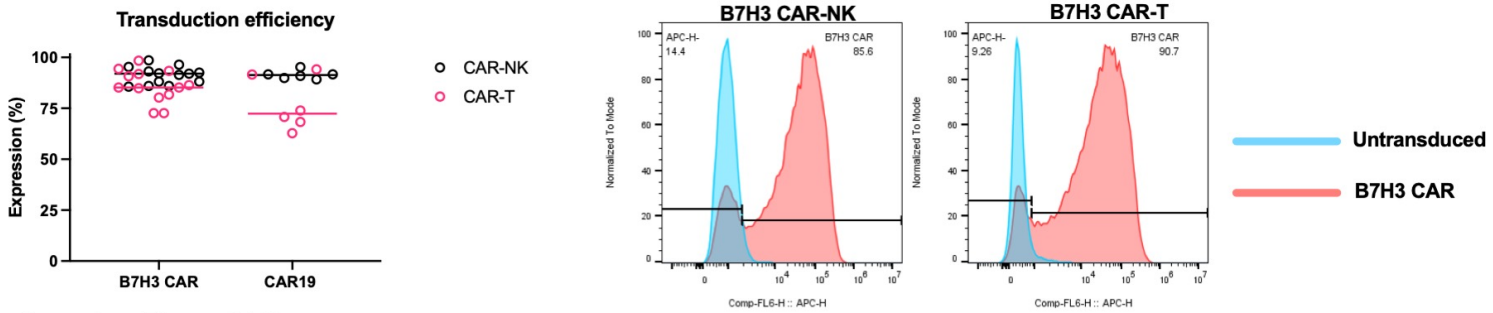
Supplementary Data

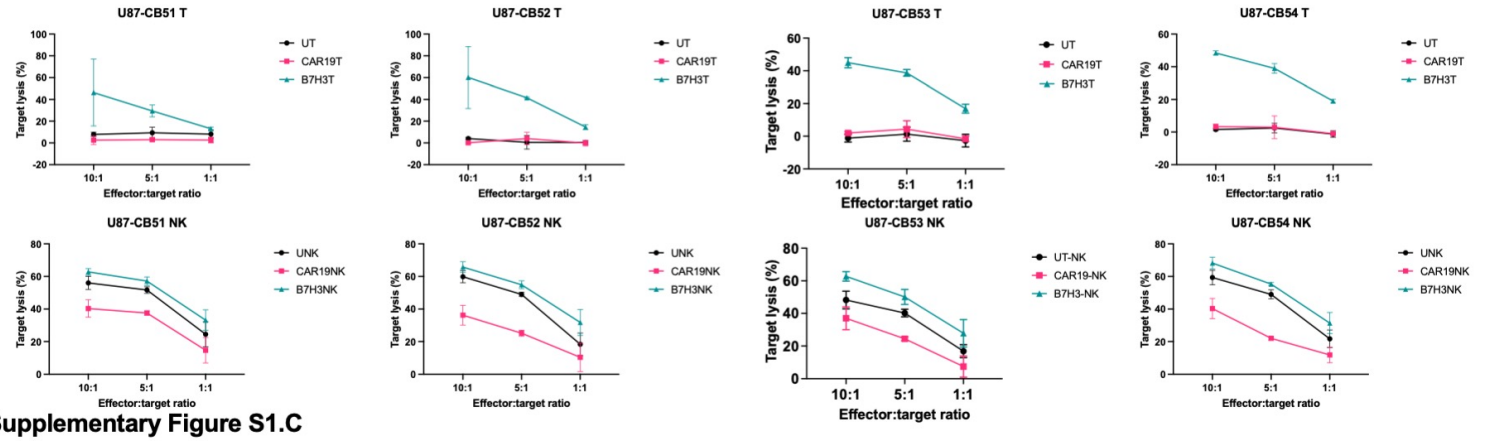
Functional Exhaustion Limits CAR-NK Therapy in Glioblastoma but Can Be Mitigated by Early Repeated Dosing

Kajal Chaudhry, Nethaji Muniraj, Haili Lang, Ashley Geiger, Gage Paul, Ehsan Dowlati, Danielle K. Sohai, Joshua L. Terao, Sridevi Yadavilli, Carlos E. Sanchez, Eric Yvon, Christopher Lazaraski, Eugene Hwang, Matthias Holdhoff, Barbara Savoldo, Conrad Russell Y. Cruz,* Catherine M. Bollard,*

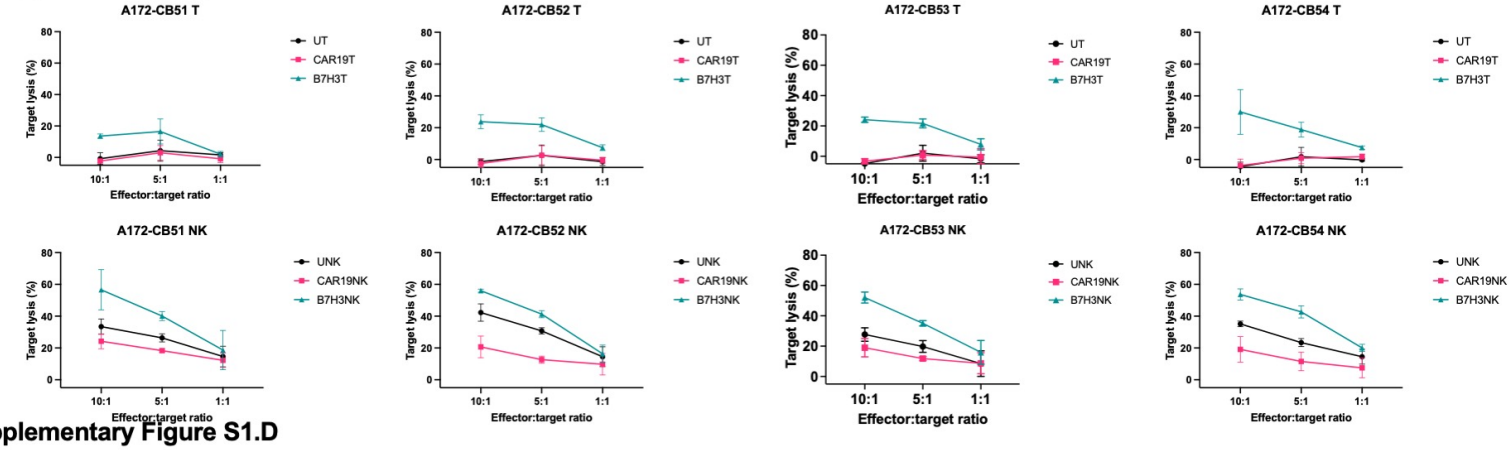
Supplementary Figure S1.A



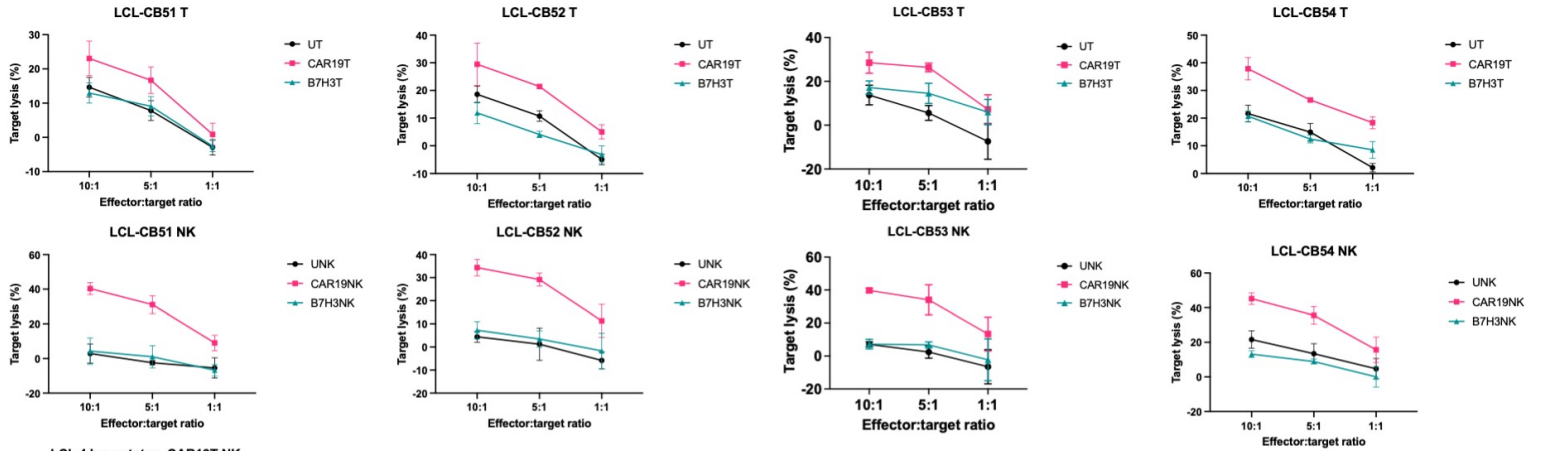
Supplementary Figure S1.B

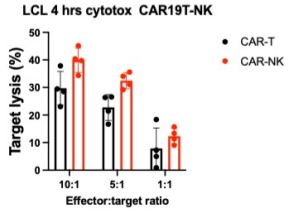


Supplementary Figure S1.C



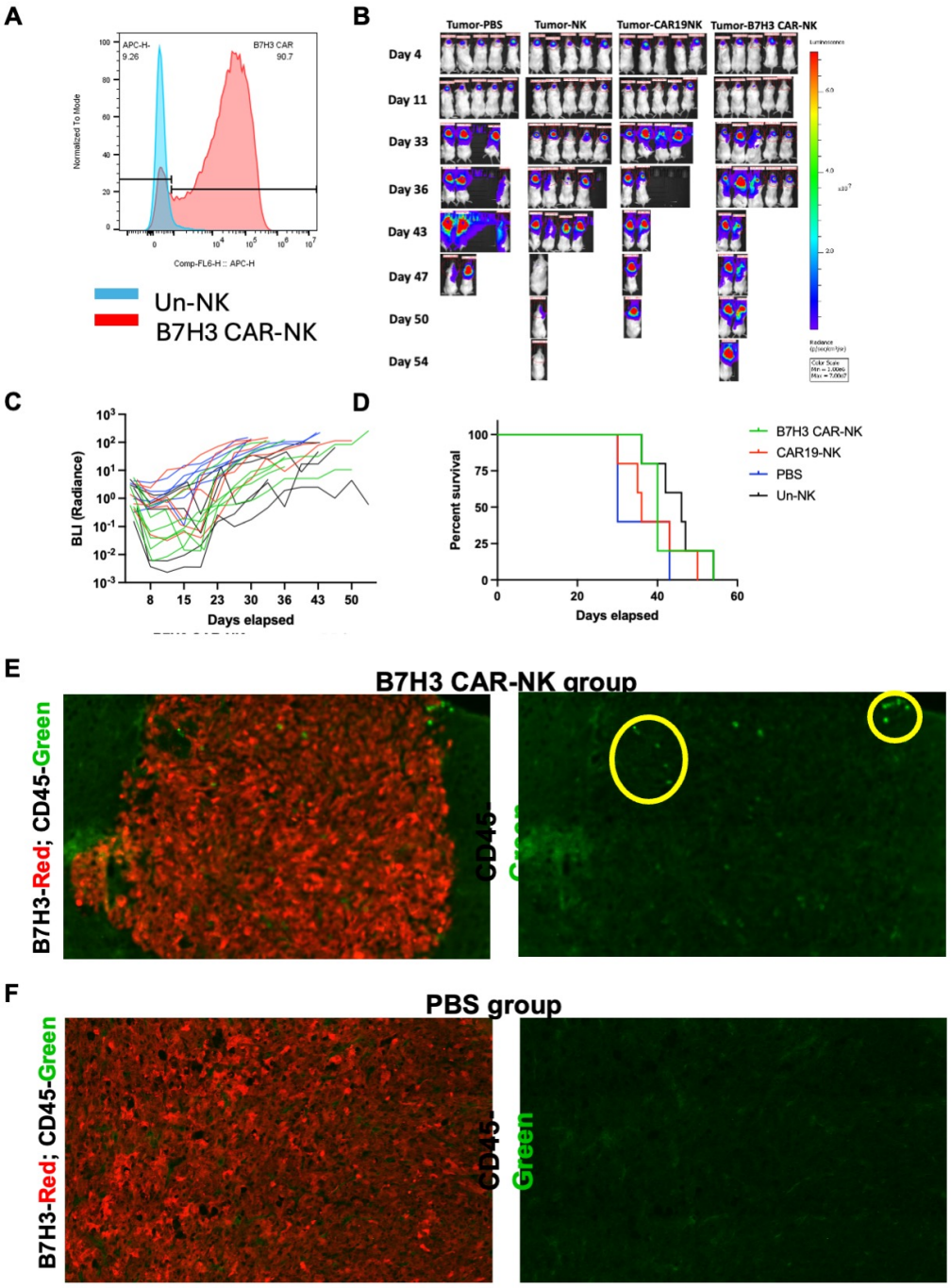
Supplementary Figure S1.D





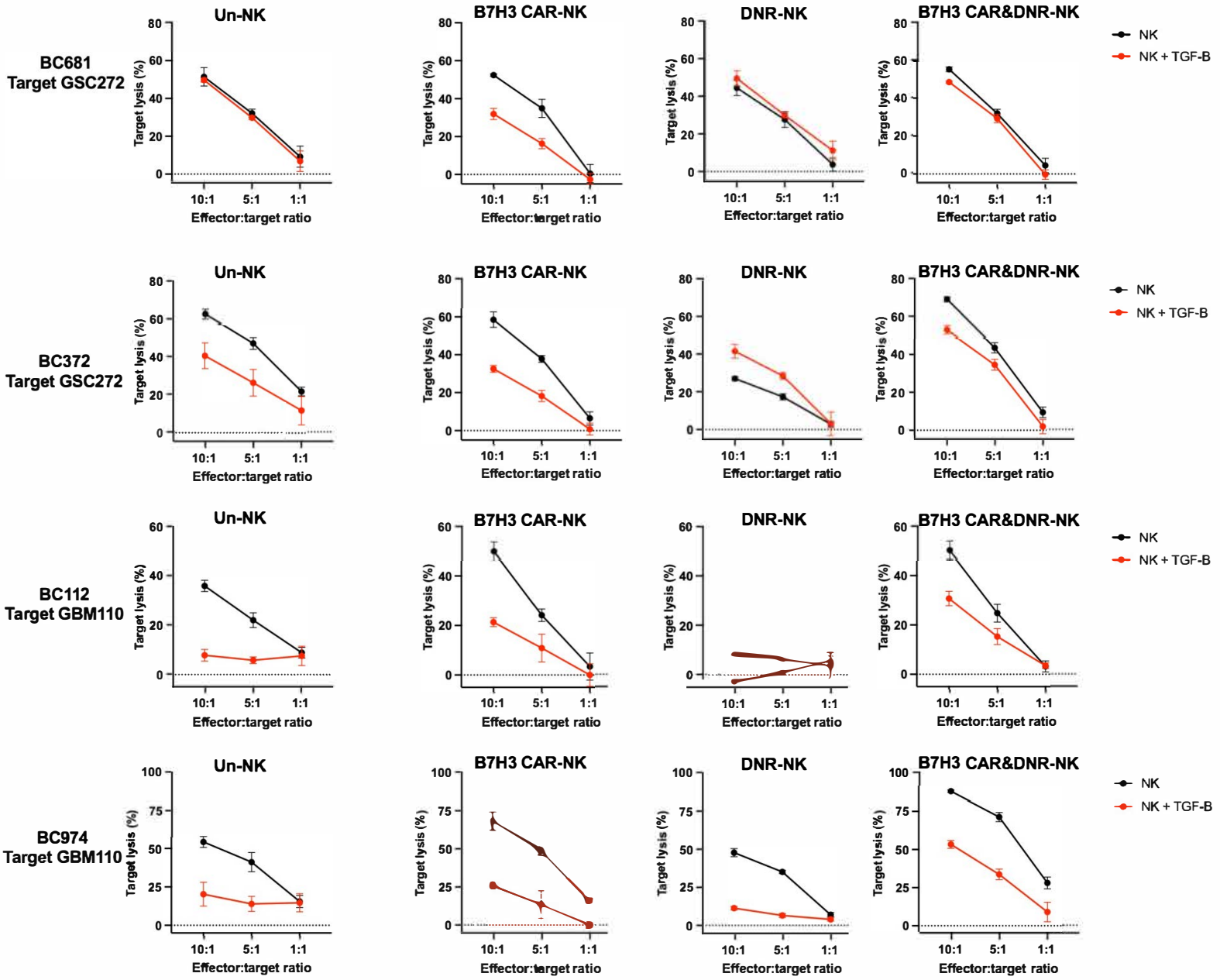
Supplementary Figure S1. CAR expression and short-term cytotoxicity of CAR-NK and CAR-T cells against specific and non-specific targets. The cytotoxic function of CAR-NK and CAR-T cells was evaluated using short-term killing assays against multiple tumor cell lines. U87 MG and A172 tumor lines served as targets for B7H3 CAR-expressing effectors, while Raji and LCL lines were used for CAR-19 effectors. The PHA blasts derived from the same donors were employed to evaluate alloreactivity of CAR-NK and CAR-T products. **(A)** CAR expression in NK and T cells was determined using flow cytometry. **(B)** Short-term (4-hour) cytotoxicity of CAR-NK and CAR-T cells against the U87 MG cell line (individual donors). **(C)** Short-term (4-hour) cytotoxicity of CAR-NK and CAR-T cells against the A172 cell line (individual donors). **(D)** Short-term (4-hour) cytotoxicity of CAR-NK and CAR-T cells against the LCL cell line (individual donors). **(E)** Short-term (4-hour) cytotoxicity of CAR-NK and CAR-T cells against the Raji cell line (individual donors). **(F)** Short-term (4-hour) cytotoxicity of CAR-NK and CAR-T cells against autologous PHA blast. **(G)** Copies of CAR transgene and CD45 gene, as detected using ddPCR, in brain and spleen samples treated with various B7H3 CAR-expressing and untransduced NK and T cells at the end point of the study. n = 5-6 individual mice per treatment group. A log2 scale was used to better delineate the data.

Supplementary Figure S2



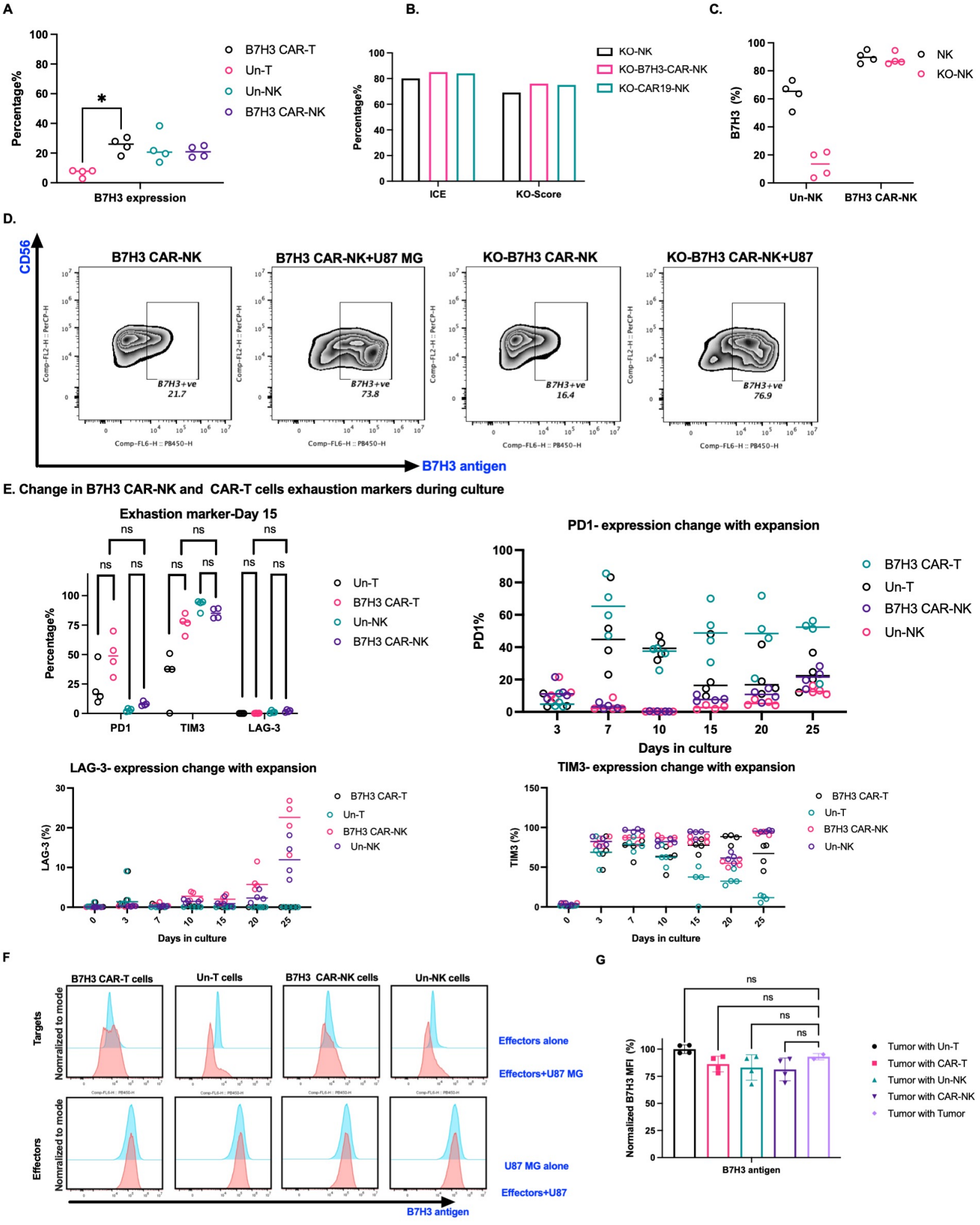
Supplementary Figure S2. NK cell persistence in vivo. NSG mice were intracranially injected with 5 × 10^4 tumor cells on day 1. NK cells: Un-NK, CAR19-NK, and B7H3 CAR-NK (2 × 10^6 cells per injection) or PBS were administered intracranially at the same stereotactic coordinates 5 days post tumor implantation. **(A)** Representative flow cytometry analysis of B7H3 CAR-expression on NK cells. **(B)** Tumor progression was monitored by bioluminescence imaging, and radiance was quantified using Living Image software (PerkinElmer). **(C)** Radiance values for individual mice are shown. **(D)** Kaplan-Meier survival analysis of tumor-engrafted NSG mice treated with CAR-NK cells or control NK cells. Survival differences were analyzed using the log-rank (Mantel-Cox) test. (n = 6 mice per group). **(E-F)** Brain tumor sections collected at the experimental endpoint were subjected to immunofluorescence staining using an anti-CD45 (FITC) antibody to detect human immune cells. Tumor regions were identified by mCherry fluorescence. Representative images show the localization of $CD45^+$ cells within $mCherry^+$ tumor areas.

Supplementary Figure S3



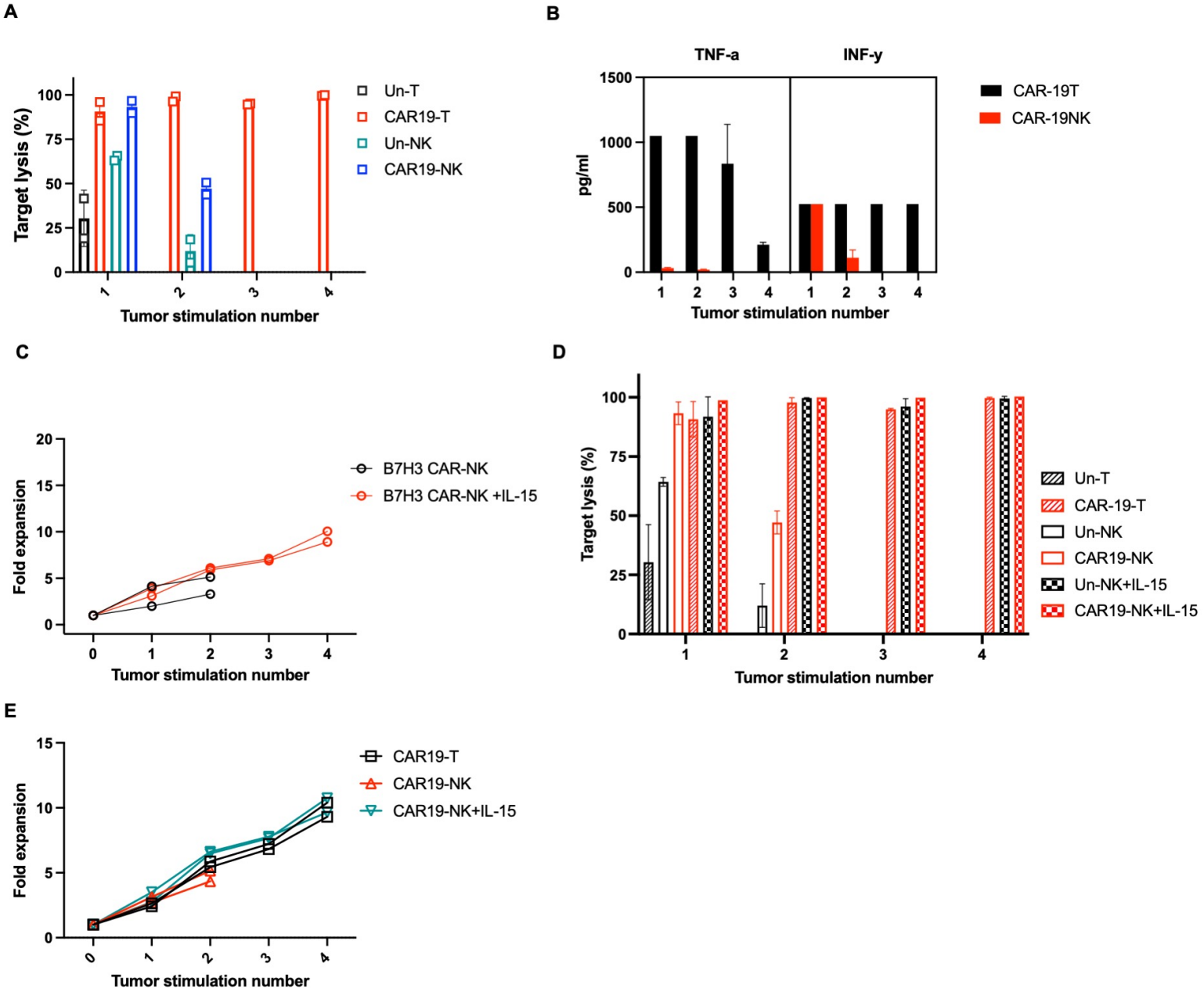
Supplementary Figure S3. DNR rescues CAR-NK cytolytic activity. Cytolytic activity of CAR-NK cells against U87 MG targets was evaluated following exposure to exogenous TGF-β. Different NK cell products (Un-NK, B7H3 CAR-NK, DNR-NK, and B7H3 CAR&DNR-N) from donors were cultured with or without exogenous TGF-β (5 ng/mL) for 5 days prior to assessing cytotoxic function in short-term killing assays against B7H3⁺ tumor cell lines (GSC272 and GBM110). Data are presented as mean ± SD.

Supplemental Figure S4.



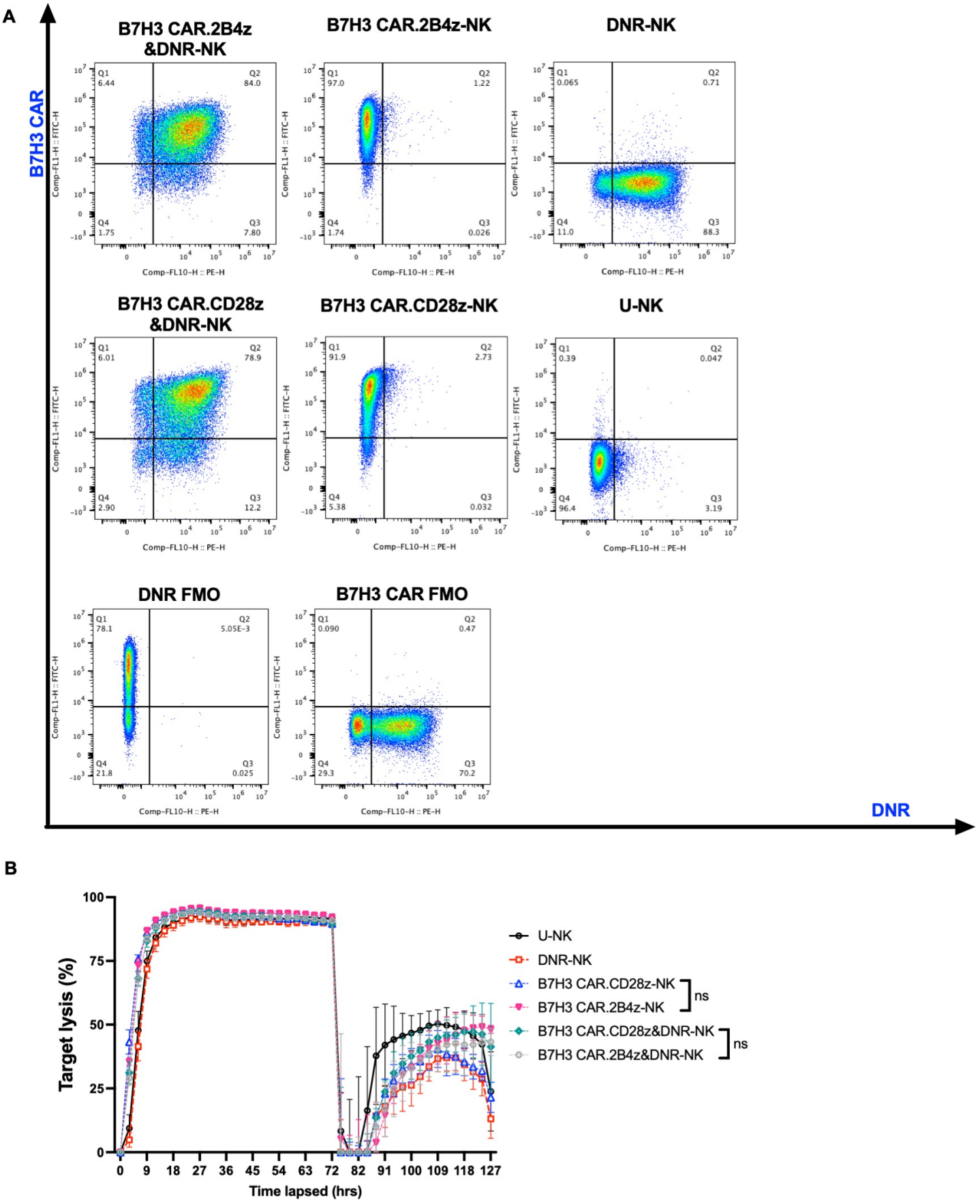
Supplemental Figure S4. (A) B7H3 antigen level in transduced and untransduced NK and T cells. **(B)** Representative data of the ICE analysis of the B7H3 antigen knockout. **(C)** B7H3 antigen levels in B7H3-KO NK cells. **(D)** B7H3 antigen trogocytosis in CAR-NK cells. **(E)** Changes in exhaustion markers in B7H3 CAR-NK and CAR-T cells during culturing. (F) B7H3 antigen expression (MFI) on targets and effectors after coculturing. **(G)** Normalized B7H3 MFI on targets after coculturing.

Supplementary Figure 5



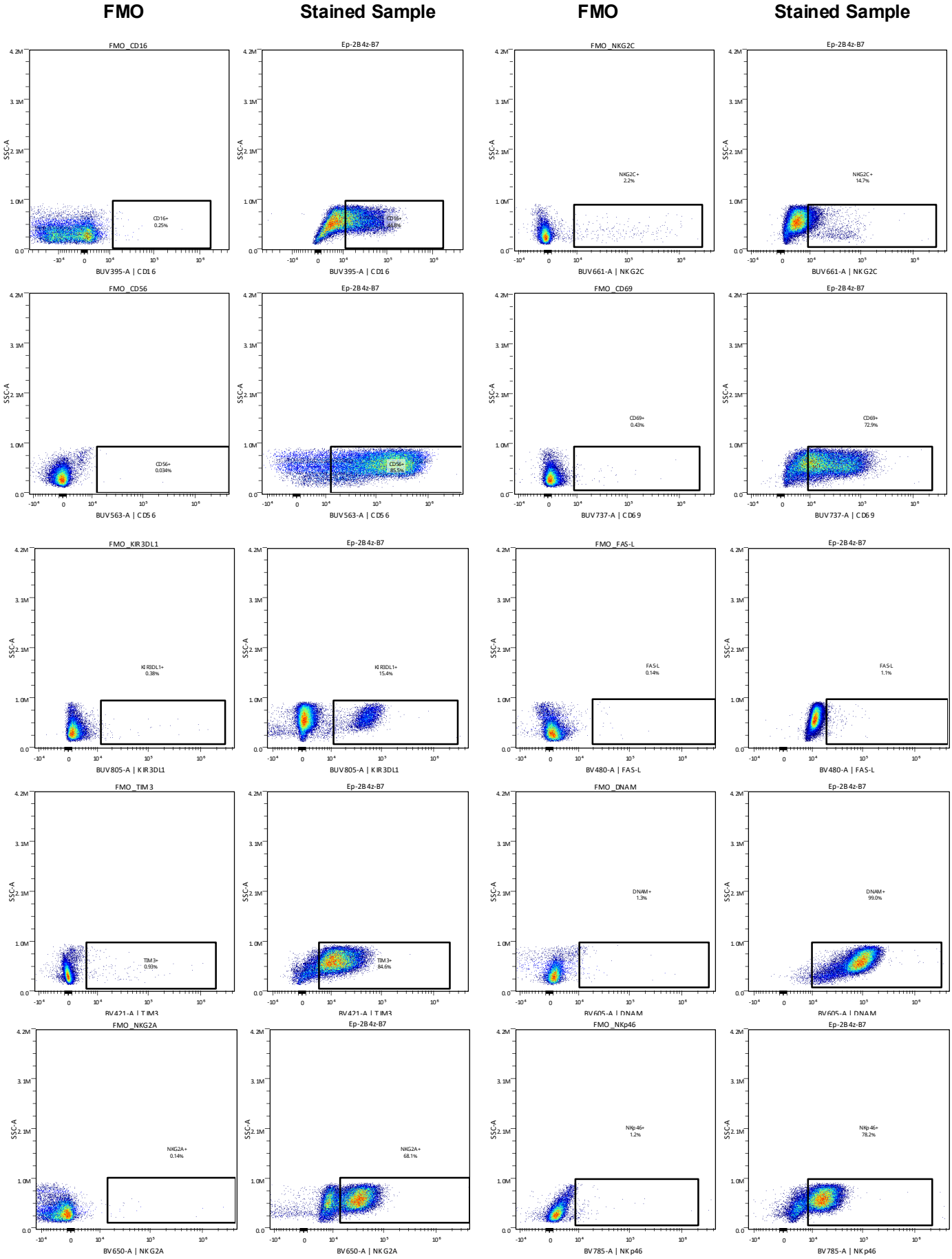
Supplementary Figure S5. IL-15 prevents NK cell exhaustion and revitalizes serial killing. (A) Raji cell lysis by CAR19 transduced NK and T cells. Data are presented as mean ± SD. (n= 2 donors). **(B)** TNF-α and IFN-γ secretion by CAR19 transduced NK and T cells after tumor rechallenges. Data are presented as mean ± SD. (n=2 donors). **(C)** CAR19-NK cell and CAR19-T cell proliferation upon repeated tumor challenges (n=2 donors individually plotted). **(D)** Raji cell lysis by CAR19-NK with and without exogenous IL-15. CAR19-T cells were used as a control. Data are presented as mean ± SD. (n=2 donors). (E) CAR19-NK cell proliferation upon repeated tumor challenges in the presence of exogenous IL-15. CAR-19-T cells were used as a control. (n=2 donors individually plotted).

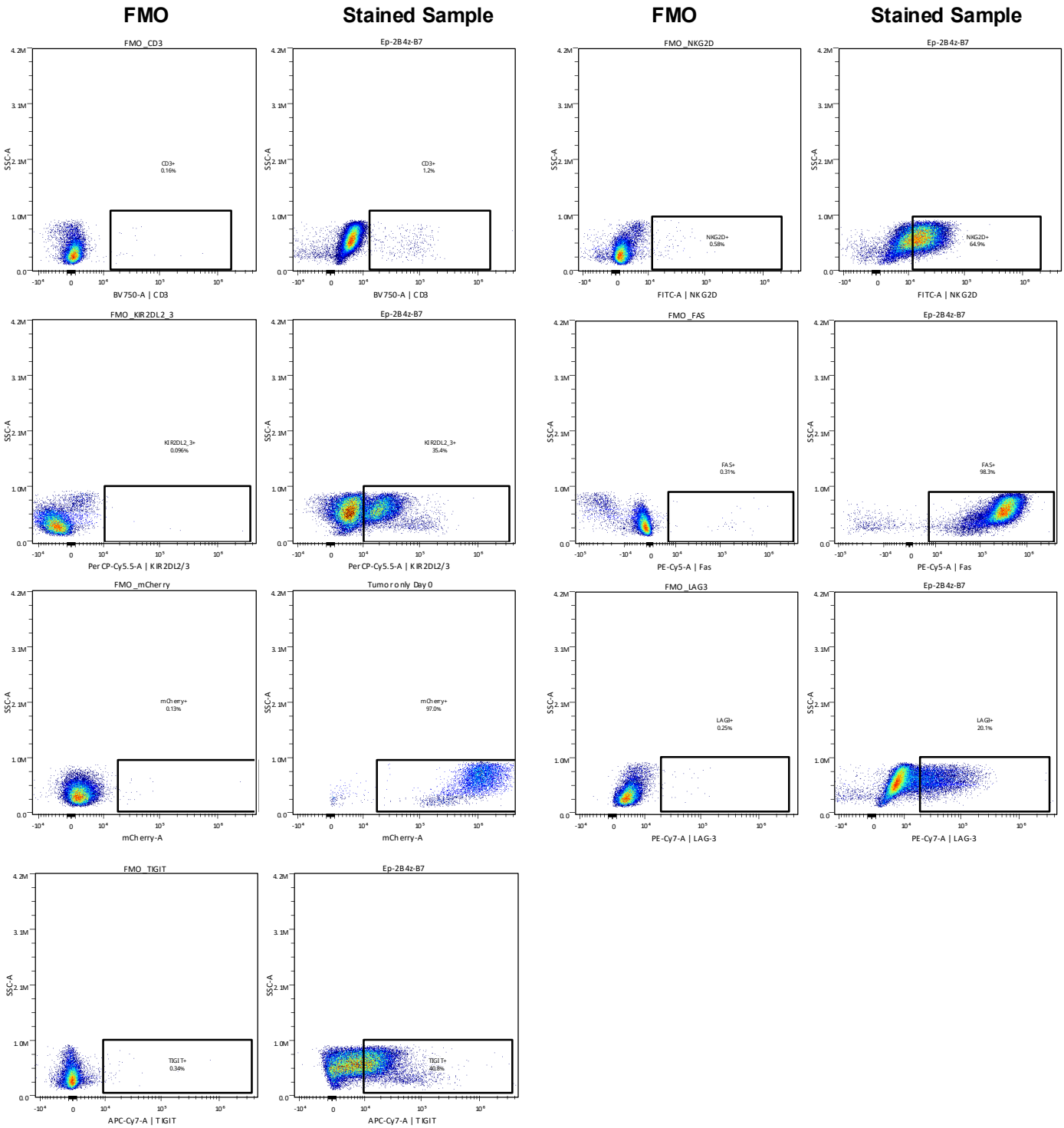
Supplementary Figure 6.



Supplementary Figure S6. 2B4 cell-specific costimulatory domain failed to increase NK's weakening serial killing activity. (A) Representative flow cytometric data showing B7H3/2B4z CAR expression in NK cells after transduction. (n = 2 donors). (B) B7H3/CD28z, B7H3/2B4z CAR-transduced NK cells and control NK cells were cocultured with U87 MG tumor cells at a 2:1 ratio, with restimulation every 3-5 days against fresh tumor cells until they no longer killed and/or expanded NK cells (n=2 donors). xCelligence-based cytotoxicity assay. Data were analyzed by the Kruskal-Wallis test and presented as mean ± SD.

Supplementary Figure 7





Supplementary Figure S7. FMO gating controls for the spectral NK-cell panel. Representative flow cytometry plots (FSC-H vs. indicated fluorophore) pairing each FMO control (left) with the stained sample (right). Low FMO backgrounds (<3%) confirm marker-specific staining and accurate unmixing.