Supplementary Information

Supplementary Materials and Methods

Mutant CD33 CAR constructs: Amino acid residues ED237-239 and EEE248-250 of the 4-1BB domain (GenBank: AAA53133), reported to bind TRAF family members [1, 2] were changed to AAA. Residues Y72, Y83, Y111, Y123, Y142 and Y153 of the CD3ζ domain (GenBank: NP932170), comprising the ITAMs that bind to ZAP70 [3] were changed to phenylalanines.

Flow cytometry: Immunophenotypic analysis was performed using anti-human CD4 (clone OKT4), CD3 (UCHT1), CD33 (WM53), CD62L (DREG56), CD95 (DX2), CD69 (FN50), 4-1BB (4B4), TIM-3 (F38-2E2), LAG-3 (3DS223H), TNFα, (MAb11), and Grzb, (GB11) from eBioscience; CD8 (HIT8a), CD19 (HIB19), CD45 (HI30), CCR7 (G043H7), CD45RA (HI100), CD45RO (UCHL1), CD25 (BC96), PD-1 (EH12.2H7), IL2 (MQ1-17H12), and IFNγ, (4S.B3) from Biolegend; p-S6 Ribosomal Protein S235/236 (D57.2.2E), p-4E-BP1 Thr37/46 (236B4), p-AKT S473 (D9E), and p-ERK 1/2 (197G2) from Cell Signaling Technologies. For intracellular cytokine staining, T cells were stimulated with MOLM-13-CD19 cells at a 1:1 ratio, and incubated for 24 and 48 hours. Brefeldin A (eBioscience) was added and cells were stained using a fixation and permeabilization buffer set (eBioscience) 4 hours later. For phosphoflow, unstimulated cells and cells treated with 50 ng/ml PMA and 1 μg/ml ionomycin for 10 minutes were fixed with 1% paraformaldehyde and permeabilized with cold 90% methanol prior to staining.

RNA-sequencing: Paired-end sequencing was performed using the HighSeq platform with 100bp read length. Reads were aligned to the human GRCh37-lite using SJCRH's Strongarm pipeline. [4, 5] Counts per gene were obtained using htseq-count version 0.6.1 [6] with Gencode vM5 [7] level 1 and 2 gene annotations. Counts were normalized with VOOM [8] and analyzed with LIMMA [9] within the R statistical environment. Significance was defined as having a false discovery rate (FDR) <0.05. VOOM normalized counts were analyzed with QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN).

Study approval: Animal experiments were performed in AAALAC-accredited, SPF facilities in the SJCRH Animal Resource Center following national, state and institutional guidelines. Protocols were approved by the SJCRH Animal Care and Use Committee. Human cells were acquired from anonymized platelet collections of consenting healthy donors and approved by the SJCRH Institutional Review Board.

Statistical analysis: Significance was determined using Graphpad Prism 6. For Kaplan-Meier survival analyses, significance was determined with the log-rank (Mantel-Cox) test. For quantification of tumor, CAR and control T cell numbers in single transfer experiments, significance was determined by one way ANOVA with Tukey's multiple comparison post-test. For co-transfer experiments assessing CAR and control T cell numbers, significance was determined by ratio paired t-test. For assessment of T_N, T_{CM}, T_{EM}, and T_{EFF} populations, significance was determined by two-way ANOVA with Tukey's multiple comparison post-test. In

43 experiments where the ratio of control to CAR-T cells was compared to an input ratio of 1:1,

44 significance was determined using a single-sample t-test with a theoretical mean of 1.

Supplementary Figure Legends

Supplementary Figure 1. The MOLM-13-CD19 cell line was incubated with CD33 CAR, CD19 CAR or vector-transduced control T cells at the indicated ratios for 24 or 48 hours. Tumor cells were quantified by flow cytometry and normalized to cultures without added T cells.

Supplementary Figure 2. (A) NSG mice were injected with MOLM-13-CD19 tumor cells and treated with CD33 CAR, CD19 CAR, or control T cells. Organs were harvested 18 days after transfer. Total numbers of $CD3^+CD8^+$ CAR or control T cells are shown. (B) CD33 CAR-T cells (GFP) and control T cells (RFP) were co-transferred in equal numbers into NSG mice in the presence or absence of tumor. Liver and spleen were harvested 5 days after transfer. Absolute number of $CD4^+$ CAR and control T cells in the presence or absence of tumor at day 5 is shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Supplementary Figure 3. (A) Histograms showing differentiation marker expression on $CD8^+$ CD33 CAR (solid line) or control (dashed line) T cells 6 or 12 days after activation. (B) Summary data of differentiation marker expression on $CD8^+$ CD33 CAR or control T cells 6 and 12 days after activation. (C) Representative dot plots showing gating strategy for $CD8^+$ T_{SCM} subset of CAR and control T cells. (D) Naïve $CD8^+CD45RA^+CD45RO^-CCR7^+CD95^-$ T cells were sorted from donor samples and transduced with CD33 CAR or control vector. Composition of T_N, T_{CM}, T_{EM}, and T_{EFF} subsets was determined 9 days after activation. (D) Representative dot plots and summary data for the composition of T_N, T_{CM}, T_{EM}, and T_{EFF} $CD8^+$ T cell subsets in CD19 CAR and control T cells 6 and 12 days after activation. (E) Activation and exhaustion marker expression on $CD8^+$ CD33 CAR

and control T cells 12 days after activation (F) Percent of T_{SCM} cells among $CD8^+$ CD19 CAR and control T cells 6 and 12 days after activation. (G) Percent of T_{SCM} cells 6 and 12 days after *ex vivo* activation. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Supplementary Figure 4. (A-B) CD33 CAR and control T cells were mixed 1:1 and transferred with or without MOLM-13-CD19 cells into NSG mice. (A) Differentiation marker expression levels on $CD4^+$ CAR-T cells in the liver and spleen 5 days after transfer. (B) Composition and (C) total numbers of $CD4^+$ T_N, T_{CM}, T_{EM} and T_{EFF} subsets in the liver and spleen 5 days after transfer. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Supplementary Figure 5. (A-B) Ingenuity Pathway Analysis (IPA) of genes differentially expressed (FDR<0.2) between $CD8^+$ CD33 CAR and control T cells generated from three unique healthy donors, evaluated 12 days after activation. (A) -log (p-value) of selected canonical pathways identified by IPA analysis as upregulated in $CD8^+$ CD33 CAR-T cells relative to control T cells. (B) Heat maps show genes uniquely dysregulated in $CD8^+$ CD33 CAR-T cells, segregated by pathway. (C) Representative histograms and summary data of phosphoflow staining of p-AKT and pERK in control and CD33 CAR-T cells 12 days after stimulation. CAR-T cells were stimulated with PMA/ionomycin for 10 minutes as a positive control. *** p<0.001, **** p<0.0001.

Supplementary Figure 6. Representative dot plots and summary data of CCR7 expression and proliferation as monitored by CellTrace Violet in $CD8^+$ CAR-T cells with or without inhibitor

treatment. Bar graph indicates percentage of proliferating $CD8^+$ cells that are $CCR7^+$. * $p<0.05$, **** $p<0.0001$.

Supplementary Figure 7. 5 days after activation, CD33 CAR-T cells were treated with PI3K inhibitor LY294002 (LY) as indicated for 4 days, followed by flow cytometric analysis. (A) Composition of $CD8^+$ T_N, T_{CM}, T_{EM} and T_{EFF} subsets. (B) Numbers of $CD8^+$ inhibitor treated and untreated CD33 CAR-T cells and control T cells, normalized to the number of untreated CAR-T cells. ** $p<0.01$.

Supplementary Figure 8. (A) MOLM-13-CD19 cells were incubated with CD33 CAR-T cells with or without LY294002 (LY) treatment, or control T cells at indicated ratios. Tumor cells were quantified and normalized to cultures without added T cells. (B) CD33 CAR-T cells with or without LY treatment, or control T cells were incubated 1:1 with MOLM-13-CD19 tumor cells for 48h prior to intracellular staining. (C) Mice were injected with MOLM-13-CD19 tumor cells and untreated or LY-treated CD33 CAR-T cells. Organs were harvested 14 days after transfer. Total cell numbers of $CD8^+$ T_N, T_{CM}, T_{EM} and T_{EFF} subsets are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Supplementary References

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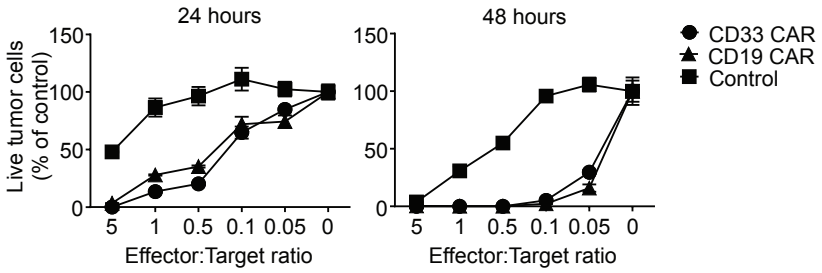
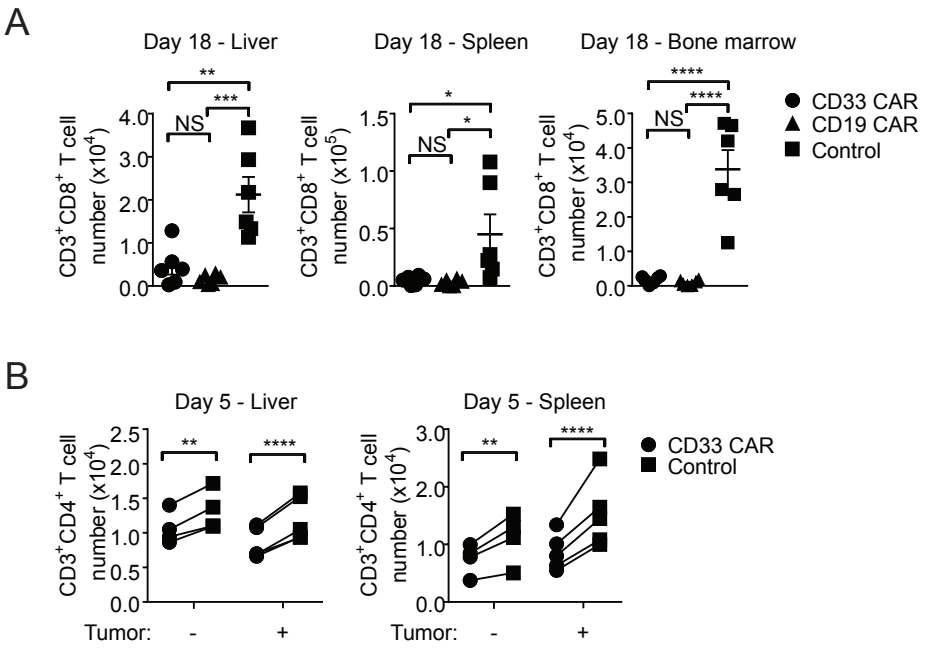
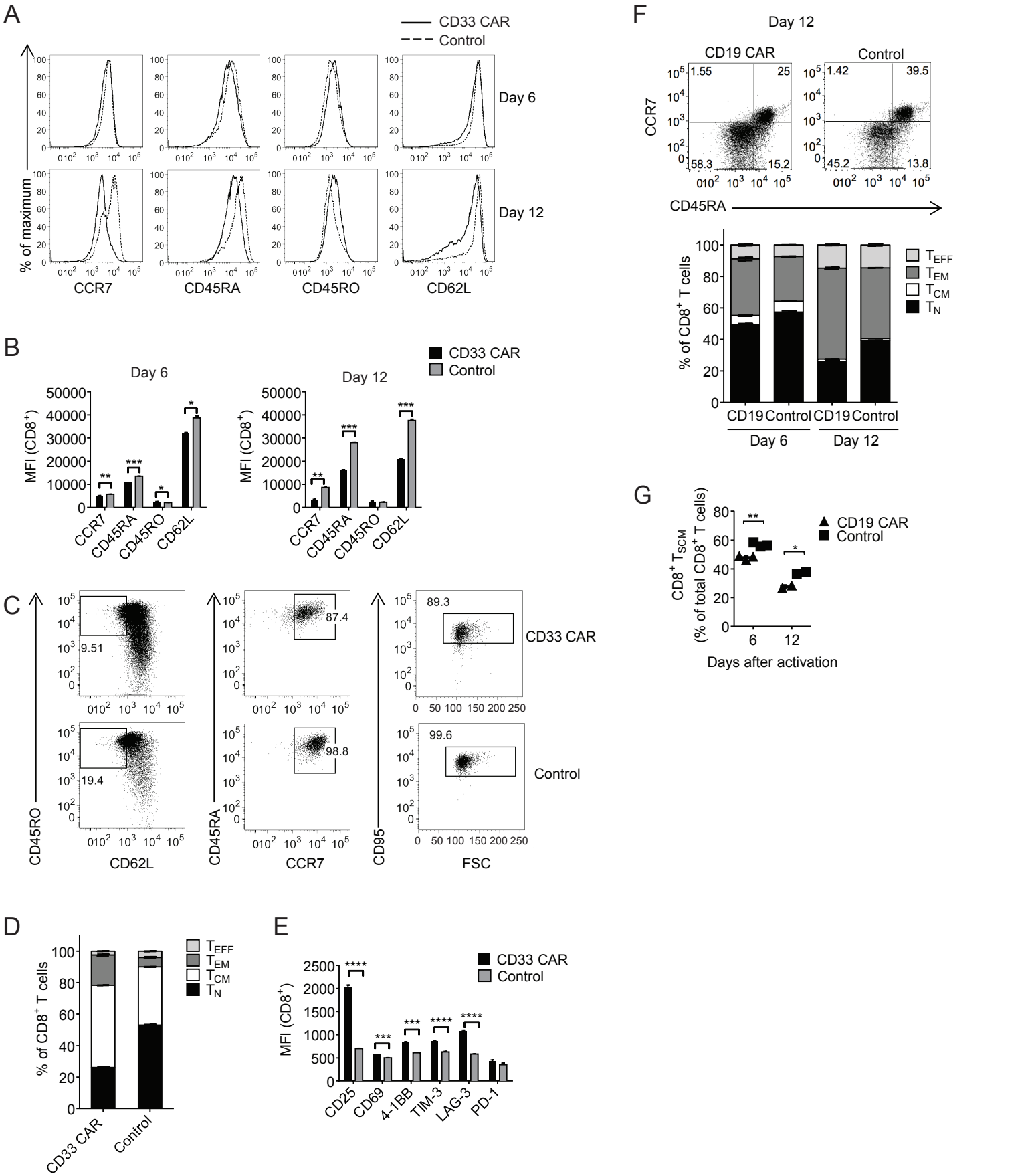
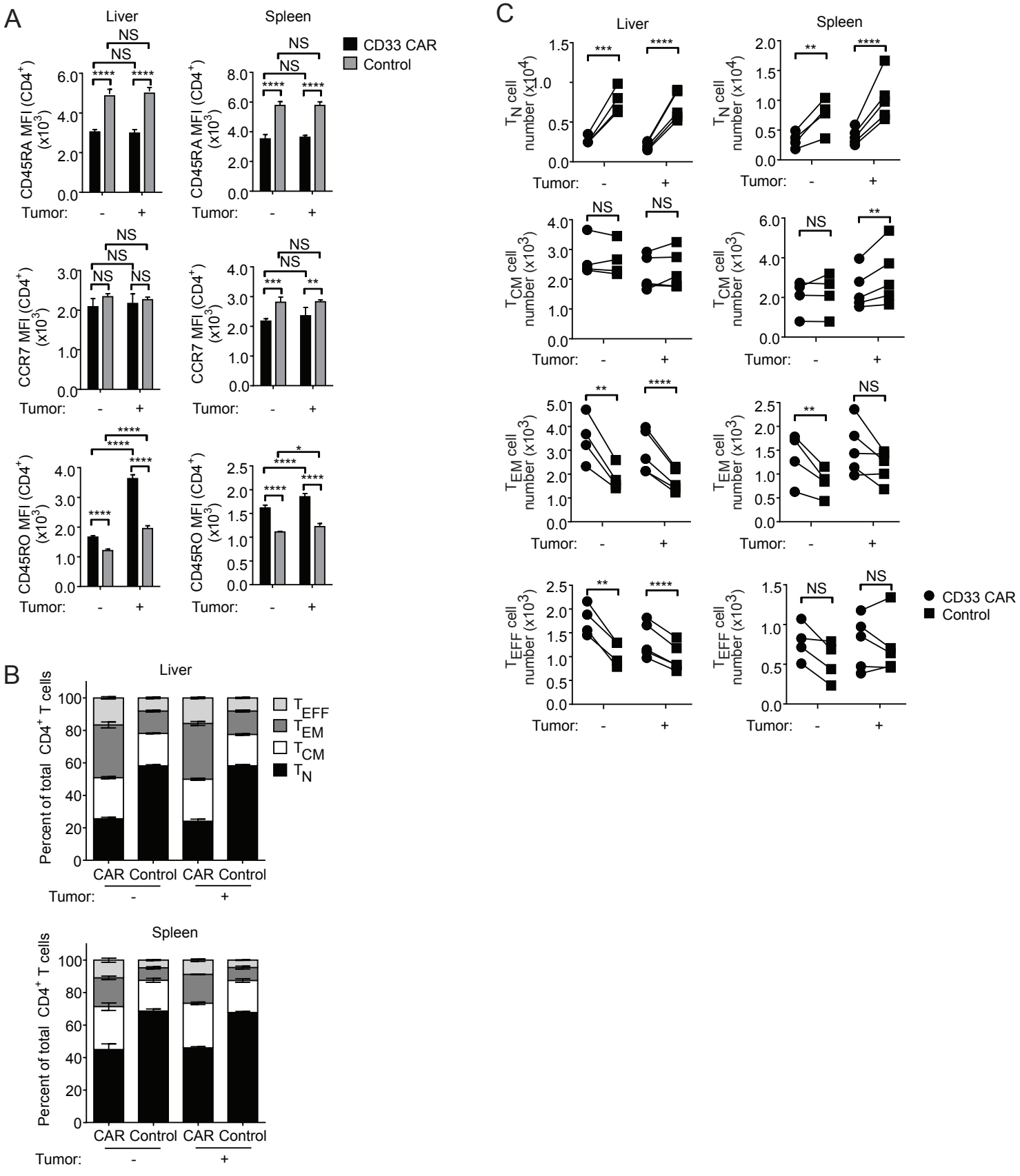
Supplementary Figure 1



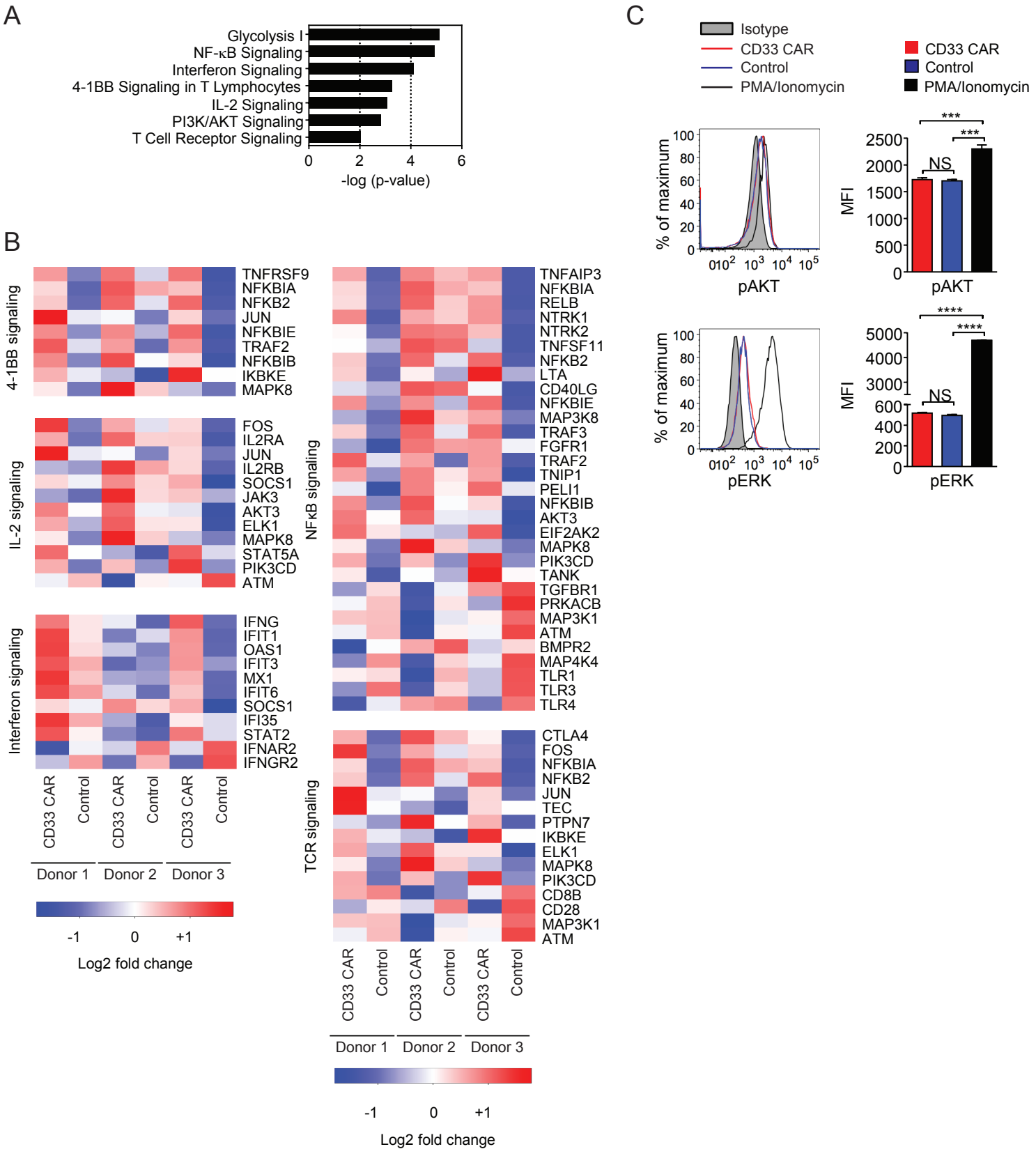
Supplementary Figure 2




A
CD33 CAR
Control
Day 6
Day 12
% of maximum
CCR7
CD45RA
CD45RO
CD62L
B
CD33 CAR
Control
Day 6
Day 12
MFI (CD8+)
CCR7
CD45RA
CD45RO
CD62L
C
CD45RO
CD62L
CD45RA
CCR7
CD95
FSC
CD33 CAR
Control
9.51
19.4
87.4
98.8
89.3
99.6
D
% of CD8+ T cells
TEFF
TEM
TCM
TN
CD33 CAR
Control
E
CD33 CAR
Control
MFI (CD8+)
CD25
CD69
4-1BB
TIM-3
LAG-3
PD-1
F
Day 12
CD19 CAR
Control
CCR7
CD45RA
1.55
25
58.3
15.2
1.42
39.5
45.2
13.8
% of CD8+ T cells
TEFF
TEM
TCM
TN
CD19
Control
Day 6
Day 12
G
CD8+ TSCM
(% of total CD8+ T cells)
CD19 CAR
Control
Days after activation

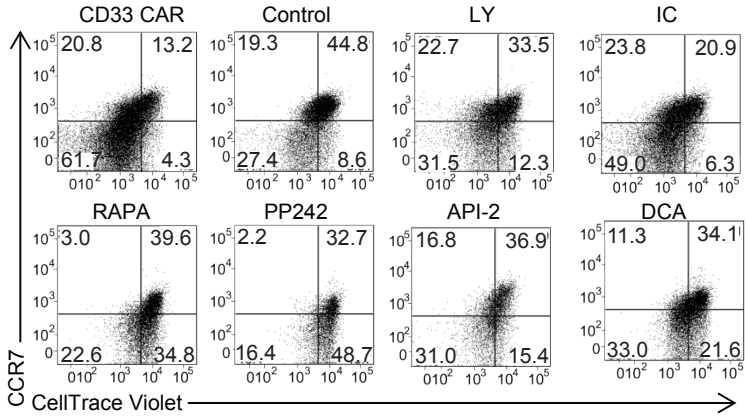

A
Liver
Spleen
CD33 CAR
Control
CD45RA MFI (CD4+) (x10^3)
CCR7 MFI (CD4+) (x10^3)
CD45RO MFI (CD4+) (x10^3)
Tumor: - +
NS
****
***
**
*
B
Liver
Spleen
Percent of total CD4+ T cells
T_EFF
T_EM
T_CM
T_N
CAR Control CAR Control
Tumor: - +
C
Liver
Spleen
T_N cell number (x10^4)
T_CM cell number (x10^3)
T_EM cell number (x10^3)
T_EFF cell number (x10^3)
Tumor: - +
CD33 CAR
Control

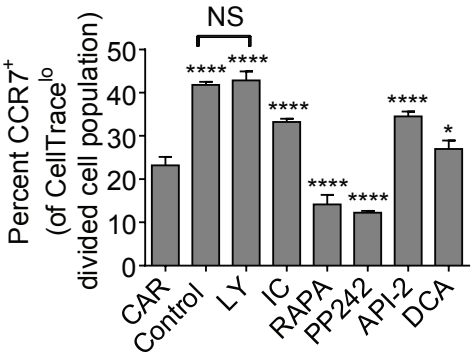
Supplementary Figure 5



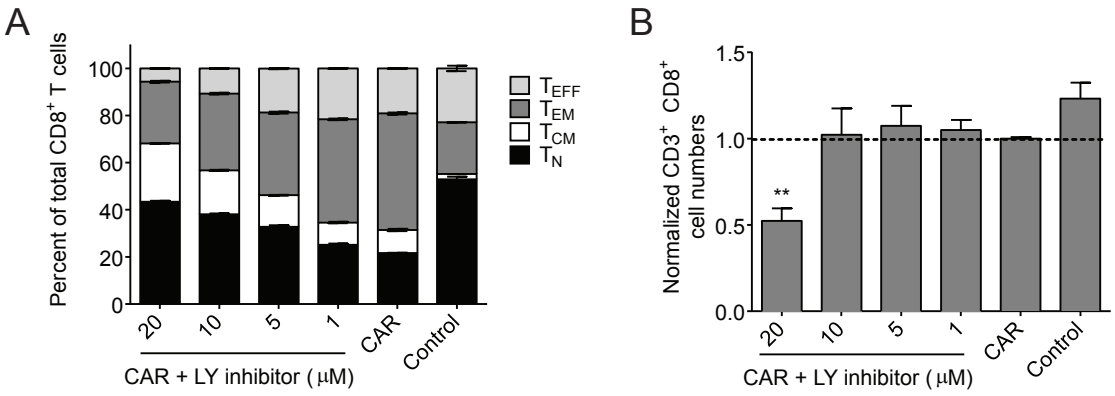
Supplementary Figure 6

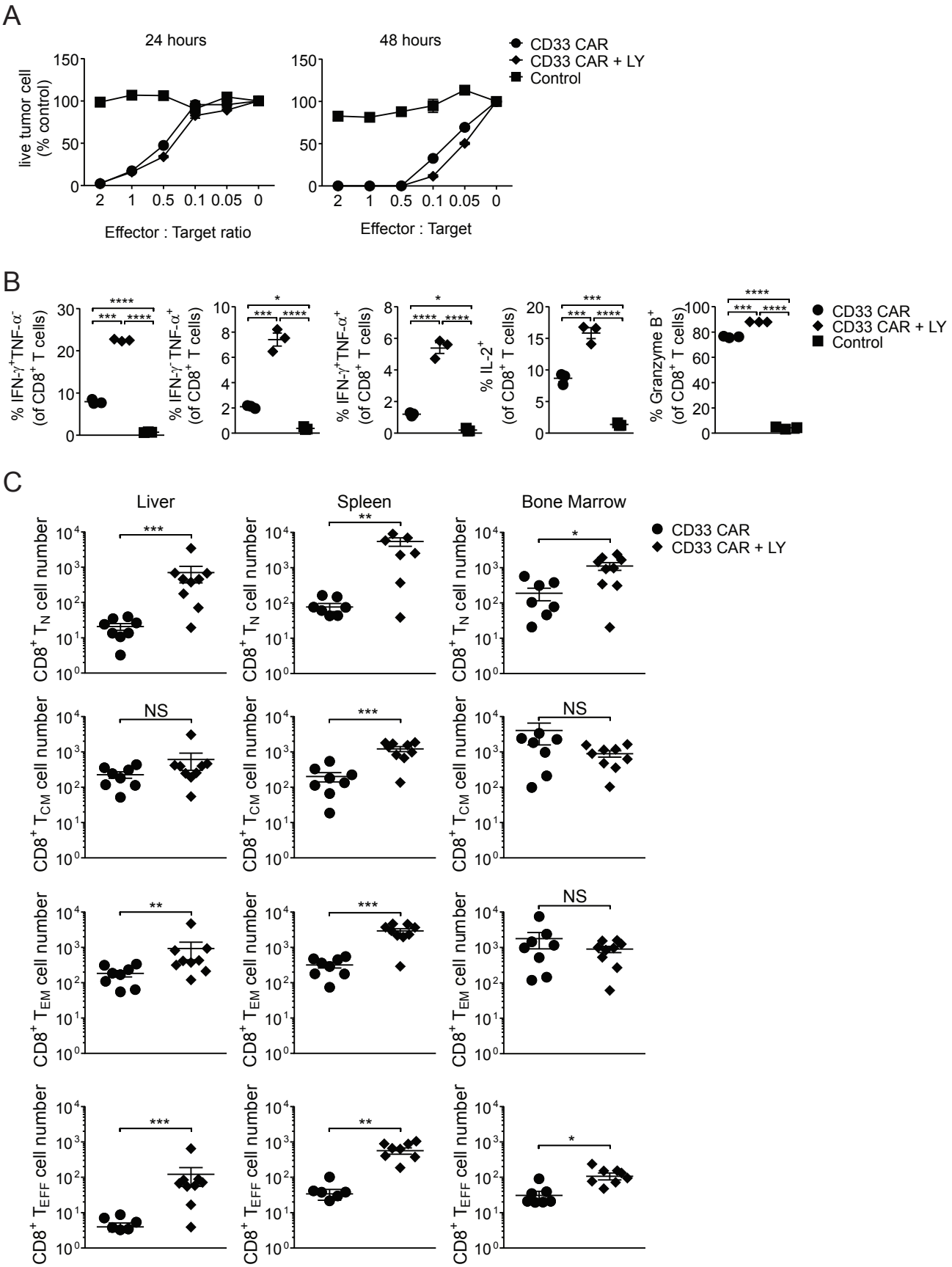
A





Supplementary Figure 7




A
24 hours
48 hours
CD33 CAR
CD33 CAR + LY
Control
live tumor cell (% control)
Effector : Target ratio
Effector : Target
B
% IFN-γ+TNF-α-
% IFN-γ-TNF-α+
% IFN-γ+TNF-α+
% IL-2+
% Granzyme B+
(of CD8+ T cells)
C
Liver
Spleen
Bone Marrow
CD8+ TN cell number
CD8+ TCM cell number
CD8+ TEM cell number
CD8+ TEFF cell number
NS