

IMMUNOBIOLOGY AND IMMUNOTHERAPY

Integration of ζ -deficient CARs into the $CD3\zeta$ gene conveys potent cytotoxicity in T and NK cells

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KEY POINTS

- Integration of ζ -deficient CARs into the $CD3\zeta$ gene allows generation of functional TCR-ablated CAR-T cells for allogeneic off-the-shelf use.
- $CD3\zeta$ -editing platform allows CAR redirection of NK cells without affecting their canonical functions.

Chimeric antigen receptor (CAR)-redirected immune cells hold significant therapeutic potential for oncology, autoimmune diseases, transplant medicine, and infections. All approved CART therapies rely on personalized manufacturing using undirected viral gene transfer, which results in nonphysiological regulation of CAR-signaling and limits their accessibility due to logistical challenges, high costs and biosafety requirements. Random gene transfer modalities pose a risk of malignant transformation by insertional mutagenesis. Here, we propose a novel approach utilizing CRISPR-Cas gene editing to redirect T cells and natural killer (NK) cells with CARs. By transferring shorter, truncated CAR-transgenes lacking a main activation domain into the human $CD3\zeta$ ($CD247$) gene, functional CAR fusion-genes are generated that exploit the endogenous $CD3\zeta$ gene as the CAR's activation domain. Repurposing this T/NK-cell lineage gene facilitated physiological regulation of CAR expression and redirection of various immune cell types, including conventional T cells, $TCR\gamma/\delta$ T cells, regulatory T cells, and NK cells. In T cells, $CD3\zeta$

in-frame fusion eliminated TCR surface expression, reducing the risk of graft-versus-host disease in allogeneic off-the-shelf settings. $CD3\zeta$ - $CD19$ -CAR-T cells exhibited comparable leukemia control to $TCR\alpha$ chain constant ($TRAC$)-replaced and lentivirus-transduced CAR-T cells in vivo. Tuning of $CD3\zeta$ -CAR-expression levels significantly improved the in vivo efficacy. Notably, $CD3\zeta$ gene editing enabled redirection of NK cells without impairing their canonical functions. Thus, $CD3\zeta$ gene editing is a promising platform for the development of allogeneic off-the-shelf cell therapies using redirected killer lymphocytes.

Introduction

The adoptive transfer of immune cells is a powerful tool to combat chronic diseases, such as cancer. Guiding lymphocytes to specifically bind and respond to antigens can be used to redirect the antitumor efficacy of cytotoxic T cells¹ and natural

killer (NK) cells² as well as promote tissue-specific immunosuppression through regulatory T cells (T_{reg}).^{3,4} To overcome the limitations associated with low frequencies of certain antigen-specific T cells in patients, gene transfer of chimeric antigen receptors (CAR) can be used to install the desired antigen-specificity to large numbers of cells needed for

adoptive cell transfer and treatment success in severe disease. Autologous CAR-T cells are an approved treatment for B-cell malignancies, such as acute B-lymphoblastic leukemia,^{1,5} B-cell lymphoma^{6,7} and multiple myeloma.⁸

The TCR/CD3-complex is the endogenous antigen-receptor in T cells. It consists of a TCR α and a corresponding TCR β chain which engage antigenic peptides presented by MHC molecules, as well as the accessory proteins CD3 γ , CD3 δ , CD3 ϵ and CD3 ζ which transduce the TCR signal downstream. While all CD3 proteins are required for TCR/CD3 assembly, biosynthesis of CD3 ζ is the rate-limiting step in TCR/CD3 complex formation.⁹ Further, the intracellular domain of CD3 ζ is sufficient to drive TCR-like activation in chimeric receptors.^{10,11} Therefore, all clinically approved (second-generation) CARs use the intracellular domain of CD3 ζ as their primary TCR-activation-like effector domain. CARs further comprise an extracellular antigen-binding domain, a hinge domain, a transmembrane domain and an additional intracellular costimulatory domain, such as CD28 or 4-1BB. CARs without a main activation domain do not induce cytotoxicity, but have been proposed to boost T-cell function by providing costimulation.¹²

Most clinical CAR-T-cell products are generated by transduction with viral vectors which randomly integrate their cargo into the genome and drive CAR expression through strong promoters, such as EF1 α .^{5-8,13-16} Positional effects and epigenetic silencing of transgenic expression cassettes have been linked to inconsistent CAR expression levels.^{17,18} While previous trials with virally transduced T cells were safe in most patients,¹⁹ gene transfer with (semi)random integration poses the risk of malignant transformation as highlighted by cases of clonal expansion after disruption of tumor suppressor genes *TET2*²⁰ or *CBL21* by CAR provirus as well as by the development of CAR⁺ T-cell lymphoma after treatment with products generated via Piggy-Bac transposase technology^{22,23} and lentiviral (LV) vectors.²⁴

Targeted gene transfer using gene editing can improve the consistency of redirected T-cell products by predictable antigen receptor expression.^{17,25,26} To this end, a programmable nuclease, such as CRISPR-Cas, is introduced into the T cells alongside a DNA repair template to exploit homology-directed DNA repair (HDR) for site-specific integration of the CAR-transgene. Multiple genomic sites have been proposed to redirect T cells with CARs, including the protein-coding genes *TCR α chain constant (TRAC)*,^{17,27-29} *PDCD1* (encoding PD-1)^{28,30} and *GAPDH*³¹ as well as genomic safe harbor (GSH) loci, such as the (intragenic) human AAV-integration site (*hAAVS1*)³⁰ or the extragenic GSH 6 (*eGSH6*) locus.¹⁸ TRAC has emerged as the gold-standard for gene-edited CAR-T cells. One reason is the improved cell functionality associated with the temporary downregulation of the CAR after target engagement.¹⁷ This mirrors the natural regulation of the human TCR and protects from overt differentiation and T-cell exhaustion.¹⁷ An additional advantage is that the integration of CAR-transgenes into TRAC disrupts the TCR/CD3-complex. This creates CAR⁺ TCR⁻ T cells which lack TCR-mediated alloreactivity, thereby offering a route towards safer application of CAR-T cells in allogeneic settings.³²

In this study, we demonstrate virus-free CAR redirection via in-frame integration of truncated, CD3 ζ -deficient CAR-transgenes (*truncCARs*) into an early exon of the CD3 ζ -gene. Our knock-in

strategy produces fusion genes composed of the exogenous *truncCAR*-transgene (encoding an antigen binder, a hinge, a transmembrane as well as a costimulatory domain but no main activation domain) and the endogenous CD3 ζ -gene. This reduces the required transgene size and exploits the CD3 ζ promoter for physiological CAR-regulation. CD3 ζ -gene editing can also be used for redirection of regulatory T cells, TCR γ/δ T cells and most notably primary human NK cells which cannot be redirected by TRAC-targeting.

Material and methods

Cell culture

The study was performed in accordance with the declaration of Helsinki (Charité ethics committee approval EA4/091/19). Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors via density gradient centrifugation from peripheral blood. T cells were enriched by magnetic cell separation (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) using CD3-microbeads and cultured in T-cell medium, a 1:1 mixture of RPMI (Gibco, Thermo Fisher Scientific, Waltham, MA) and Click's (Fujifilm Irvine Scientific, Santa Ana, CA) media supplemented with 10% fetal calf serum, interleukin-7 (IL-7) (10 ng/mL, Sartorius CellGenix, Freiburg, Germany) and IL-15 (5 ng/mL, Sartorius CellGenix). NK cells were enriched from the CD3-negative fraction using an NK isolation kit (Miltenyi) and cultured in NK MACS Medium (Miltenyi) supplemented with 10% fetal calf serum, IL-2 (500 IU/mL) and IL-15 (5 ng/mL).

Genetic engineering

Targeted virus-free CAR-integration was performed as recently described.^{33,34} In short, human T or NK cells were transfected with precomplexed CRISPR-Cas9 ribonucleoproteins and double-stranded DNA (dsDNA) (DNA/sgRNA sequences; supplemental Table 1, available on the *Blood* website). The dsDNA served as template for HDR and consisted of the (CAR/*truncCAR*) transgene flanked by 400 bp homology arms. Cells were resuspended in 20 μ L P3 Electroporation Buffer (Lonza, Cologne, Germany) and electroporated with 1 μ g of HDR-template and 1.38 μ L of ribonucleoproteins consisting of synthetic modified single guide RNA (sgRNA, 100 μ M, Integrated DNA Technologies [IDT], Coralville, IA), 15-50 kDa poly(L-glutamic acid)³⁵ (100 μ g/ μ L, Sigma-Aldrich, St Louis, MO) and recombinant SpCas9 protein (61 μ M, IDT) in a 0.96:1:0.8 volume ratio using the 4D-Nucleofector (Lonza). T cells activated for 48 hours on α CD3/CD28-coated tissue culture plates were electroporated at a density of 5×10^4 cells/ μ L of buffer using program EH-115. Primary human NK cells were expanded in NK medium using NK activation/expansion beads (Miltenyi) for 6-7 days and electroporated using program DA-100. The NK-92 cell line was electroporated at 2.5×10^4 cells/ μ L with the program CA-137. 10 minutes postelectroporation, T cells were transferred into medium supplemented with 0.5 μ M HDR-Enhancer v2 (IDT). For LV controls, activated T cells were transduced 1 day post-T-cell isolation while being kept on α CD3/CD28 coated tissue culture well plates for another day. After editing, cells were expanded in G-Rex 6-well plates (Wilson Wolf, St. Paul, MN).

Off-target analysis with CAST-Seq

The assay was performed using genomic DNA isolated from T cells 12 days after nucleofection as previously described.^{36,37}

Flow cytometry

Assessment of CAR⁺ rate, cytotoxicity, intracellular cytokine production, exhaustion, phenotype and CAR-regulation was performed on a Cytoflex LX device (Beckman Coulter) using the panels stated in supplemental Table 2 and as previously described.³³ Activation-induced cell death of HER2-CAR-T cells was assessed after stimulation with plate-bound anti-Fc antibody (10 µg/mL, Jackson ImmunoResearch, West Grove, PA) via staining for annexin V Alexa Fluor 647 (Biolegend, San Diego, CA) and 7AAD (Biolegend). NK-cell degranulation was assessed after 4 hours of coculture with target cells in the presence of monensin A (1 µM, Golgistop, Becton Dickinson, Franklin Lakes, NJ) and BV785-conjugated anti-CD107a antibody (Biolegend). NK-cell-mediated antibody-dependent cellular cytotoxicity (ADCC) was assessed after 16 hours of coculture with CD20⁺ bGal⁻ Jeko-1 cells in the presence of anti-CD20 or anti-bGal antibody (Invivogen, San Diego, CA).

Live cell imaging

In vitro tumor control of HER2-CAR-T cells was assessed via live cell imaging of GFP-expressing cancer cells on an Incucyte device (Sartorius).

Animal experiments

In brief, immunodeficient mice were infused with 0.5×10^6 Nalm-6 cells (expressing *luciferase*) via tail vein injection. Four days later, 0.5×10^6 or 1×10^6 TCR-deficient CD19-CAR-T cells were infused intravenously. CAR-T cells were generated either via targeted integration of a CAR or a *truncCAR* into the *TRAC* or *CD3ζ*-gene, respectively, or by LV gene transfer and consecutive *TRAC*-knockout (KO). Tumor burden was assessed as previously reported³⁸ using bioluminescence imaging. The staff carrying out the mice experiments were blinded for the T-cell conditions. Mice were sacrificed according to study protocol either at ethical end points (models 1 + 3) or 5 weeks after tumor inoculation (model 2) according to the respective study protocols. For more detailed study protocols refer to supplementary Methods.

Data analysis, statistics and presentation

Flow cytometry data was analysed with FlowJo Software (BD). Prism 9 (GraphPad) was used to create graphs and perform statistics. Illustrations were created on BioRender.com.

The study with material from human participants was performed in accordance with the declaration of Helsinki (Charité ethics committee approval EA4/091/19). The in vivo CAR-T-cell potency studies were performed in accordance with the German animal welfare act and the EU-directive 2010/63. Animal studies 1 and 3 were approved by local authorities (Landesamt für Gesundheit und Soziales, Berlin, Germany) under the permission A0010/19. Model 2 was approved by the Lower Saxony Office for Consumer Protection and Food Safety-LAVES (permit number 16/2222).

Results

Integration of truncated CD3ζ-deficient (*trunc*) CARs in CD3ζ enables redirection of T cells

We performed targeted delivery of a 1419-bp-sized CD19-specific *truncCAR* (CD19-IgG1-CD28) into *CD3ζ* (exon 2,

beginning of intracellular domain) and *TRAC* (exon 1) using CRISPR-Cas9 (Figure 1A). As additional control, we integrated a full-length 2015-bp-sized CAR (CD19-IgG1-CD28-CD3ζ) into *TRAC* as recently described.³³ Transgene expression in primary human T cells was confirmed by flow cytometry (Figure 1B). Like *TRAC*-editing, CAR-integration into the *CD3ζ*-gene disrupted TCR/CD3 surface expression in the majority of cells. In a VITAL-assay,³⁹ which monitors relative antigen-specific cytotoxicity, *TRAC*-edited *truncCAR*-T cells did not elicit any antigen-specific cytotoxicity as expected due to the lack of a main activation domain (Figure 1C). In contrast, *CD3ζ*-edited *truncCAR*-T cells effectively lysed CD19⁺ cells similar to *TRAC*-edited T cells transfected with the full-length CAR (Figure 1C), confirming the generation of functionally active *truncCAR*-CD3ζ fusion protein after insertion of CAR moieties into the endogenous *CD3ζ*-gene.

Off-target assessment of CD3ζ gene editing

To ensure high precision of CRISPR-Cas9-mediated *CD3ζ*-targeting, we performed off-target assessment with CAST-Seq³⁶ which did not reveal any chromosomal translocations. The analysis revealed only the expected on-target aberrations including a very rare 15 Mb deletion between *CD3ζ* and a potential off-target site located on the same chromosome (supplemental Figure 1).

CD19-specific CD3ζ-*truncCAR* and *TRAC*-CAR-T cells have comparable CAR-regulation and antileukemia activity in vivo

We next compared CD19-CAR-expression levels and anti-leukemia potential of *CD3ζ*-*truncCAR*-T cells, *TRAC*-CAR-T cells and lentivirus-transduced (LV) *TRAC*-KO CAR-T cells in vitro. CAR-expression levels in *CD3ζ*-*truncCAR*-T cells were lower than in *TRAC*-integrated and LV counterparts (Figure 1D). Compared to *TRAC*-CAR-T cells, *CD3ζ*-*truncCAR*-T cells and LV CAR-T cells displayed significantly reduced dose-dependent killing in a 6-hour VITAL assay at some effector:target cell ratios (Figure 1E). Upon CD19⁺ Nalm-6 target cell engagement, *CD3ζ*-*truncCAR* and *TRAC*-CAR-T cells downregulated the CAR for 12-24 hours before returning to their relative baseline levels (Figure 1F). In contrast, LV CAR-T cells upregulated CAR-expression in response to stimulation and exceeded their baseline levels after 48 hours. Previous studies demonstrated that physiological control of CAR-expression in the *TRAC* locus enhances their antitumor performance in vivo.¹⁷ Therefore, we evaluated the antitumor efficacy of the differently engineered T cells (LV, *TRAC*, *CD3ζ*-*truncCAR*) in 2 independent, blinded xenograft models of acute lymphoblastic leukemia using immunodeficient mice. In both experiments, 0.5×10^6 luciferase-labeled CD19⁺ Nalm-6 tumor cells were administered systemically prior to the infusion of TCR-deficient CAR-T cells 4 days later. In mouse model 1 (Figure 1G; supplemental Figure 2a), mice received 14-day expanded cryopreserved CAR-T cells at a dose of 1×10^6 CAR⁺ cells. All 3 CAR-T treatments slowed tumor growth to a similar extent (control: L1CAM-CAR⁴⁰). In vivo efficacy was also observed in mouse model 2 (supplemental Figure 2b). Here, fresh, 14-day expanded CAR-T cells were administered at a dose of 0.5×10^6 CAR⁺ cells. Five weeks after tumor inoculation, mice treated with *TRAC*- and *CD3ζ*-edited CAR-T cells had significantly lower leukemia burden than animals which received lentivirus-transduced CAR-T cells (supplemental Figure 2b).

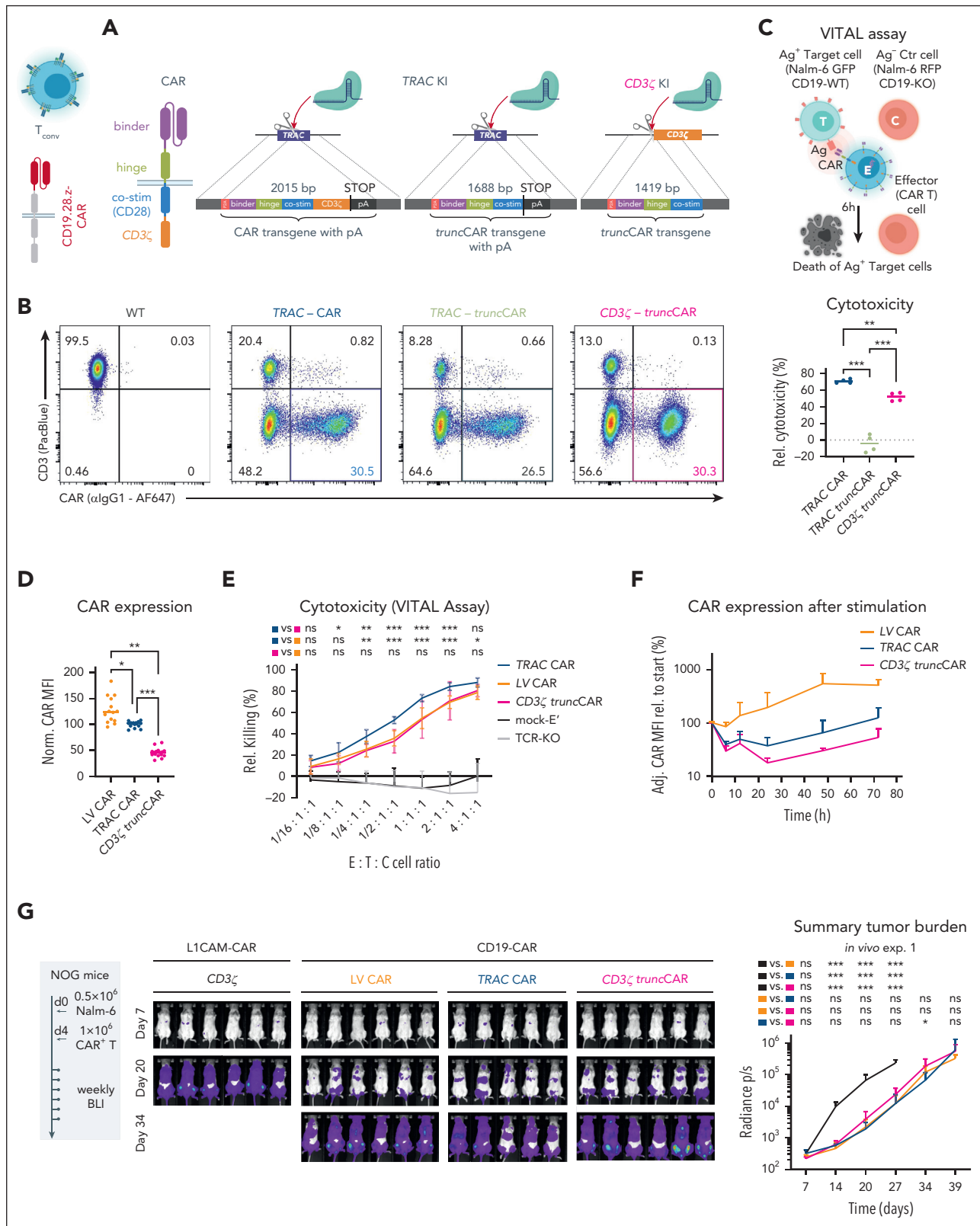


Figure 1. Integration of a truncated CD19-specific CAR into CD3 ζ , but not TRAC, conveys cytotoxicity in conventional T cells toward CD19⁺ leukemia cells. (A) full-length second-generation CAR protein (left) and virus-free knock-in strategies to integrate a full-length CAR into TRAC or a truncated CAR (*truncCAR*) into TRAC or CD3 ζ . (B) Flow cytometry dot plots after knock-in. Transgene integration into TRAC or CD3 ζ disrupts expression of the TCR/CD3 complex. (C) Relative cytotoxicity in coculture with (CD19⁺) Nalm-6 target cells and CD19 KO Nalm-6 control cells (VITAL assay). Calculation of relative cytotoxicity according to formula stated in methods section. (n = 2 biological replicates each in 2 technical replicates; ordinary one-way ANOVA followed by Holm-Šidák multiple comparison test with a single pooled variance). (D-G) Functional testing of CD3 ζ *truncCAR*, T cells in comparison to TRAC CAR and LV CAR-T cells. (D) Mean fluorescence intensity (MFI) determined by flow cytometry as a measure of cellular CAR expression and normalized to each donor's mean CAR MFI in the TRAC condition. (n = 7 biological replicates each in 2-5 technical replicates; mixed-effects analysis with

Tightly-controlled HER2-CAR expression from CD3 ζ avoids antigen-independent differentiation, but leads to low cytokine production

To test CD3 ζ -editing for another CAR-target antigen, we generated HER2-specific CAR-T cells via integration of a *truncCAR* into CD3 ζ .⁴¹⁻⁴⁴ As controls, we integrated the full-length HER2-CAR into *TRAC*, or into the safe-harbor locus *hAAVS1* driven by an exogenous LTR/EF1 α -promoter. CD3 ζ -edited HER2-*truncCAR*-T cells demonstrated the lowest CAR-expression level (supplemental Figure 3a). *TRAC*-edited T cells displayed unexpectedly high HER2-CAR-surface density, exceeding the LTR/EF1 α -driven CAR expression from the *hAAVS1* locus and CD19-CAR expression from *TRAC*. Phenotype analysis demonstrated antigen-independent differentiation in an expression level dependent manner (supplemental Figure 3b). *TRAC*-HER2-CAR-T cells expressed the highest levels of inhibitory receptors PD-1, Lag-3 and Tim-3 after 2 weeks expansion (supplemental Figure 3c). In contrast, CD3 ζ -HER2-*truncCAR*-T cells displayed differentiation and exhaustion marker profiles mirroring the CAR⁻ T-cell fraction which indicates reduced or absent tonic signaling. Further, CD3 ζ -edited HER2-*truncCAR*-T cells showed less activation-induced cell death than *TRAC*- or *AAVS1*-edited CAR-T cells after CAR stimulation using plate-bound anti-CAR antibody (supplemental Figure 3d). CD3 ζ -*truncCAR*-T cells showed similar cytotoxicity toward 3 different HER2⁺ tumor cell lines when compared to *TRAC*-HER2-CAR-T cells (supplemental Figure 3e). However, HER2-CD3 ζ -*truncCAR*-T cells secreted less TNF α and IFN γ when cocultured with tumor cells (supplemental Figure 3f), indicating that lower HER2-CAR expression may reduce tonic signaling but potentially impairs other functions.

Increasing CAR expression from the CD3 ζ locus improves cytokine production and antitumor efficacy

We hypothesized that the reduced effector functions of CD19- and HER2-specific CAR-T cells generated via CD3 ζ -*truncCAR* integration is caused by the lower amounts of CAR molecules available for synapse formation. Optimization of the 2A-cleavage peptide by the addition of a GSG-linker has been shown to increase protein expression in multi-cistronic transgenes.^{45,46} In the CD3 ζ -*truncCAR* condition, an optimized GSG-P2A (Figure 2A) increased CD19-CAR expression even above the *TRAC*-CAR condition (Figure 2B). This modification increased CAR-mediated cytotoxicity (Figure 2C) and intracellular cytokine production to levels similar to *TRAC*-CAR-T cells (Figure 2D; supplemental Figure 4).

We next evaluated the impact of the different CD19-CAR-expression levels during repeated leukemia challenges (Figure 2E-H) which were performed once per week at a CAR⁺

T cell to tumor cell ratio of 1:1. After serial coculture, all 3 conditions retained their physiological CAR-expression dynamics, but basal CAR expression did not differ anymore between CD3 ζ -*truncCAR*^{GSG} and *TRAC*, while the original CD3 ζ -*truncCAR* cells still showed lower CAR expression (Figure 2E). Interestingly, all 3 conditions showed similar cytotoxicity (Figure 2F) and proliferation (Figure 2G). CD3 ζ -edited conditions displayed slightly lower expression of inhibitory markers in the CD8 compartment after serial leukemia rechallenges (Figure 2H; detailed analysis in supplemental Figure 5). Serial coculture resulted in a similar shift towards a more differentiated phenotype in all conditions (supplemental Figure 6a) with a trend towards a CD8 polarization in the CD3 ζ -*truncCAR*^{GSG} condition (supplemental Figure 6b). Of note, the differences in cytokine production were preserved (supplemental Figure 6c).

Finally, we assessed the in vivo antitumor efficacy in a Nalm-6 mouse model (Figure 2I). Here, ex vivo expansion of CAR-T cells was shortened to 6 days due to a preferable phenotype with a high proportion of central memory (T_{CM}) and naive-like (T_N) cells and a physiological CD4/CD8 ratio (supplemental Figure 7). *TRAC*-CAR-T cells and CD3 ζ -*truncCAR* both resulted in a similarly prolonged, statistically significant survival compared to mock-electroporated T cells. Expression-tuned CD3 ζ -*truncCAR*^{GSG}-T cells showed the highest survival benefit which was statistically significant to the other treatment groups.

CD3 ζ -targeting allows redirection of more immune cell types than *TRAC*-editing

Nonconventional T cells and NK cells have emerged as important CAR carriers for adoptive cell transfer.^{2,3,47-49} To test the suitability of CD3 ζ -editing for different cell therapy applications, we compared CD3 ζ -*truncCAR* and *TRAC*-CAR integration in TCR γ/δ T cells, T_{reg} and primary NK cells (Figure 3). Like *TRAC*, CD3 ζ is expressed in all TCR α/β T cells and gene editing of the respective loci led to similar frequencies of HLA-A2-specific CARs in T_{reg} cells (Figure 3A). Furthermore, CD3 ζ is expressed in other immune cells which do not express *TRAC* and should therefore not be targetable by in-frame *TRAC* integration, notably TCR γ/δ T cells and NK cells. To our surprise, *TRAC*-editing in TCR γ/δ T cells resulted in substantial CAR⁺/TCR γ/δ ⁺ double-positive fractions, suggesting mRNA transcription of the *TRAC*-gene in TCR γ/δ T cells (Figure 3B). As expected for NK cells, *truncCAR* integration into CD3 ζ , but not *TRAC*, led to detectable CAR expression. Therefore, CD3 ζ -gene editing may serve as a universal approach to redirect different conventional and nonconventional T cells as well as NK cells with CARs (Figure 3C).

Figure 1 (continued) Geisser-Greenhouse correction + Holm-Šidák multiple comparison test with individual variances computed for each comparison). (E) Relative cytotoxicity towards CD19⁺ cells assessed in a 6-hour VITAL assay. (mock-E: mock-electroporated controls without ribonucleoproteins/HDR templates) (n = 4 biological replicates each in 1-3 technical replicates; two-way ANOVA followed by Holm-Šidák multiple comparison test with a single pooled variance) (F) Changes in CAR-expression levels (MFI normalized to start) after target cell encounter. (*TRAC* and LV in 4 biological replicates; CD3 ζ in 2 biological replicates). (G) Acute lymphoblastic leukemia xenograft mouse model using luciferase-labeled Nalm-6 (CD19⁺) tumor cells. 4 days post Nalm-6 administration, 1 × 10⁶ cryopreserved, 14-day expanded TCR-deleted CAR⁺ T cells were injected systemically. Tumor burden was assessed via bioluminescence imaging. (n = 5-6; 2-way ANOVA with Geisser-Greenhouse correction of log-transformed bioluminescence imaging data followed by Holm-Šidák multiple comparison test, with individual variances computed for each comparison). Asterisks in this and all further figures represent different P values calculated in the respective statistical tests (not significant [ns], P > .05; *P < .05; **P < .01; ***P < .001).

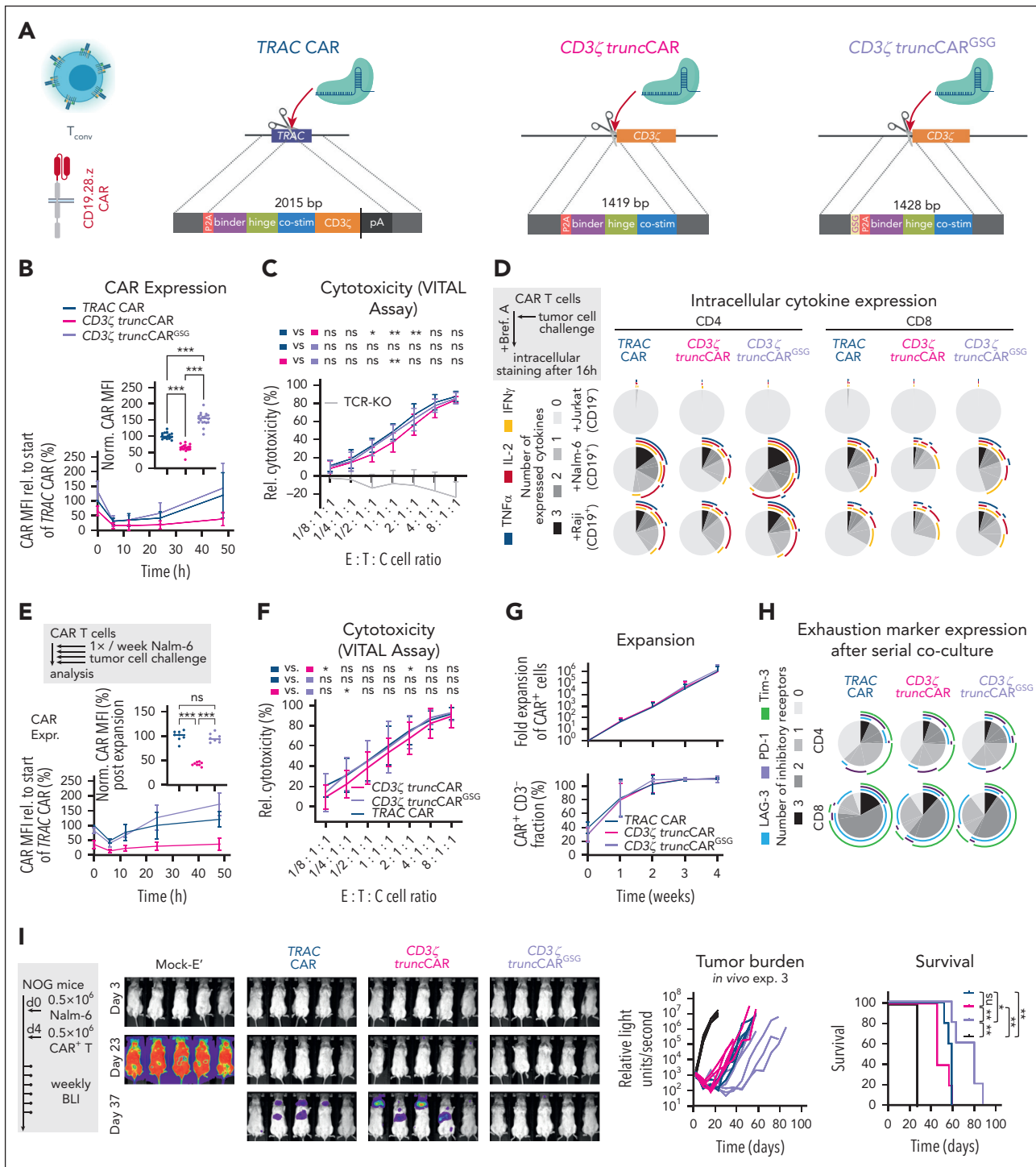


Figure 2. Evaluation of an optimized CD3 ζ truncCAR transgene and its impact on CAR-T cell function in vitro. (A) dsDNA templates for targeted delivery of a CAR or truncCAR respectively into TRAC (left) or CD3 ζ (middle), as in Figure 1A, and for targeted delivery of a GSG-P2A-linker-modified truncCAR into CD3 ζ (right). (B) Top: Mean fluorescence intensity (MFI) determined by flow cytometry at steady state (n = 4 biological replicates in 4-6 technical replicates in 2 independent experiments, data normalized to mean of TRAC for each donor; mixed-effects analysis with Geisser-Greenhouse correction followed by Holm-Šidák multiple comparison test, with individual variances computed for each comparison). Bottom: dynamics of CAR MFI after CAR-stimulation using CD19⁺ Nalm-6 tumor cells. (n = 3-4 biological replicates in 1-2 technical replicates). (C) Relative cytotoxicity assessed in a 6-hour VITAL assay (similar to Figure 1C, n = 4 biological replicates in 3 technical replicates; two-way ANOVA followed by Holm-Šidák multiple comparison test with a single pooled variance.). (D) Cytokine expression in CAR⁺ cells in response to control (CD19⁻) cell or target (CD19⁺) cell encounter (n = 3 biological replicates). (E-H) CAR-T-cell rechallenge in serial cocultures with Nalm-6 target cells. (E) Top: CAR MFI normalized to TRAC condition at steady state (n = 2 biological replicates in 4 technical replicates; statistics as in B). Bottom: dynamics of CAR MFI after target cell engagement (n = 2-4 biological replicates in 1-2 technical replicates). (F) 6-hour VITAL assay. (n = 3 biological replicates in 3-4 technical replicates; two-way ANOVA followed by Holm-Šidák multiple comparison test with a single pooled variance.). (G) Top: relative expansion of CAR⁺ T cells. Bottom: CAR⁺ frequency within T-cell products. (n = 4 biological replicates). (H) Cell surface expression of inhibitory receptors (LAG-3, PD-1, TIM-3; means of n = 4 biological replicates). (I) In vivo CAR-T-cell efficacy tested in Nalm-6 acute lymphoblastic leukemia xenograft mouse model (n = 5-6 mice/group; multiple log-rank tests).

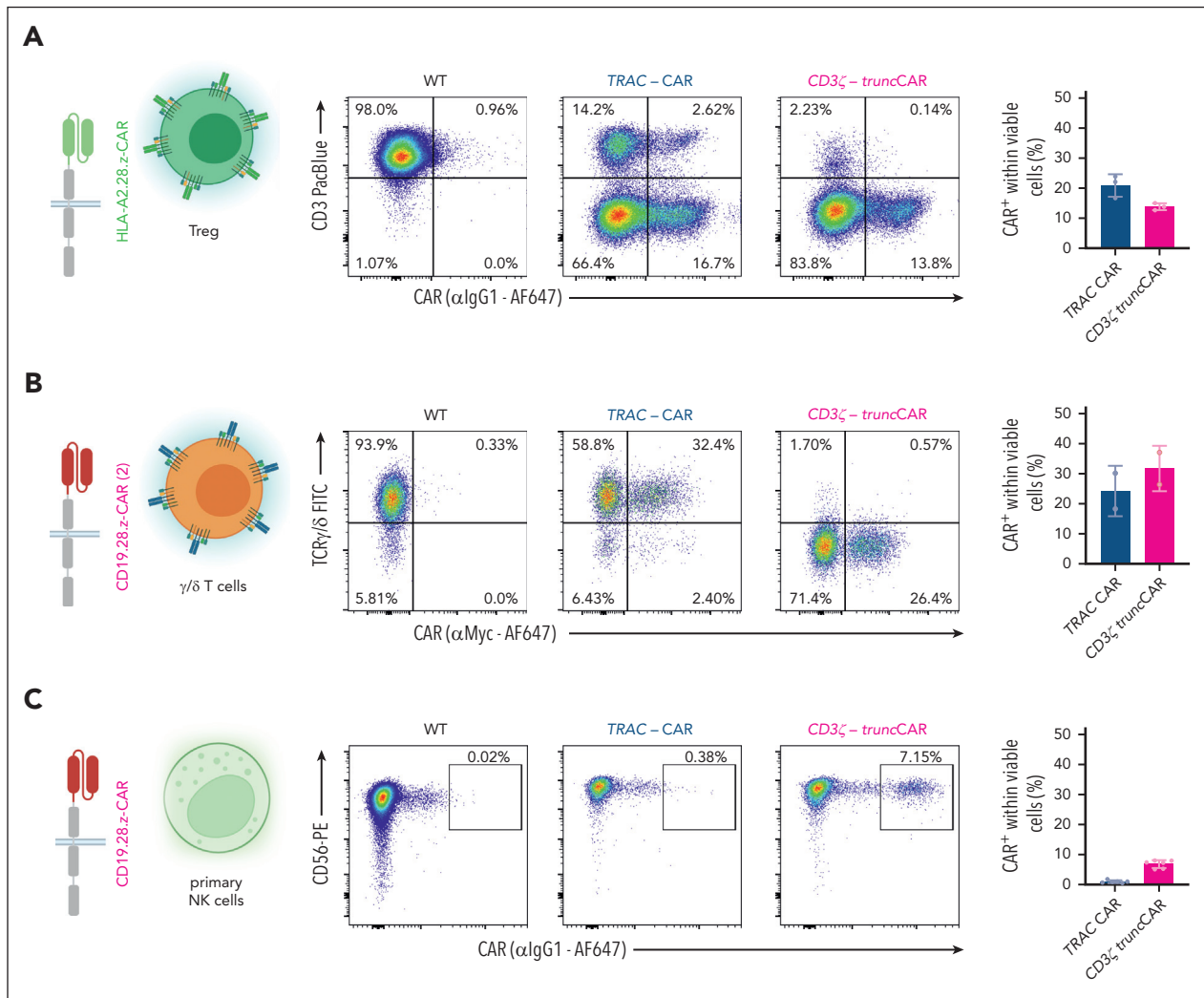


Figure 3. CD3 ζ truncCAR-integration facilitates CAR expression in different nonconventional T-cell subtypes and NK cells. (A) HLA-A2 CAR-integration in regulatory T-cells, n = 3 biological replicates. (B) CD19-CAR integration in TCR γ/δ T cells. TRAC integration generates CAR⁺/TCR γ/δ ⁺ double positive T cells, n = 2 biological replicates. (C) Integration of a CD19-CAR in primary human NK cells, n = 6 biological replicates.

CD3 ζ -KO does not impede canonical functions of primary NK cells

In NK cells, CD3 ζ is an adapter protein which assembles with activating killer cell immunoglobulin-like receptors (KIR) and Fc-receptors, such as CD16.⁴⁹ These cells dynamically balance inhibitory and activating signals, favoring the elimination of target cells upon detecting elevated activating KIR signaling (triggered by stress or cancer markers such as MICA/B) or when CD16 mediates ADCC. Our knock-in approach impedes the expression of free CD3 ζ -protein, which could potentially impair NK-cell activation and disturb canonical NK functions. To investigate these potential downsides, we disrupted CD3 ζ in primary human NK cells, either via CRISPR-Cas9-mediated KO or CD3 ζ -GFP-reporter knock-in that disrupts CD3 ζ (Figure 4A). Measuring cytotoxicity (Figure 4B) and degranulation (Figure 4C) in simple cocultures, we did not observe major differences regarding missing-self activation, cancer-directed activation, and alloreactivity. Importantly, gene editing of CD3 ζ did not alter CD16 expression. (Figure 4D). We also did not detect differences in anti-CD20-antibody-induced ADCC towards the CD20⁺ cell line Jeko-1 (Figure 4E) which is

partially resistant to NK-cell cytotoxicity (supplemental Figure 8).

CD3 ζ -truncCAR knock-in conveys cytotoxicity in primary NK cells and NK-92 cells

Using PBMC-derived NK cells, we next sought to characterize and compare CD3 ζ -truncCAR-NK cells with LV-transduced NK cells (Figure 5). CD3 ζ -truncCAR knock-in rates remained below 10% and were thus considerably lower than in T cells (Figure 5A). Despite using a high multiplicity of infection (MOI = 5) for LV CAR transfer, transduction rates were higher only in some replicates. While CAR MFI did not significantly differ between the conditions, the coefficient of variation of the CAR MFI was significantly lower after CD3 ζ -integration indicating a more controlled and predictable transgene expression after targeted CD3 ζ -integration (Figure 5B). Both conditions, but not a TRAC-CAR knock-in control, showed dose-dependent CAR-mediated killing in a VITAL assay, an internally controlled coculture assay which is less biased by the NK cells' CAR-independent (background) killing (Figure 5C). CD3 ζ -truncCAR-NK cells significantly outperformed the LV control at

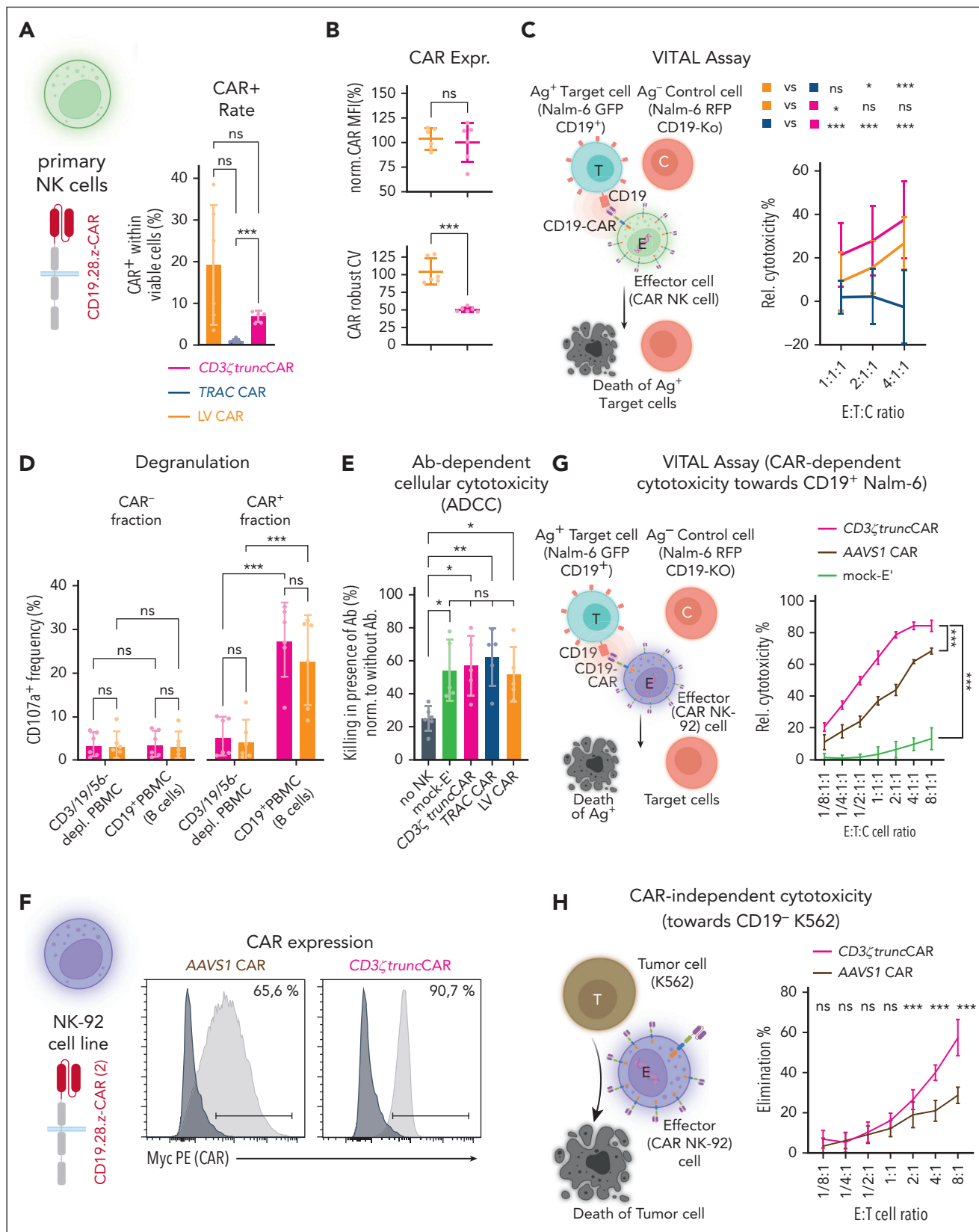


Figure 5. CD3 ζ -editing enables redirection of NK cells with CARs and does not impede canonical NK cell functions in vitro. CAR editing in primary NK cells via LV CAR transfer, TRAC-CAR or CD3 ζ -truncCAR-integration: (A) CAR⁺ frequencies after editing (n = 6 biological replicates, mixed-effects analysis with Geisser-Greenhouse correction followed by Tukey's multiple comparison test with individual variances computed for each comparison). (B) mean CAR expression (MFI) normalized to CD3 ζ -truncCAR integrated NK cells and robust coefficient of variation in CAR⁺ cells (n = 6 biological replicates; t test). (C) CAR-dependent cytotoxicity detected in a VITAL assay (data normalized to mock-electroporated (wildtype) NK cells; n = 6 biological replicates each in 3-4 technical replicates; 2-way ANOVA followed by Tukey's multiple comparison test

hAAVS1, CD3 ζ -truncCAR-NK-92 cells displayed higher CAR-mediated cytotoxicity (Figure 5G) and superior (CAR-independent) missing-self activation towards the MHC-I-deficient cell line K562 (Figure 5H). As NK-92 cells do not express CD16, ADCC was not studied.

Discussion

Here, we propose a strategy for site-specific CAR gene transfer to T and NK cells. Truncated CAR transgenes lacking a TCR-like effector domain were precisely inserted into the CD3 ζ -gene. Via in-frame integration, a complete CAR fusion gene (comprising an exogenous truncated CAR transgene and the endogenous CD3 ζ -gene) is formed resulting in surface expression of functional CAR proteins. In T cells, this prevents TCR/CD3 complex assembly and brings the CAR under the transcriptional regulation of the CD3 ζ -promoter. Despite its function as a signal transducer of activating NK-cell receptors, CD3 ζ can be edited to generate functional CAR-NK cells without affecting their canonical functions.

The CD3 ζ -locus is a CAR-integration site which limits necessary transgene size and shares features and advantages with the TRAC knock-in in T cells.^{17,27} Like in the TRAC approach, CD3 ζ -editing causes TCR-ablation, because the CAR's CD3 ζ -domain cannot rescue TCR/CD3 expression in CD3 ζ -KO T cells.⁵³ Together, this circumvents alloreactivity in T cells and should minimize the risk for GvHD if residual TCR⁺ T cells are efficiently depleted before allogeneic application of CD3 ζ -edited CAR-T cells.^{54,55} Therefore, the CD3 ζ -approach may be preferentially suited for allogeneic applications. Further, the physiological TCR-like CAR downregulation after antigen-engagement (achieved via TRAC- or CD3 ζ -integration) may enable transient resting, preventing terminal differentiation and exhaustion.^{17,56} When considering autologous manufacturing, transgene expression from TCR/NK-cell lineage genes, such as TRAC or CD3 ζ , provides a safety advantage because it should prevent the inadvertent CAR expression in B-cell leukemic blasts which can cause B-ALL relapse.⁵⁷

CAR-expression level influences CAR-T-cell performance, differentiation and exhaustion in preclinical and clinical settings.^{17,58,59} For viral gene transfer, CAR surface density may be modulated by variation of viral titers, aiming for different transgene copy numbers, as well as promoters⁶⁰ or transgene designs.⁵⁸ Exogenous promoters required for CAR expression after random integration can cause unphysiological CAR upregulation after antigen-encounter (Figure 2C) leading to cellular exhaustion.¹⁷ The promoters and respective 5'- or 3'-UTR could also contribute to the differences in transgene expression when comparing CD3 ζ - or TRAC-editing. However, we have also observed transgene-related differences (CD19-CAR vs

HER2-CAR; see supplemental Figure 3) that were locus-dependent which warrants further investigation. We show that basal CD19-CAR expression can be increased by insertion of a GSG-linker before the 2A-self-cleavage peptide (Figure 2). Increasing the CD19-CAR expression in CD3 ζ -truncCAR^{GSG}-T cells was associated with enhanced cytokine production after antigen-engagement and improved antileukemia activity in vivo (Figure 2). Modulation of both, steady-state CAR expression and dynamic CAR regulation, may impact the activation threshold of the CAR-T cells. A lower CAR expression may be beneficial to mitigate antigen-independent differentiation of CARs prone to tonic signaling or reduce on-target off-tumor toxicity when targeting tumor-associated antigens upregulated in the tumor but not completely absent in normal tissue.⁶¹ Of note, all CARs used in this study employed the CD28 costimulatory domain. Future studies should revisit the contribution of other costimulatory domains to select the most efficacious CAR version for the targeted disease.

Serendipitously, TRAC-integration resulted in the generation of large fractions of CAR⁺/TCR $\gamma\delta$ ⁺ double-positive T cells (Figure 3), despite the apparent absence of a TRAC gene-product in this cell type. We hypothesize that this unexpected outcome arises from the interconnection between the genomic sites encoding the TCR α and TCR δ chains.⁶² Further investigation is warranted to explore potential synergies between CARs and certain $\gamma\delta$ -TCRs in this distinct cell type.⁴⁸

Unlike TRAC, CD3 ζ -editing can be applied not only to all T-cell subsets but also to NK cells (Figure 3). Deleterious mutations of CD3 ζ have been found to be a cause for severe combined immunodeficiency, and patient NK cells were hypo-responsive in tumor cocultures and after CD16 stimulation.^{63,64} This raised concerns regarding the impact of CD3 ζ -editing on the functionality of resulting CAR-NK cells. However, in this study, CD3 ζ -disruption in primary human NK cells from healthy donors did not impair crucial immune functions such as ADCC, cytotoxicity or degranulation (Figure 4). These findings align with previous research indicating that FcR γ compensates CD3 ζ -loss after knockout, thereby enabling ADCC by primary NK cells.⁶⁵

This study is the first to demonstrate nonviral CRISPR-Cas-mediated knock-in for functional redirection of primary human NK cells with CARs. In comparison to CAR-T cells, CAR-NK cells have a favorable safety profile as they lack alloreactivity and show a reduced incidence of severe cytokine release syndrome and neurotoxicity.² CAR-NK cells can be combined with monoclonal antibodies for synergistic activity when targeting heterogeneous tumors. For example, the CD19-specific CAR-NK cells generated by CD3 ζ -editing (Figure 5) may be combined with the CD20-targeting antibody rituximab to overcome antigen-escape and relapse by CD19-negative cancer cells.

Figure 5 (continued) with a single pooled variance). (D) Degranulation as indicator of NK effector function via flow cytometric detection of CD107a (n = 6 biological replicates; two-way ANOVA followed by Holm-Sidak multiple comparison test with a single pooled variance). (E) ADCC of primary (CAR) NK cells against CD20⁺ bGal⁻ Jeko-1 cells. Bars represent killing for each condition in the presence of a CD20-targeting monoclonal antibody (0.5 μ g/mL) normalized to the respective condition without supplemented antibody (n = 5 biological replicates; mixed-effects analysis with Geisser-Greenhouse correction followed by Tukey's multiple comparison test with individual variances computed for each comparison). (F-H) CD19-CAR (2) transfer to NK-92 cells via AAVS1 integration of a CMV promoter-controlled, full-length CAR or CD3 ζ integration of a truncCAR. CAR⁺ fractions were enriched using MACS. (F) CAR expression in flow cytometry histograms. (G) CAR-dependent cytotoxicity in a 4-hour VITAL-assay (n = 6 technical replicates; two-way ANOVA with Tukey's multiple comparison test with a single pooled variance). (H) CAR-independent cytotoxicity towards the MHC I deficient, CD19⁻ K562 (control) cell line (n = 15 technical replicates; two-way ANOVA followed by Holm-Sidak multiple comparison test with a single pooled variance).

However, allogeneic CAR-NK cells are generally short-lived and do not persist. Therefore, physiological CAR regulation which improves persistence in T cells may not confer similar biological advantages in NK cells. Consequently, the primary advantages of CD3 ζ -editing in NK cells may be related to manufacturing and cost-aspects of miniaturized nonviral vectors. Before testing in suitable *in vivo* models and future clinical translation, the efficacy of nonviral reprogramming of primary NK cells should be further increased, for example by using pharmacological enhancers³³ and/or end-modified ssDNA donor templates.⁶⁶

CD3 ζ -editing for CAR gene transfer should be combined with other edits to enhance the functionality of CAR-T/NK-cell products for autologous and allogeneic use. First clinical trials demonstrated that TCR-deleted allogeneic CAR-T cells can induce remissions in heavily pretreated B-ALL and B-lymphoma patients, but additional gene editing was needed to circumvent immunological barriers of HLA-mismatches between CAR-T-cell donor and patient.^{54,67,68} Therefore, CD3 ζ -editing would benefit from those modifications to improve the efficacy of allogeneic CAR-T cells.^{54,69} Future studies may investigate the combination of CD3 ζ -editing with additional KOs to improve functionality,^{70,71} safety⁷² as well as persistence^{67,73,74} of allogeneic T and NK cells. The respective additional edits required to improve the functionality of NK cells^{75,76} may differ from the ones proposed for T cells.^{71,77} Finally, complex editing may require the combination of nuclease-assisted gene transfer with other gene silencing modalities such as base editing^{78,79} to reduce the risk for genomic rearrangements with unknown biological impact.^{54,77,80}

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Authorship

Contribution: J.K. designed the study, planned and performed experiments, analyzed results, interpreted the data, and wrote the manuscript; C.F., V.D., and W.D., planned and performed experiments, analyzed results, interpreted the data, and edited the manuscript; V.G., M. Stein, T.Z., S.S., C.E.P., L.A., and J.A. performed experiments and analyzed results; C.F.-G. performed and interpreted CAST-seq and provided respective sections for the manuscript; M. Suzuki, J.H., and R.S. planned experiments, interpreted data, and edited the manuscript; H.A. provided materials (HER2-CAR transgenes⁴¹), interpreted the data and edited the manuscript; A.K., M.A.-e.-E., and A.P. provided reagents, interpreted data and edited the manuscript; T.C. supervised work on CAST-seq, provided reagents, interpreted data, and edited the manuscript; H.-D.V., P.R., and M.S.-H. supervised parts of the study, provided reagents, interpreted data, and edited the manuscript; D.L.W. designed and led the study, planned experiments, analyzed results, interpreted data, and wrote the manuscript; and all authors reviewed and approved the manuscript in its final form.

Conflict-of-interest disclosure: J.K., H.-D.V., P.R., M.S.-H., and D.L.W. are listed as inventors on a patent application related to the work presented in this manuscript. J.A. and J.H. are employees of Experimental Pharmacology & Oncology Berlin Buch GmbH. H.-D.V. is founder and CSO at CheckImmune GmbH. P.R., H.-D.V., and D.L.W. are cofounders of the startup TCBalance Biopharmaceuticals GmbH focused on regulatory T-cell therapy. R.S. is a founding shareholder and scientific advisor of BioSynGen/ Zelltechs Pte Ltd (Republic of Singapore). The remaining authors declare no competing financial interests.

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Footnotes

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HER2-CARs were previously published.⁴¹ Other CAR/HDR-templates and sgRNA sequences are provided in supplemental Table 1. Plasmids encoding CD3 ζ -HDR-templates will be distributed through Addgene (pUC19-HDRT-CD3 ζ -truncCAR^{GSG} Addgene ID: 215758; pUC19-HDRT-CD3 ζ -truncCAR^{GSG} Addgene-ID: 215759). All other data may be requested from the corresponding author, Dimitrios L. Wagner (dimitrios-l.wagner@charite.de).

The online version of this article contains a data supplement.

There is a [Blood Commentary](#) on this article in this issue.

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REFERENCES

- Kalos M, Levine BL, Porter DL, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med*. 2011;3(95):95ra73.
- Liu E, Marin D, Banerjee P, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. *N Engl J Med*. 2020;382(6):545-553.
- MacDonald KG, Hoeppli RE, Huang Q, et al. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J Clin Invest*. 2016;126(4):1413-1424.
- Roemhild A, Otto NM, Moll G, et al. Regulatory T cells for minimising immune suppression in kidney transplantation: phase I/IIa clinical trial. *BMJ*. 2020;371:m3734.
- Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371(16):1507-1517.
- Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017;377(26):2531-2544.
- Schuster SJ, Svoboda J, Chong EA, et al. Chimeric antigen receptor T cells in refractory B-cell lymphomas. *N Engl J Med*. 2017;377(26):2545-2554.
- Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *N Engl J Med*. 2019;380(18):1726-1737.
- Geisler C, Kuhlmann J, Rubin B. Assembly, intracellular processing, and expression at the cell surface of the human alpha beta T cell receptor/CD3 complex. Function of the CD3-zeta chain. *J Immunol*. 1989;143(12):4069-4077.
- Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*. 1991;64(5):891-901.
- Moingeon P, Lucich JL, McConkey DJ, et al. CD3 zeta dependence of the CD2 pathway of activation in T lymphocytes and natural killer cells. *Proc Natl Acad Sci U S A*. 1992;89(4):1492-1496.
- Omer B, Cardenas MG, Pfeiffer T, et al. A costimulatory CAR improves TCR-based cancer immunotherapy. *Cancer Immunol Res*. 2022;10(4):512-524.
- Majzner RG, Ramakrishna S, Yeom KW, et al. GD2-CAR T cell therapy for H3K27M-mutated diffuse midline gliomas. *Nature*. 2022;603(7903):934-941.
- Mougiakakos D, Krönke G, Völkl S, et al. CD19-targeted CAR T cells in refractory systemic lupus erythematosus. *N Engl J Med*. 2021;385(6):567-569.
- Mackensen A, Müller F, Mougiakakos D, et al. Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus. *Nat Med*. 2022;28(10):2124-2132.
- Müller F, Boeltz S, Knitza J, et al. CD19-targeted CAR T cells in refractory antisynthetase syndrome. *Lancet*. 2023;401(10379):815-818.
- Eyquem J, Mansilla-Soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*. 2017;543(7643):113-117.
- Odak A, Yuan H, Feucht J, et al. Novel extragenic genomic safe harbors for precise therapeutic T cell engineering. *Blood*. 2023;141(22):2698-2712.
- Scholler J, Brady TL, Binder-Scholl G, et al. Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. *Sci Transl Med*. 2012;4(132):132ra53.
- Fraietta JA, Nobles CL, Sammons MA, et al. Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells. *Nature*. 2018;558(7709):307-312.
- Shah NN, Qin H, Yates B, et al. Clonal expansion of CAR T cells harboring lentivector integration in the CBL gene following anti-CD22 CAR T-cell therapy. *Blood Adv*. 2019;3(15):2317-2322.
- Micklethwaite KP, Gowrishankar K, Gloss BS, et al. Investigation of product-derived lymphoma following infusion of piggyBac-modified CD19 chimeric antigen receptor T cells. *Blood*. 2021;138(16):1391-1405.
- Bishop DC, Clancy LE, Simms R, et al. Development of CAR T-cell lymphoma in 2 of 10 patients effectively treated with piggyBac-modified CD19 CAR T cells. *Blood*. 2021;138(16):1504-1509.
- Harrison SJ, Nguyen T, Rahman M, et al. CAR+ T-cell lymphoma post ciltacabtagene autoleucel therapy for relapsed refractory multiple myeloma [abstract]. *Blood*. 2023;142(suppl 1):6939.
- Wagner DL, Koehl U, Chmielewski M, Scheid C, Striepecke R. Review: sustainable clinical development of CAR-T cells-switching from viral transduction towards CRISPR-cas gene editing. *Front Immunol*. 2022;13:865424.
- Müller TR, Jarosch S, Hammel M, et al. Targeted T cell receptor gene editing provides predictable T cell product function for immunotherapy. *Cell Rep Med*. 2021;2(8):100374.
- MacLeod DT, Antony J, Martin AJ, et al. Integration of a CD19 CAR into the TCR alpha chain locus streamlines production of allogeneic gene-edited CAR T cells. *Mol Ther*. 2017;25(4):949-961.
- Dai X, Park JJ, Du Y, et al. One-step generation of modular CAR-T cells with AAV-Cpf1. *Nat Methods*. 2019;16(3):247-254.
- Wiebking V, Lee CM, Mostrel N, et al. Genome editing of donor-derived T-cells to generate allogeneic chimeric antigen receptor-modified T cells: optimizing $\alpha\beta$ T cell-depleted haploidentical hematopoietic stem cell transplantation. *Haematologica*. 2021;106(3):847-858.
- Zhang J, Hu Y, Yang J, et al. Non-viral, specifically targeted CAR-T cells achieve high safety and efficacy in B-NHL. *Nature*. 2022;609(7926):369-374.
- Allen AG, Khan SQ, Margulies CM, et al. A highly efficient transgene knock-in technology in clinically relevant cell types. *Nat Biotechnol*. 2024;42(3):458-469.
- Torikai H, Reik A, Liu P-Q, et al. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood*. 2012;119(24):5697-5705.
- Kath J, Du W, Pruene A, et al. Pharmacological interventions enhance virus-free generation of TRAC-replaced CAR T cells. *Mol Ther Methods Clin Dev*. 2022;25:311-330.
- Roth TL, Puig-Saus C, Yu R, et al. Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature*. 2018;559(7714):405-409.
- Nguyen DN, Roth TL, Li PJ, et al. Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency. *Nat Biotechnol*. 2020;38(1):44-49.
- Turchiano G, Andrieux G, Klermund J, et al. Quantitative evaluation of chromosomal rearrangements in gene-edited human stem cells by CAST-Seq. *Cell Stem Cell*. 2021;28(6):1136-1147.e5.
- Rhiel M, Geiger K, Andrieux G, et al. T-CAST: an optimized CAST-Seq pipeline for TALEN confirms superior safety and efficacy of obligate-heterodimeric scaffolds. *Front Genome*. 2023;5:1130736.
- Braun T, Pruene A, Darguzyte M, et al. Non-viral TRAC-knocked-in CD19KICAR-T and gp350KICAR-T cells tested against Burkitt lymphomas with type 1 or 2 EBV infection: in vivo cellular dynamics and potency. *Front Immunol*. 2023;14:1086433.
- Hermans IF, Silk JD, Yang J, et al. The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. *J Immunol Methods*. 2004;285(1):25-40.
- Künkele A, Taraseviciute A, Finn LS, et al. Preclinical assessment of CD171-directed CAR T-cell adoptive therapy for childhood neuroblastoma: CE7 epitope target safety and product manufacturing feasibility. *Clin Cancer Res*. 2017;23(2):466-477.
- Textor A, Listopad JJ, Wührmann LL, et al. Efficacy of CAR T-cell therapy in large tumors relies upon stromal targeting by IFN γ . *Cancer Res*. 2014;74(23):6796-6805.
- Ahmed N, Brawley VS, Hegde M, et al. Human epidermal growth factor receptor 2 (HER2)-specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. *J Clin Oncol*. 2015;33(15):1688-1696.
- Ahmed N, Brawley V, Hegde M, et al. HER2-specific chimeric antigen receptor-modified virus-specific T cells for progressive

- glioblastoma. *JAMA Oncol.* 2017;3(8):1094-1101.
44. Hegde M, Joseph SK, Pashankar F, et al. Tumor response and endogenous immune reactivity after administration of HER2 CAR T cells in a child with metastatic rhabdomyosarcoma. *Nat Commun.* 2020; 11(1):3549.
 45. Yang S, Cohen CJ, Peng PD, et al. Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. *Gene Ther.* 2008;15(21):1411-1423.
 46. Liu Z, Chen O, Wall JBJ, et al. Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Sci Rep.* 2017; 7(1):2193.
 47. Noyan F, Zimmermann K, Hardtke-Wolenski M, et al. Prevention of allograft rejection by use of regulatory T cells with an MHC-specific chimeric antigen receptor. *Am J Transplant.* 2017;17(4):917-930.
 48. Deniger DC, Switzer K, Mi T, et al. Bispecific T-cells expressing polyclonal repertoire of endogenous $\gamma\delta$ T-cell receptors and introduced CD19-specific chimeric antigen receptor. *Mol Ther.* 2013;21(3):638-647.
 49. Daher M, Rezvani K. Outlook for new CAR-based therapies with a focus on CAR NK cells: what lies beyond CAR-engineered T cells in the race against cancer. *Cancer Discov.* 2021; 11(1):45-58.
 50. Naeimi Kararoudi M, Tullius BP, Chakravarti N, et al. Genetic and epigenetic modification of human primary NK cells for enhanced antitumor activity. *Semin Hematol.* 2020;57(4):201-212.
 51. Robbins GM, Wang M, Pomeroy EJ, Moriarty BS. Nonviral genome engineering of natural killer cells. *Stem Cell Res Ther.* 2021;12(1):350.
 52. Klingemann H. The NK-92 cell line-30 years later: its impact on natural killer cell research and treatment of cancer. *Cytotherapy.* 2023; 25(5):451-457.
 53. Barden M, Holzinger A, Velas L, et al. CAR and TCR form individual signaling synapses and do not cross-activate, however, can cooperate in T cell activation. *Front Immunol.* 2023;14:1110482.
 54. Qasim W. Genome edited allogeneic donor "universal" chimeric antigen receptor T Cells. *Blood.* 2023;141(8):835-845.
 55. Kath J, Du W, Martini S, et al. CAR NK-92 cell-mediated depletion of residual TCR+ cells for ultrapure allogeneic TCR-deleted CAR T-cell products. *Blood Adv.* 2023;7(15):4124-4134.
 56. Weber EW, Parker KR, Sotillo E, et al. Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. *Science.* 2021;372(6537): eaba1786.
 57. Ruella M, Xu J, Barrett DM, et al. Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. *Nat Med.* 2018;24(10):1499-1503.
 58. Gomes-Silva D, Mukherjee M, Srinivasan M, et al. Tonic 4-1BB costimulation in chimeric antigen receptors impedes T Cell survival and is vector dependent. *Cell Rep.* 2017;21(1): 17-26.
 59. Rodriguez-Marquez P, Calleja-Cervantes ME, Serrano G, et al. CAR density influences antitumoral efficacy of BCMA CAR T cells and correlates with clinical outcome. *Sci Adv.* 2022;8(39):eabo0514.
 60. Ho J-Y, Wang L, Liu Y, et al. Promoter usage regulating the surface density of CAR molecules may modulate the kinetics of CAR-T cells in vivo. *Mol Ther Methods Clin Dev.* 2021;21:237-246.
 61. Flugel CL, Majzner RG, Krenciute G, et al. Overcoming on-target, off-tumour toxicity of CAR T cell therapy for solid tumours. *Nat Rev Clin Oncol.* 2023;20(1):49-62.
 62. Attaf M, Legut M, Cole DK, Sewell AK. The T cell antigen receptor: the Swiss army knife of the immune system. *Clin Exp Immunol.* 2015; 181(1):1-18.
 63. Roberts JL, Lauritsen JPH, Cooney M, et al. T-B+NK+ severe combined immunodeficiency caused by complete deficiency of the CD3 ζ subunit of the T-cell antigen receptor complex. *Blood.* 2007; 109(8):3198-3206.
 64. Valés-Gómez M, Estes G, Aydogmus C, et al. Natural killer cell hyporesponsiveness and impaired development in a CD247-deficient patient. *J Allergy Clin Immunol.* 2016;137(3):942, 5.e4.
 65. Dahlvang JD, Dick JK, Sangala JA, et al. Ablation of SYK kinase from expanded primary human NK cells via CRISPR/Cas9 enhances cytotoxicity and cytokine production. *J Immunol.* 2023;jj2200488.
 66. Shy BR, Vykunta VS, Ha A, et al. High-yield genome engineering in primary cells using a hybrid ssDNA repair template and small-molecule cocktails. *Nat Biotechnol.* 2023; 41(4):521-531.
 67. Qasim W, Zhan H, Samarasinghe S, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med.* 2017;9(374):eaaaj2013.
 68. Benjamin R, Graham C, Yallop D, et al. Genome-edited, donor-derived allogeneic anti-CD19 chimeric antigen receptor T cells in paediatric and adult B-cell acute lymphoblastic leukaemia: results of two phase 1 studies. *Lancet.* 2020;396(10266): 1885-1894.
 69. Wagner DL, Fritsche E, Pulsipher MA, et al. Immunogenicity of CAR T cells in cancer therapy. *Nat Rev Clin Oncol.* 2021;18(6): 379-393.
 70. Prinzing B, Zebley CC, Petersen CT, et al. Deleting DNMT3A in CAR T cells prevents exhaustion and enhances antitumor activity. *Sci Transl Med.* 2021;13(620):eabh0272.
 71. Carnevale J, Shifrut E, Kale N, et al. RAS2A2 ablation in T cells boosts antigen sensitivity and long-term function. *Nature.* 2022; 609(7925):174-182.
 72. Wiebking V, Patterson JO, Martin R, et al. Metabolic engineering generates a transgene-free safety switch for cell therapy. *Nat Biotechnol.* 2020;38(12):1441-1450.
 73. Torikai H, Reik A, Soldner F, et al. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood.* 2013;122(8):1341-1349.
 74. Kagoya Y, Guo T, Yeung B, et al. Genetic ablation of HLA class I, class II, and the T-cell receptor enables allogeneic T cells to be used for adoptive T-cell therapy. *Cancer Immunol Res.* 2020;8(7):926-936.
 75. Pomeroy EJ, Hunzeker JT, Kluesner MG, et al. A genetically engineered primary human natural killer cell platform for cancer immunotherapy. *Mol Ther.* 2020;28(1):52-63.
 76. Daher M, Basar R, Gokdemir E, et al. Targeting a cytokine checkpoint enhances the fitness of armored cord blood CAR-NK cells. *Blood.* 2021;137(5):624-636.
 77. Diorio C, Murray R, Naniog M, et al. Cytosine base editing enables quadruple-edited allogeneic CAR-T cells for T-ALL. *Blood.* 2022;140(6):619-629.
 78. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature.* 2016; 533(7603):420-424.
 79. Gaudelli NM, Komor AC, Rees HA, et al. Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage. *Nature.* 2017;551(7681):464-471.
 80. Glaser V, Flugel C, Kath J, et al. Combining different CRISPR nucleases for simultaneous knock-in and base editing prevents translocations in multiplex-edited CAR T cells. *Genome Biol.* 2023;24(1):89.
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