory markers and (**B**) exhaustion markers were determined 10 days after stimulation. Paired percent positive or MFI (n = 11 donors) are shown. *P* value was shown above. **C**, NKTs were treated once with 5 μM TWS119 or DMSO on day 7 after antigenic stimulation. CD62L expression was examined by flow cytometry 5 days later. A representative histogram (left) and mean CD62L+ percentage for all donors (n = 6, right) are shown. Each symbol denotes an individual donor. $^{**}P < 0.01$, paired Student's *t* test for paired result from each donor. **D,** Following primary stimulation, NKTs were cultured with IL-2 or IL-2/IL-21 for 12 days. PBS treatment or 500 ng/ml Wnt3a were administered on days 0, 1, and 2 after antigenic stimulation. CD62L expression was analyzed by flow cytometry at the end of expansion. Representative histogram and mean of all donors (n = 3) is shown. Each symbol denotes an individual donor, $^{*}P < 0.05$, paired Student's *t* test. **E,** NKTs were cultured as described in (**D**). DMSO treatment or 5 μM TWS119 were administered on day 7 after antigenic stimulation. CD62L expression was analyzed by flow cytometry at the end of expansion. Representative histogram and mean of all donors (n = 3) is shown. Each symbol denotes an individual donor, $^{*}P < 0.05$, paired Student's *t* test. **F,** Following primary stimulation, NKTs were treated with 3 doses of 500 ng/ml Wnt3a in early (days 0-2) or late (days 6-8) expansion or throughout (days 0, 3 and 8) expansion. CD62L expression of one donor was examined by flow cytometry on day 12 after antigenic stimulation.

Supplementary Figure S4



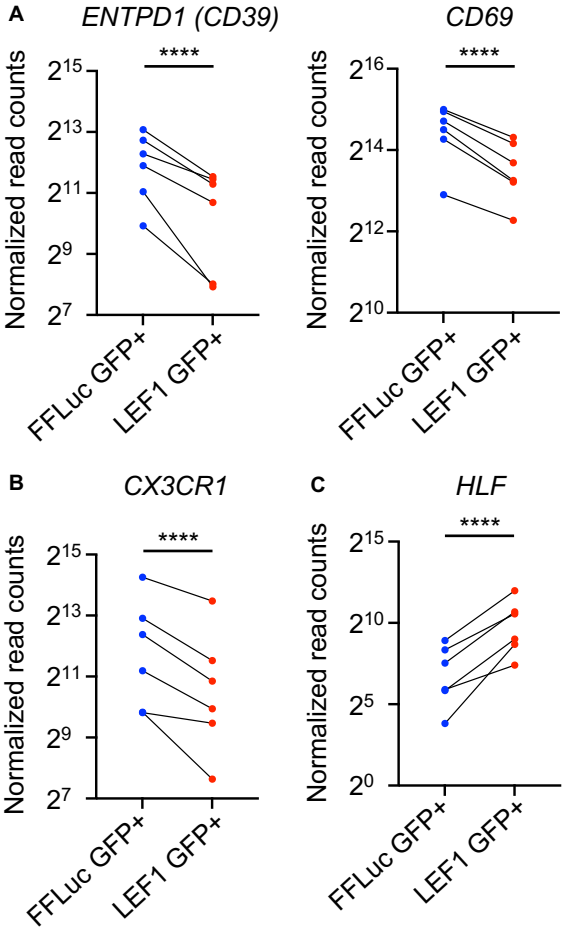
Supplementary Figure S4. Knockout and overexpression of LEF1 in NKTs. **A,** NKTs were electroporated with Cas9 with or without gRNA targeting LEF1 on day 10 of *ex vivo* expansion. CRISPR/Cas9-mediated knockout of LEF1 was evaluated using intracellular flow cytometry on day 3 after electroporation. NKTs were gated into CD62L+ and CD62L- populations, and LEF1 expression was evaluated. Representative histograms and mean ± SEM of LEF1+ percentage for all donors (n = 3) are shown. **B,** TIM-3 expression was measured 10 days after secondary stimulation. Representative donor histogram and mean ± SEM TIM3 MFI (n = 3 donors) are shown. $^{**}P < 0.01$, $^{*}P < 0.05$, ns: not significant, paired Student's *t* test for paired result from each donor. **C,** NKTs were transduced with GFP.LEF1 or GFP.FFLuc two days after secondary stimulation with αGalCer-pulsed aAPCs. On day 12 of expansion, GFP expression was examined by flow cytometry, and LEF1 expression was analyzed separately by intracellular flow cytometry. Representative dot-plots are shown (n = 3 donors). **D,** NKTs were transduced with GFP.LEF1 construct or GFP.FFLuc two days after secondary stimulation and NKT mitochondrial mass was determined by MitoTracker staining using flow cytometry 10 days after secondary stimulation. Representative histograms (left) and mean ± SEM MitoTracker MFI (n = 6) are shown. $^{**}P < 0.01$, paired Student's *t* test for paired result from each donor. **E,** Oxygen consumption rates (OCR) of GFP.FFLuc- and GFP.LEF1-transduced NKTs, with normalized transduced cell number, were measured by Seahorse assay under basal conditions and in response to oligomycin, FCCP, and rotenone/antimycin A (Rot/Ant). Shown are representative results from two donors tested. $^{***}P < 0.001$, AUC analysis. **F,** Basal, maximal, and spare respiratory capacity were calculated from (**E**). Shown are representative results from two donors tested. $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired Student's *t* test

Supplementary Figure S5



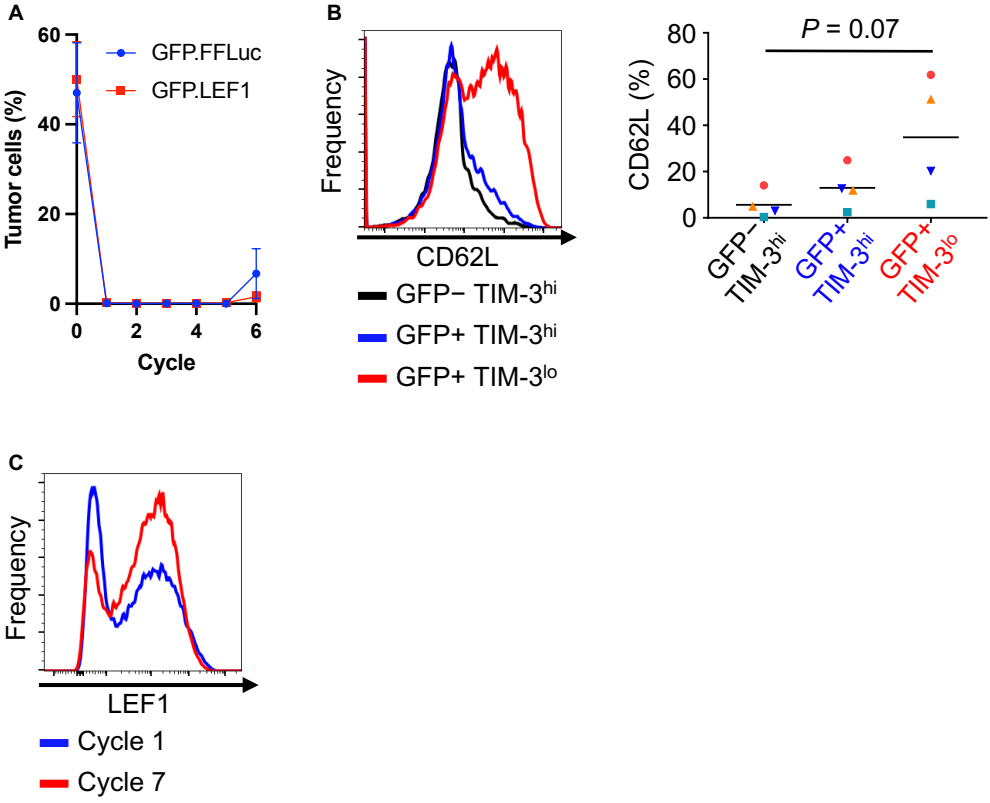
Supplementary Figure S5. Ectopic expression of LEF1 imparts a quiescent and less NK-like transcriptional signature in NKTs. **A-D,** NKTs were transduced with GFP.LEF1 or GFP.FFLuc two days after secondary stimulation with αGalCer-pulsed aAPCs. On day 12 of expansion, NKTs were FACS sorted based on GFP expression. RNA was isolated from sorted GFP+ NKTs and subjected to bulk RNA-seq analysis. (**A-B**) GSEA plots showing enrichment for (**A**) a less NK-like and (**B**) a less T_{EM}-like signature caused by LEF1 overexpression. (**C-D**)**,** GSEA plots showing enrichment for (**C**) effector, and (**D**) activated T cell signatures in GFP.FFLuc-NKTs compared to GFP.LEF1-NKTs.

Supplementary Figure S6



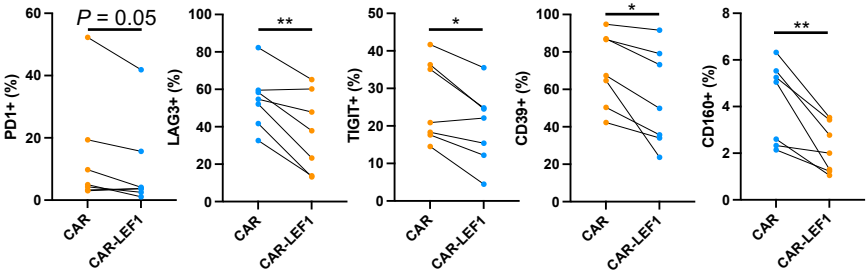
Supplementary Figure S6. LEF1 overexpression changes expression of markers to a pattern associated with stemness. **A-C,** Ten days after secondary stimulation, GFP+ cells were FACS sorted from GFP.FFluc and GFP.LEF1 NKTs. RNA isolated from the sorted cells was processed for bulk RNA sequencing analysis. Normalized read counts of FFLuc GFP+ and LEF1 GFP+ cells from each donor were shown. Expression of (**A**) CD39 and CD69, (**B**) CX3CR1, and (**C**) HLF are shown. FDR < 0.0001.

Supplementary Figure S7



Supplementary Figure S7. LEF1 overexpression in serial stimulation of NKTs. **A-C,** NKTs transduced with GFP.FFLuc and GFP.LEF1 were repeatedly challenged with CD1d+ J32 leukemia cells at a 1:1 ratio every 3 days. **A**) Percentage of live tumor cells at baseline (cycle 0) and the end of each cycle was mean ± SEM of all donors (n = 4). **B**) After the fifth cycle of killing, NKTs were rested for 6 days following antigenic stimulation. CD62L expression in NKTs gated into GFP- TIM-3^{hi}, GFP+ TIM-3^{hi}, and GFP+ TIM-3^{lo} populations was assessed by flow cytometry. Shown are representative results (left) and mean of all donors tested (n = 4 donors, right). Each symbol denotes an individual donor. One-way ANOVA with Sidak's post hoc test. **C**) LEF1 overexpression kinetics were followed for 7 cycles of repeated stimulations in NKTs. LEF1 expression was examined by intracellular flow cytometry 3 days after each stimulation. Shown are results at indicated timepoints from one donor tested.

Supplementary Figure S8



Supplementary Figure S8. LEF1 incorporation resists exhaustion caused by GD2.CAR overexpression. Two days after secondary stimulation with αGalCer-pulsed aAPCs, NKTs were transduced with parental or LEF1-containing CAR.GD2 constructs and exhaustion markers were assessed eight days after by flow cytometry in CAR+-gated NKTs. Mean ± SEM of percent positive or MFI (n = 7 donors) are shown. **P* < 0.05, ***P* < 0.01, paired Student's *t* test for paired result from each donor.