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

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Abstract:
Chimeric antigen receptor (CAR)-redirected immune cells hold significant therapeutic potential for oncology, autoimmune diseases, transplant medicine, and infections. All approved CAR-T therapies rely on personalized manufacturing using undirected viral gene transfer, which results in non-physiological 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 CD3ζ (CD247) gene, functional CAR fusion-genes are generated that exploit the endogenous CD3ζ 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, TCRγ/δ T-cells, regulatory T-cells, and NK-cells. In T-cells, CD3ζ in-frame fusion eliminated TCR surface expression, reducing the risk of graft-versus-host disease in allogeneic off-the-shelf settings. CD3ζ-CD19-CAR-T-cells exhibited comparable leukemia control to T cell receptor alpha constant (TRAC)-replaced and lentivirus-transduced CAR-T-cells in vivo. Tuning of CD3ζ-CAR-expression levels significantly improved the in vivo efficacy. Notably, CD3ζ gene editing enabled redirection of NK-cells without impairing their canonical functions. Thus, CD3ζ gene editing is a promising platform for the development of allogeneic off-the-shelf cell therapies using redirected killer lymphocytes.-

Conflict of interest: COI declared - see note

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Clinical trial registration information (if any):

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

Short title: CD3-zeta editing for redirection of T and NK cells

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Data Sharing Statement
HER2-CARs were previously published[38]. Other CAR/HDR-templates and sgRNA sequences are provided in **Suppl. Table 1**. Plasmids encoding *CD3ζ*-HDR-templates will be distributed through Addgene (pUC19-HDRT-*CD3ζ-trunc*CAR^{GSG} Addgene ID: 215758; pUC19-HDRT-*CD3ζ-trunc*CAR^{GSG} Addgene-ID: 215759). All other data may be requested from the corresponding author.

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Key points

- Integration of ζ-deficient CARs into *CD3ζ* gene allows generation of functional TCR-ablated CAR-T cells for allogeneic off-the-shelf use
- *CD3ζ*-editing platform allows CAR redirection of NK-cells without affecting their canonical functions

Keywords
Chimeric Antigen Receptors, T cells, CAR T cells, CAR NK-cells, CAR Treg, CRISPR-Cas, Gene editing, Non-viral gene transfer, CD3-zeta, CD247

I. Abstract (234/250 words):

Chimeric antigen receptor (CAR)-redirected immune cells hold significant therapeutic potential for oncology, autoimmune diseases, transplant medicine, and infections. All approved CAR-T therapies rely on personalized manufacturing using undirected viral gene transfer, which results in non-physiological 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 *CD3ζ (CD247)* gene, functional CAR fusion-genes are generated that exploit the endogenous *CD3ζ* 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, TCRγ/δ T-cells, regulatory T-cells, and NK-cells. In T-cells, *CD3ζ* in-frame fusion eliminated TCR surface expression, reducing the risk of graft-versus-host disease in allogeneic off-the-shelf settings. *CD3ζ*-CD19-CAR-T-cells exhibited comparable leukemia control to *T cell receptor alpha constant* (*TRAC*)-replaced and lentivirus-transduced CAR-T-cells *in vivo*. Tuning of *CD3ζ*-CAR-expression levels significantly improved the *in vivo* efficacy. Notably, *CD3ζ* gene editing enabled redirection of NK-cells without impairing their canonical functions. Thus, *CD3ζ* gene editing is a promising platform for the development of allogeneic off-the-shelf cell therapies using redirected killer lymphocytes.

II. Introduction (634 Words)

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 anti-tumor efficacy of cytotoxic T-cells[1] and natural killer (NK) cells[2] 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 TCRα and a corresponding TCRβ chain which engage antigenic peptides presented by MHC molecules, as well as the accessory proteins CD3γ, CD3δ, CD3ε and CD3ζ which transduce the TCR signal downstream. While all CD3 proteins are required for TCR/CD3 assembly, biosynthesis of CD3ζ is the rate-limiting step in TCR/CD3 complex formation[9]. Further, the intracellular domain of CD3ζ is sufficient to drive TCR-like activation in chimeric receptors[10,11]. Therefore, all clinically approved (second-generation) CARs use the intracellular domain of CD3ζ 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 co-stimulatory 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 co-stimulation[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 EF1α[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 *CBL*[21] by CAR provirus the development of CAR^+ T-cell lymphoma after treatment with products generated via PiggyBac 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 *TCRα chain constant* (*TRAC*)[17,27–29], *PDCD1 (*encoding PD-1)[28,30] and *GAPDH*[31] as well as intra-/extragenic genomic safe harbor (GSH) loci, such as the human AAV-integration site (*hAAVS1*)[30] and *eGSH6*[18], respectively. *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 allo-reactivity, thereby demonstrating a route towards safe application of CAR-T-cells in allogeneic settings[32].

In this study, we demonstrate virus-free CAR redirection via in-frame integration of truncated, CD3ζ-deficient *CAR*-transgenes (*trunc*CARs) into an early exon of the *CD3ζ*-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 co-stimulatory domain but no main activation domain) and the endogenous *CD3ζ*-gene. This reduces the required transgene size and exploits the *CD3ζ* promoter for physiological CAR-regulation. *CD3ζ*-gene editing can also be used for redirection of regulatory T-cells, TCRγ/δ T-cells and most notably primary human NK-cells which cannot be redirected by *TRAC*-targeting.

III. Material and methods (740 Words)

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 (FCS), 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 the NK isolation Kit (Miltenyi) and cultured in NK MACS Medium (Miltenyi) supplemented with 10% FCS, IL-2 (500 IU/ml) and IL-15 (5ng/ml).

Genetic engineering

Targeted virus-free CAR-integration was performed as recently described[33]. In short, human T or NK-cells were transfected with precomplexed CRISPR-Cas9 ribonucleoproteins (RNP) and double-stranded DNA (dsDNA) (DNA/sgRNA Sequences: **Suppl. Table 1**). The dsDNA served as template for HDR and consisted of the (CAR/truncCAR) transgene flanked by 400bp homology arms. Cells were resuspended in 20µl P3 Electroporation Buffer (Lonza, Cologne, Germany) and electroporated with 1µg HDR-template and 1.38µl RNP consisting of synthetic modified single guide RNA (sgRNA, 100 µM, Integrated DNA Technologies (IDT), Coralville, IA), 15-50kDa poly(L-glutamic acid)[34] (100µg/µl, Sigma-Aldrich, St. Louis, MO) and recombinant SpCas9 protein (61µM, IDT) in a 0.96:1:0.8 volume ratio using the 4D-Nucleofector (Lonza). T-cells activated for 48h on αCD3/CD28-coated tissue culture plates were electroporated at a density of $5x10^4$cells/µl 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 line was electroporated at $2.5x10^4$cells/µl with the program CA-137. 10min post-electroporation, T-cells were transferred into medium supplemented with 0.5µM HDR-Enhancer v2 (IDT). For LV controls, activated T-cells were transduced 1 day post T-cell isolation while being kept on αCD3/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[35,36].

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 **Suppl. 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 4h of co-culture with target cells in the presence of Monensin A (1µM, Golgistop, Beckton Dickinson, Franklin Lakes, NJ) and BV785-conjugated anti-CD107a antibody (Biolegend). NK-cell-mediated antibody-dependent cellular cytotoxicity (ADCC) was assessed after 16h of co-culture 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
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, LaGeSo Berlin, Germany) under the permission A0010/19. Study 2 was approved by the Lower Saxony Office for Consumer Protection and Food Safety – LAVES (permit number 16/2222). In brief, immunodeficient mice were infused with $0.5x10^6$ Nalm-6 cells (expressing *luciferase*) via tail vein injection. Four days later, $0.5x10^6$ or $1x10^6$ TCR-deficient CD19-CAR-T-cells were infused intravenously. CAR-T-cells were generated either via targeted integration of a CAR or a *trunc*CAR into the *TRAC* or *CD3ζ*-gene, respectively, or by LV gene transfer and consecutive *TRAC*-knock-out (KO). Tumor burden was assessed as previously reported[37] 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 endpoints (models 1+3) or five 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, LaGeSo

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).

IV. Results (1691 Words)

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

We performed targeted delivery of a 1419bp-sized CD19-specific *trunc*CAR (CD19-IgG1-CD28) into *CD3ζ* (exon 2, beginning of intracellular domain) and *TRAC* (exon 1) using CRISPR-Cas9 (**Fig. 1a**). As additional control, we integrated a full-length 2015bp-sized CAR (CD19-IgG1-CD28-CD3ζ) into *TRAC* as recently described[33]. Transgene expression in primary human T-cells was confirmed by flow cytometry (**Fig. 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 *trunc*CAR-T-cells did not elicit any antigen-specific cytotoxicity as expected due to the lack of a main activation domain (**Fig. 1c**). In contrast, *CD3ζ*-edited *trunc*CAR-T-cells effectively lysed $CD19^+$ cells similar to *TRAC*-edited T-cells transfected with the full-length CAR (**Fig. 1c**), confirming the generation of functionally active *trunc*CAR-CD3ζ fusion protein after insertion of CAR moieties into the endogenous *CD3ζ*-gene.

Off-target assessment of CD3ζ-editing

To ensure high precision of CRISPR-Cas9-mediated *CD3ζ*-targeting, we performed off-target assessment with CAST-Seq[35] which did not reveal any chromosomal translocations. The analysis revealed only the expected on-target aberrations including a very rare 15Mb deletion between *CD3ζ* and a potential off-target site located on the same chromosome (**Suppl. Fig. 1**).

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

We next compared CD19-CAR-expression levels and anti-leukemia potential of *CD3ζ-trunc*CAR-T-cells, *TRAC*-CAR-T-cells and lentivirus-transduced (LV) *TRAC*-KO CAR-T-cells *in vitro*. CAR-expression levels in *CD3ζ-trunc*CAR-T-cells were lower than in *TRAC*-integrated and LV counterparts (**Fig. 1d**). Compared to *TRAC*-CAR-T-cells, *CD3ζ-trunc*CAR-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 (**Fig. 1e).** Upon $CD19^+$ Nalm-6 target cell engagement, *CD3ζ-trunc*CAR and *TRAC*-CAR-T-cells downregulated the CAR for 12-24h before returning to their relative baseline levels (**Fig. 1f**). In contrast, LV CAR-T-cells upregulated CAR-expression in response to stimulation and exceeded their baseline levels after 48h. Previous studies demonstrated that physiological control of CAR-expression in the *TRAC* locus enhances their anti-tumor performance *in vivo*[17]. Therefore, we evaluated the anti-tumor efficacy of the differently engineered T-cells (LV, *TRAC*, *CD3ζ-trunc*CAR) in two independent, blinded xenograft models of acute lymphoblastic leukemia using immunodeficient mice. In both experiments, $0.5x10^6$ luciferase-labeled $CD19^+$ Nalm-6 tumor cells were administered systemically prior to the infusion of TCR-deficient CAR-T-cells four days later. In mouse model 1 (**Fig. 1g, Suppl. Fig. 2a**), mice received 14-day expanded cryopreserved CAR-T-cells at a

dose of $1x10^6$ CAR^+ cells. All three CAR-T treatments slowed tumor growth to a similar extent (control: L1CAM-CAR[40]). *In vivo* efficacy was also observed in mouse model 2 (**Suppl. Fig. 2b**). Here, fresh, 14-day expanded CAR-T-cells were administered at a dose of $0.5x10^6$ 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 (**Suppl. Fig. 2b**).

Tightly-controlled HER2-CAR-expression from CD3ζ avoids antigen-independent differentiation, but leads to low cytokine production

To test *CD3ζ*-editing for another CAR-target antigen, we generated HER2-specific CAR-T-cells via integration of a *trunc*CAR into *CD3ζ* [41–43]. As controls, we integrated of the HER2-CAR into *TRAC*, or into the safe-harbor locus *hAAVS1* driven by an exogenous LTR/EF1α-promoter. *CD3ζ*-edited HER2-*trunc*CAR-T-cells demonstrated the lowest CAR-expression level (**Suppl. Fig. 3a**). *TRAC*-edited T-cells displayed unexpectedly high HER2-CAR-surface density, exceeding the LTR/EF1α-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 (**Suppl. Fig. 3b**). *TRAC*-HER2-CAR-T-cells expressed the highest levels of inhibitory receptors PD-1, Lag-3 and Tim-3 after two weeks expansion (**Suppl. Fig. 3c**). In contrast, *CD3ζ*-HER2-*trunc*CAR-T-cells displayed differentiation and exhaustion marker profiles mirroring the CAR^- T-cell fraction which indicates reduced or absent tonic signaling. Further, *CD3ζ*-edited HER2-*trunc*CAR-T-cells showed less activation-induced cell death than *TRAC*- or *AAVS1*-edited CAR-T-cells after CAR stimulation using plate-bound antibody (**Suppl. Fig. 3d**). *CD3ζ-trunc*CAR-T-cells showed similar cytotoxicity toward three different $HER2^+$ tumor cell lines when compared to *TRAC*-HER2-CAR-T-cells (**Suppl. Fig. 3e**). However, HER2-*CD3ζ-trunc*CAR T-cells secreted less TNFα and IFNγ when co-cultured with tumor cells (**Suppl. Fig. 3f**), indicating that lower HER2-CAR-expression may reduce tonic signaling but potentially impairs other functions.

Increasing CAR-expression from CD3ζ improves cytokine production and anti-tumor efficacy

We hypothesized that the reduced effector functions of CD19- and HER2-specific CAR T-cells generated via *CD3ζ-trunc*CAR-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[44,45]. In the *CD3ζ-trunc*CAR condition, an optimized GSG-P2A (**Fig. 2a**) increased CD19-CAR-expression even above the *TRAC*-CAR condition (**Fig. 2b**). This modification increased CAR-mediated cytotoxicity (**Fig. 2c**) and intracellular cytokine production to levels similar to *TRAC*-CAR-T-cells (**Fig. 2d, Suppl. Fig. 4**).

We next evaluated the impact of the different CD19-CAR expression levels during repeated leukemia challenges (**Fig. 2e-h**) which were performed once per week at a CAR^+ T-cell to tumor cell ratio of 1:1. After serial co-culture, all three conditions retained their physiological CAR-expression dynamics, but basal CAR-expression did not differ anymore between *CD3ζ-trunc*CAR^{GSG} and *TRAC*, while the original *CD3ζ-trunc*CAR cells still showed lower CAR-expression (**Fig. 2e**). Interestingly, all three conditions showed similar cytotoxicity (**Fig. 2f**) and proliferation (**Fig. 2g**). *CD3ζ*-edited conditions displayed slightly lower expression of inhibitory markers in the CD8

compartment after serial leukemia re-challenges (**Fig. 2h; detailed analysis in Suppl. Fig. 5**). Serial co-culture resulted in a similar shift towards a more differentiated phenotype in all conditions (**Suppl. Fig. 6a**) with a trend towards a CD8 polarization in the *CD3ζ-trunc*CAR^{GSG} condition (**Suppl. Fig. 6b**). Of note, the differences in cytokine production were preserved (**Suppl. Fig. 6c**).

Finally, we assessed the *in vivo* anti-tumor efficacy in a Nalm-6 mouse model (**Fig. 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 naïve-like (T_N) cells and a physiological CD4/CD8 ratio (**Suppl. Fig. 7**). *TRAC*-CAR-T-cells and *CD3ζ-trunc*CAR both resulted in a similarly prolonged, statistically significant survival compared to mock-electroporated T-cells. Expression-tuned *CD3ζ-trunc*CAR^{GSG}-T-cells showed the highest survival benefit which was statistically significant to the other treatment groups.

CD3ζ-targeting allows redirection of more immune cell types than TRAC-editing

Non-conventional T-cells and natural killer (NK)-cells have emerged as important CAR carriers for adoptive cell transfer[2,3,46–48]. To test the suitability of *CD3ζ*-editing for different cell therapy applications, we compared *CD3ζ-trunc*CAR and *TRAC*-CAR-integration in $TCR_{\gamma/\delta}$ T-cells, T_{reg} and primary NK-cells (**Fig. 3**). Like *TRAC*, *CD3ζ* is expressed in all $TCR_{\alpha/\beta}$ T-cells and gene editing of the respective loci led to similar frequencies of HLA-A2-specific CARs in T_{reg} cells (**Fig. 3a**). Furthermore, *CD3ζ* is expressed in other immune cells which do not express *TRAC* and should therefore not be targetable by in-frame *TRAC* integration, notably $TCR_{\gamma/\delta}$ T-cells and natural killer (NK) cells. Of note, *TRAC*-editing in $TCR_{\gamma/\delta}$ T-cells resulted in substantial CAR$^+$ fractions, suggesting mRNA transcription of the *TRAC*-gene in $TCR_{\gamma/\delta}$ T-cells (**Fig. 3b).** As expected for NK-cells, *trunc*CAR-integration into *CD3ζ*, but not *TRAC*, led to detectable CAR-expression. Therefore, *CD3ζ*-gene editing may serve as a universal approach to redirect different conventional and non-conventional T-cells as well as NK-cells with CARs (**Fig. 3c**).

CD3ζ-KO does not impede canonical functions of primary NK-cells

In NK-cells, CD3ζ is an adapter protein which assembles with activating killer-cell immunoglobulin-like receptors (KIR) and Fc-receptors, such as CD16[48]. 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 antibody-dependent cellular cytotoxicity (ADCC). Our knock-in approach impedes the expression of free CD3ζ-protein, which could potentially impair NK-cell activation and disturb canonical NK functions. To investigate these potential downsides, we disrupted *CD3ζ* in primary human NK-cells, either via CRISPR-Cas9-mediated KO or via *CD3ζ*-GFP-reporter knock-in that disrupts *CD3ζ* (**Fig. 4a**). Measuring cytotoxicity (**Fig. 4b**) and degranulation (**Fig. 4c)** in simple co-cultures, we did not observe major differences regarding missing-self activation, cancer-directed activation, and allo-reactivity. Importantly, gene editing of *CD3ζ* did not alter CD16 expression. (**Fig. 4d**). We also did not detect differences in anti-CD20-antibody-induced ADCC towards the CD20$^+$ cell line Jeko-1 (**Fig. 4e**) which is partially resistant to NK-cell cytotoxicity (**Suppl. Fig. 8**).

CD3ζ-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 *CD3ζ*-truncCAR-NK-cells with LV-transduced NK-cells (**Fig. 5**). *CD3ζ*-truncCAR knock-in rates remained below 10% and were thus considerably lower than in T-cells (**Fig. 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 (CV) of the CAR MFI was significantly lower after *CD3ζ*-integration indicating a more controlled and predictable transgene-expression after targeted CAR-integration (**Fig. 5b**). Both conditions, but not a *TRAC*-CAR knock-in control, showed dose-dependent CAR-mediated killing in a VITAL assay, an internally controlled co-culture assay which is less biased by the NK-cells' CAR-independent (background-) killing (**Fig. 5c**). *CD3ζ*-truncCAR-NK-cells significantly outperformed the LV control at the lowest dose and kept this trend at high doses. Analysis of the degranulation marker CD107a showed similar CAR-mediated activation of both LV and *CD3ζ-trunc*CAR NK-cells when co-cultured with CD19-expressing allogeneic B cells (**Fig. 5d**). As for *CD3ζ*-KO cells (**Fig. 4**), ADCC towards the $CD20^+$ cell line Jeko-1 was not altered for *TRAC*, LV or *CD3ζ-trunc*CAR-NK-cells compared to mock-electroporated (wildtype) NK-cells (**Fig. 5e**). Thus, *CD3ζ*-gene editing may be used to redirect primary NK-cells with CARs while retaining their canonical functions.

Low CAR gene transfer rates in primary NK-cells are a challenge in the field[49,50]. The use of immortal cell lines does not require high CAR-integration rates because the edited cells can be enriched prior to a potentially unlimited expansion. One example is the NK-cell-derived cancer cell line NK-92, which has been used as the cell source for CAR-NK therapy in multiple clinical trials[51]. To test the feasibility of our approach in NK-92 cells, we generated CD19-specific *CD3ζ-trunc*CAR-NK-92 cells and *hAAVS1*-CAR-NK-92 cells as controls (**Fig. 5f**). CAR^+ NK-92 cells were enriched via MACS. Compared to *hAAVS1*, *CD3ζ-trunc*CAR-NK-92 cells displayed higher CAR-mediated cytotoxicity (**Fig. 5g**) and superior (CAR-independent) missing-self activation towards the MHC-I-deficient cell line K562 (**Fig. 5h**). As NK-92 cells do not express CD16, ADCC was not studied.

V. Discussion (935 Words)

Here, we propose a novel 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 *CD3ζ*-gene. Via in-frame integration, a complete CAR fusion gene (comprising an exogenous truncated *CAR*-transgene and the endogenous *CD3ζ*-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 *CD3ζ*-promoter. Despite its function as a signal transducer of activating NK-cell receptors, *CD3ζ* can be edited to generate functional CAR-NK-cells without affecting their canonical functions.

The *CD3ζ*-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 *TRAC*-, *CD3ζ*-editing causes TCR-ablation, because the CAR's CD3ζ-domain cannot rescue TCR/CD3-expression in *CD3ζ*-KO T-cells[52]. Together, this circumvents alloreactivity in T-cells and should minimize the risk for GvHD if residual TCR^+ T cells are efficiently depleted prior to allogeneic application of *CD3ζ*-edited CAR-T-cells[53,54].

Therefore, *CD3ζ*-approach may be preferentially suited for allogeneic applications. Further, the physiological TCR-like CAR-downregulation after antigen-engagement (achieved via *TRAC*- or *CD3ζ*-integration) may enable transient resting, preventing terminal differentiation and exhaustion[17,55]. When considering autologous manufacturing, transgene expression from TCR/NK-cell lineage genes, such as *TRAC* or *CD3ζ*, provides a safety advantage because it should prevent the inadvertent CAR-expression in B-cell leukemic blasts which can cause B-ALL relapse[56].

CAR-expression level influences CAR-T-cell performance, differentiation and exhaustion in pre-clinical and clinical settings[17,57,58]. For viral gene transfer, CAR-surface density may be modulated by variation of viral titers, aiming for different transgene copy numbers, as well as different promoters[59] or transgene designs[57]. Exogenous promoters required for CAR-expression after random integration can cause unphysiological CAR up-regulation after antigen-encounter (**Fig. 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 *CD3ζ*- or *TRAC*-editing. However, we have also observed transgene-related differences (CD19-CAR vs HER2-CAR, see **Suppl. Fig. 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 (**Fig. 2**). Increasing the CD19-CAR-expression in *CD3ζ-trunc*CAR^{GSG}-T-cells was associated with enhanced cytokine production after antigen-engagement and improved anti-leukemia activity *in vivo* (**Fig. 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[60]. Of note, all CARs used in this study employed the CD28 co-stimulatory domain. Future studies should revisit the contribution of other co-stimulatory 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 (**Fig. 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$_\alpha$ and TCR$_\delta$ chains[61]. Further investigation is warranted to explore potential synergies between CARs and certain γ/δ-TCRs in this distinct cell type[47].

Unlike *TRAC, CD3ζ*-editing can be applied not only to all T-cell subsets but also to NK-cells (**Fig. 3**). Deleterious mutations of *CD3ζ* have been found to be a cause for severe combined immunodeficiency, and patient NK-cells were hypo-responsive in tumor co-cultures and after CD16 stimulation[62,63]. This raised concerns regarding the impact of *CD3ζ*-editing on the functionality of resulting CAR-NK-cells. However, in this study, *CD3ζ*-disruption in primary human NK-cells from healthy donors did not impair crucial immune functions such as ADCC, cytotoxicity or degranulation (**Fig. 4**). These findings align with previous research indicating that FcRγ compensates CD3ζ-loss post-knockout, thereby enabling ADCC by primary NK-cells[64].

This study is the first to demonstrate non-viral 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 heterogenous tumors. For example, the CD19-specific CAR-NK-cells generated by *CD3ζ*-editing (**Fig. 5**) may be combined with rituximab to overcome antigen-escape and relapse by CD19-negative cancer cells. 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 *CD3ζ*-editing in NK-cells may be related to manufacturing and cost-aspects of miniaturized non-viral vectors. Prior to testing in suitable *in vivo* models and future clinical translation, the efficacy of non-viral reprogramming of primary NK-cells should be further increased, for example by using pharmacological enhancers[33] and/or end-modified ssDNA donor templates[65].

CD3ζ-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 pre-treated 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[66,67,53]. Therefore, *CD3ζ*-editing would benefit from those modifications to improve the efficacy of allogeneic CAR-T-cells[53,68]. Future studies may investigate the combination of *CD3ζ*-editing with additional KOs to improve functionality[69,70], safety[71] as well as persistence[72,66,73] of allogeneic T and NK-cells. The respective additional edits required to improve the functionality of NK-cells[74,75] may differ to the ones proposed for T-cells[70,76]. Finally, complex editing may require the combination of nuclease-assisted gene transfer with other gene silencing modalities such as base editing[77,78] to reduce the risk for genomic rearrangements with unknown biological impact[53,76,79].

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VII. Author contributions

J.K. designed this study, planned, and performed experiments, analyzed results, interpreted the data, and wrote the manuscript. C.F., V.D., W.D., planned and performed experiments, analyzed results, interpreted the data, and edited the manuscript. V.G., M.St., T.Z., S.S., C.P., L.A., J.A. performed experiments and analyzed results. C.F.-G. performed and interpreted CAST-seq and provided respective sections for the manuscript. M.Su., J.H., R.S. planned experiments, interpreted data and edited the manuscript. H.A. provided materials (*HER2-CAR*-transgenes[33]), interpreted the data and edited the manuscript. A.K., M.A. 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., 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. All authors reviewed and approved the manuscript in its final form.

VIII. Conflict of Interest Disclosures

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 co-founders 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). All other co-authors report no conflict of interest related to this work.

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X. Figure Legends

Fig. 1: Integration of a truncated CD19-specific CAR into *CD3ζ*, 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 (*trunc*CAR) into *TRAC* or *CD3ζ*. (**b**) Flow cytometry dot plots after knock-in. Transgene integration into *TRAC* or *CD3ζ* disrupts expression of the TCR/CD3 complex. (**c**) Relative cytotoxicity in co-culture with (CD19$^+$) Nalm-6 target cells and CD19 knock-out 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-Šídák's multiple comparison test with a single pooled variance). (**d-g**) Functional testing of *CD3ζ trunc*CAR, 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 Geisser-Greenhouse correction + Holm-Šídák's 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 RNP/HDR templates) (n=4 biological replicates each in 1-3 technical replicates; two-way ANOVA followed by Holm-Šídák's 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; *CD3ζ* 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, 1x10^6 cryopreserved, 14-day expanded TCR-deleted CAR$^+$ T-cells were injected systemically. Tumor burden was assessed via bioluminescence imaging (BLI). (n=5-6; 2-way ANOVA with Geisser-Greenhouse correction of log-transformed BLI data followed by Holm-Šídák's 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 (ns : $p > 0.05$; * : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$).

Fig. 2: Evaluation of an optimized *CD3ζ trunc*CAR transgene and its impact on CAR-T cell function *in vitro*. (**a**) dsDNA templates for targeted delivery of a CAR or *trunc*CAR respectively into *TRAC* (left) or *CD3ζ* (middle), as in Fig. 1a, and for targeted delivery of a GSG-P2A-linker-modified *trunc*CAR into *CD3ζ* (right). (**b**) Top: Mean fluorescence intensity (MFI) determined by flow cytometry at steady (n=4 biological replicates in 4-6 technical replicates in two independent experiments, data normalized to mean of *TRAC* for each donor; mixed-effects analysis with Geisser-Greenhouse correction followed by Holm-Šídák's 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 biol. Replicates in 1-2 techn. replicates). (**c**) Relative cytotoxicity assessed in a 6-hour VITAL assay (similar to Fig. 1c, n=4 biological replicates in 3 technical replicates; two-way ANOVA followed by Holm-Šídák's 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 re-challenge in serial co-cultures 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-Šídák's multiple comparison test with a single pooled variance.). (**g**) Top: relative expansion of CAR^+ T-cells (top); 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 bio. repl.). (**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).

Fig. 3: *CD3ζ trunc*CAR-integration facilitates CAR-expression in different non-conventional T cell subtypes and NK-cells. (**a**) HLA-A2 CAR-integration in regulatory T-cells, n=3 biological replicates (**b**) CD19-CAR-integration in $TCR_{\gamma/\delta}$ T-cells. *TRAC* integration generates CAR^+/ $TCR_{\gamma/\delta}{}^+$ double positive T-cells, n=2 biological replicates (**c**) Integration of a CD19-CAR in primary human NK-cells, n=6 biological replicates

Fig. 4: *CD3ζ*-disuption does not impede canonical NK cell functions *in vitro*. (**a**) *CD3ζ* editing outcomes assessed by flow cytometry. (**b**) Cytotoxicity of primary *CD3ζ* disrupted NK-cells in simple 16h co-culture assay with K562 cells, Nalm-6 cells or allogeneic PBMC. (n=3 biological replicates); (**c**) Degranulation of primary *CD3ζ* disrupted NK-cells assessed by flow cytometry. (n=3 biological replicates; two-way ANOVA followed by Dunnett's multiple comparison test with a single pooled variance). (**d**) Expression of CD16 and *CD3ζ* in wild-type NK-92 cells and primary NK-cells after *CD3ζ*-disruption. (**e**) ADCC of primary *CD3ζ*-disrupted NK-cells against $CD20^+$ $bGal^-$ Jeko-1 cells at different concentrations of antibodies specific for CD20 (Rituximab) or bGal (n=3 biological replicates, each in 3 technical replicates).

Fig. 5: *CD3ζ*-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 *CD3ζ-trunc*CAR-integration: (**a**) CAR^+ frequencies after editing (n=6 biol. 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 *CD3ζ* -truncCAR integrated NK cells and robust coefficient of variation (robust CV) in CAR^+ cells (n=6 biol. replicates; Student's 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 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-Šídák's multiple comparison test with a single pooled variance); (**e**) antibody-dependent cellular cytotoxicity (ADCC) of primary (CAR) NK-cells against $CD20^+$ $bGal^-$ Jeko-1 cells. Bars represent killing for each condition in the presence of the CD20-targeting monoclonal antibody (0.5µg/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 promotor-controlled, full-length CAR or *CD3ζ* integration of a *trunc*CAR. CAR^+ fractions were enriched using MACS. (**f**) CAR-

892 expression in flow cytometry histograms. (**g**) CAR-dependent cytotoxicity in a 4-hour
893 VITAL-assay (n=6 technical replicates; two-way ANOVA with Tukey's multiple
894 comparison test with a single pooled variance. (**h**) CAR-independent cytotoxicity
895 towards the MHC I deficient, $CD19^-$ K562 (control) cell line (n=15 technical replicates;
896 two-way ANOVA followed by Holm-Šídák's multiple comparison test with a single
897 pooled variance).

Generation of Potent CAR-T Cells and CAR-NK Cells via Targeted Gene Transfer into the Genomic *CD3ζ* Locus

New locus for CAR integration

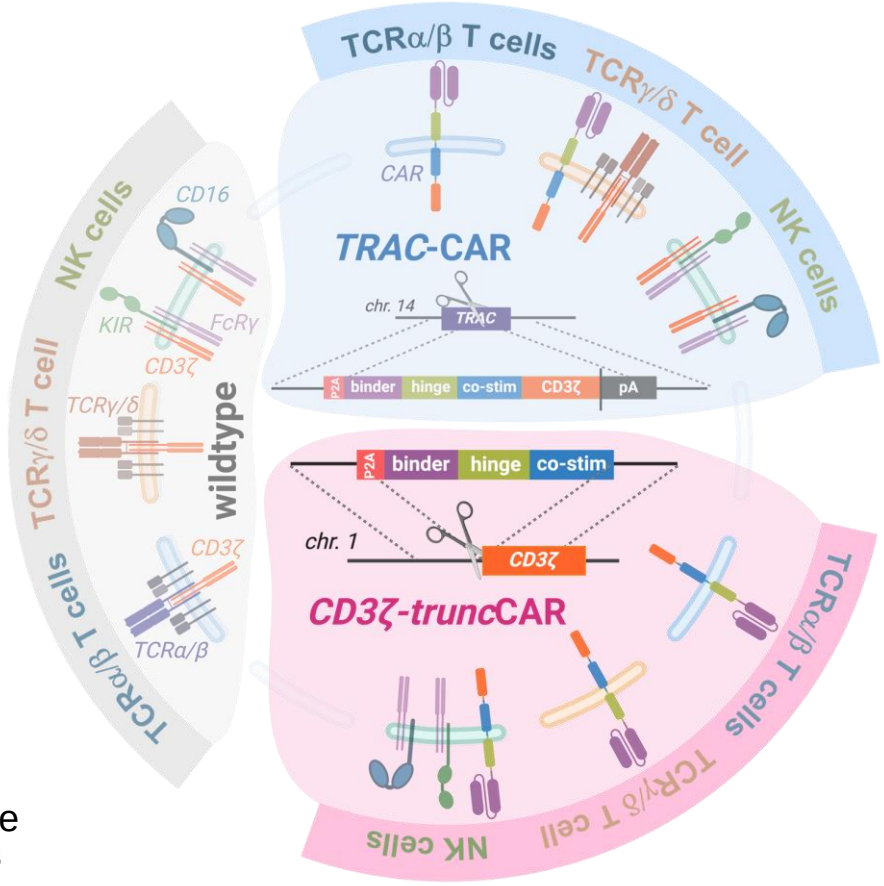
- Size reduction of transgenes via utilization of genomic *CD3ζ* as CAR effector domain
- Physiological CAR expression and regulation by *CD3ζ's* promoter and UTRs

T cells

- *CD3ζ*-CAR T cells lack TCR expression and allo-reactivity, similar to *TRAC*-editing
- Anti-leukemia activity comparable to *TRAC*-editing or lentivirus
- Effective in TCRα/β, TCRγ/δ and regulatory T cells

NK cells

- Canonical effector functions and comparable anti-tumor activity to lentiviral CAR-NK cells



Conclusion: **Non-viral CRISPR-Cas gene editing of the *CD3ζ* locus enables the development of effective CAR-T and CAR-NK cell products for autologous and allogeneic 'off-the-shelf' applications.**

Figure 1

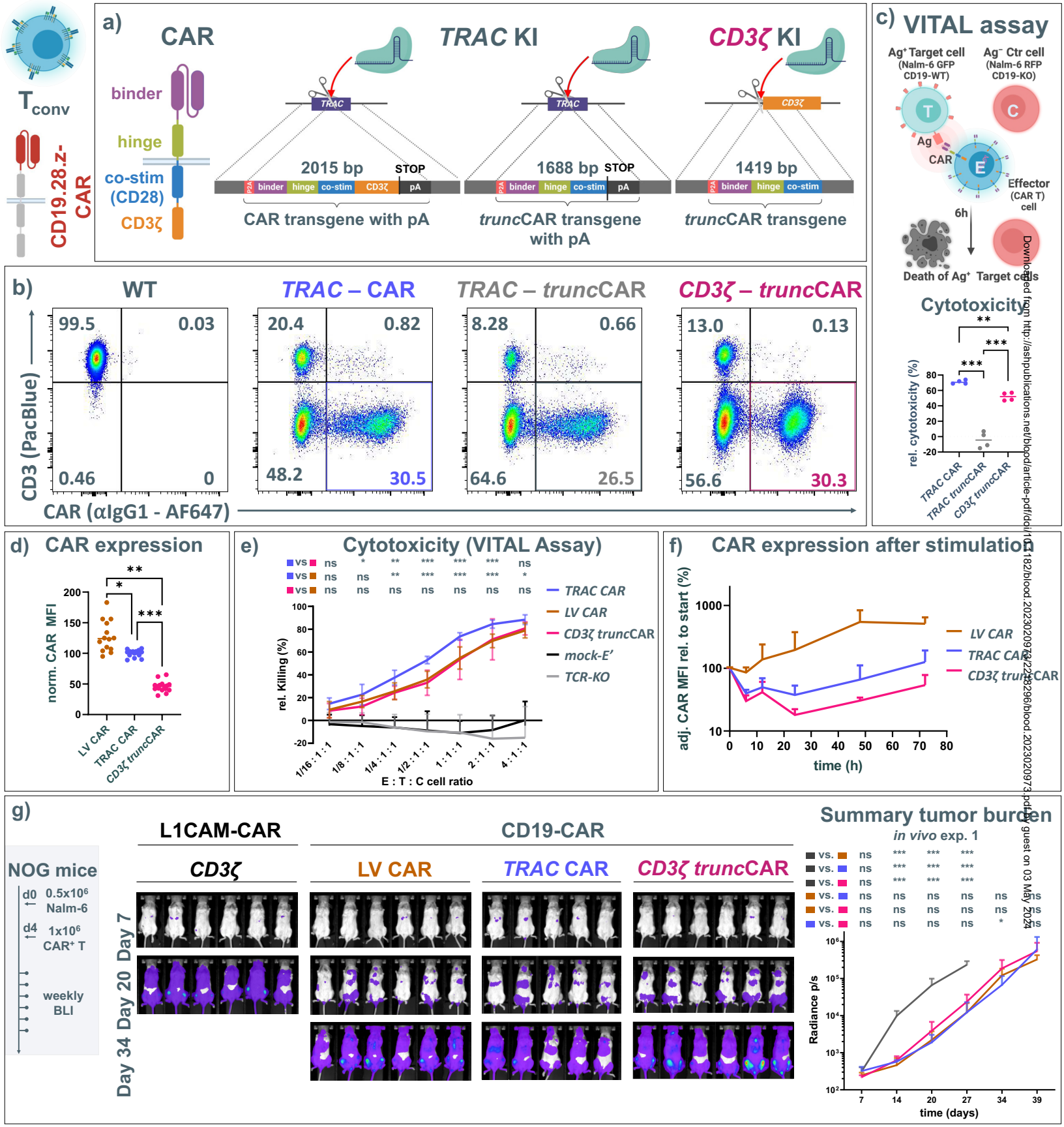


Figure 2

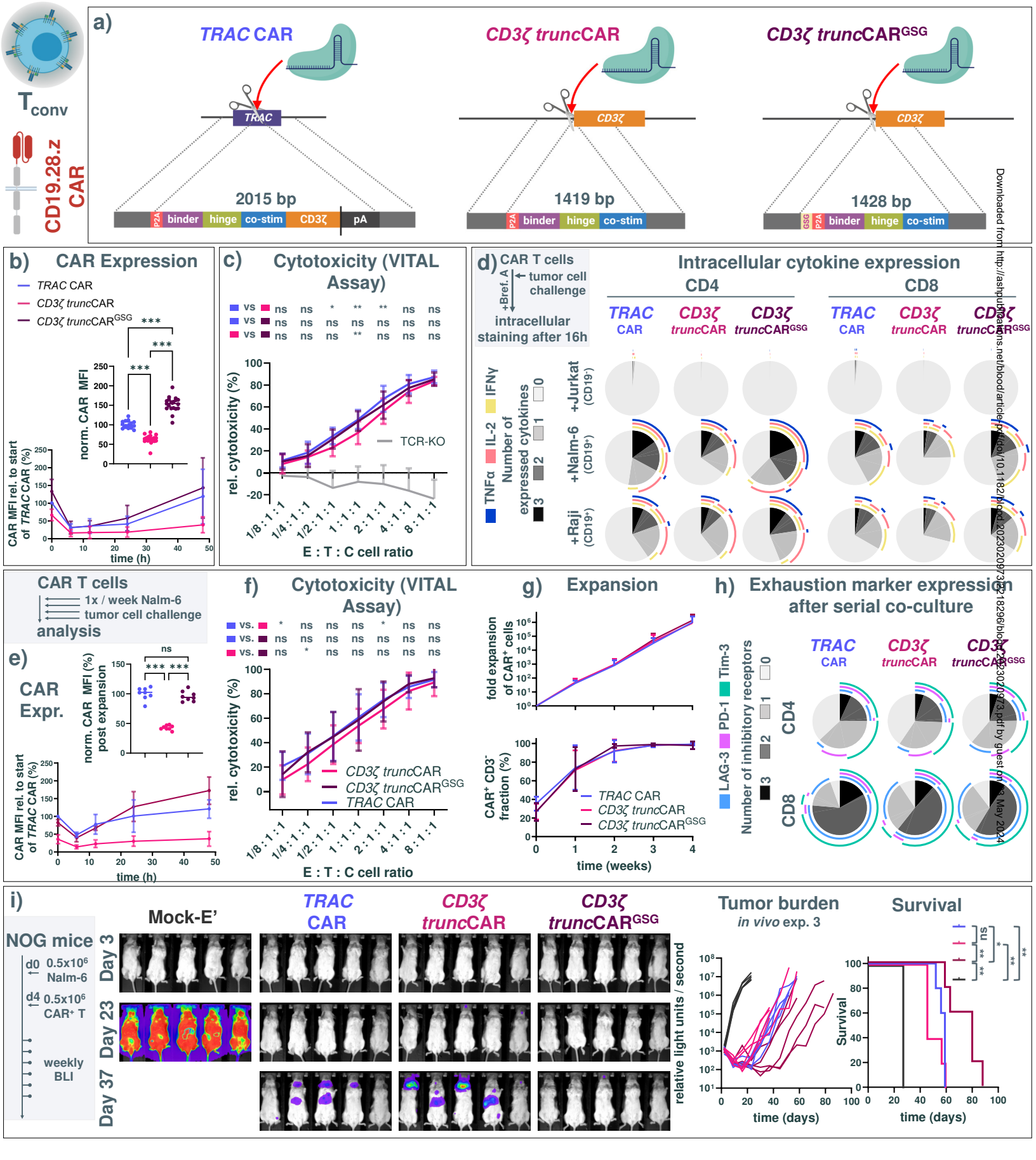


Figure 3

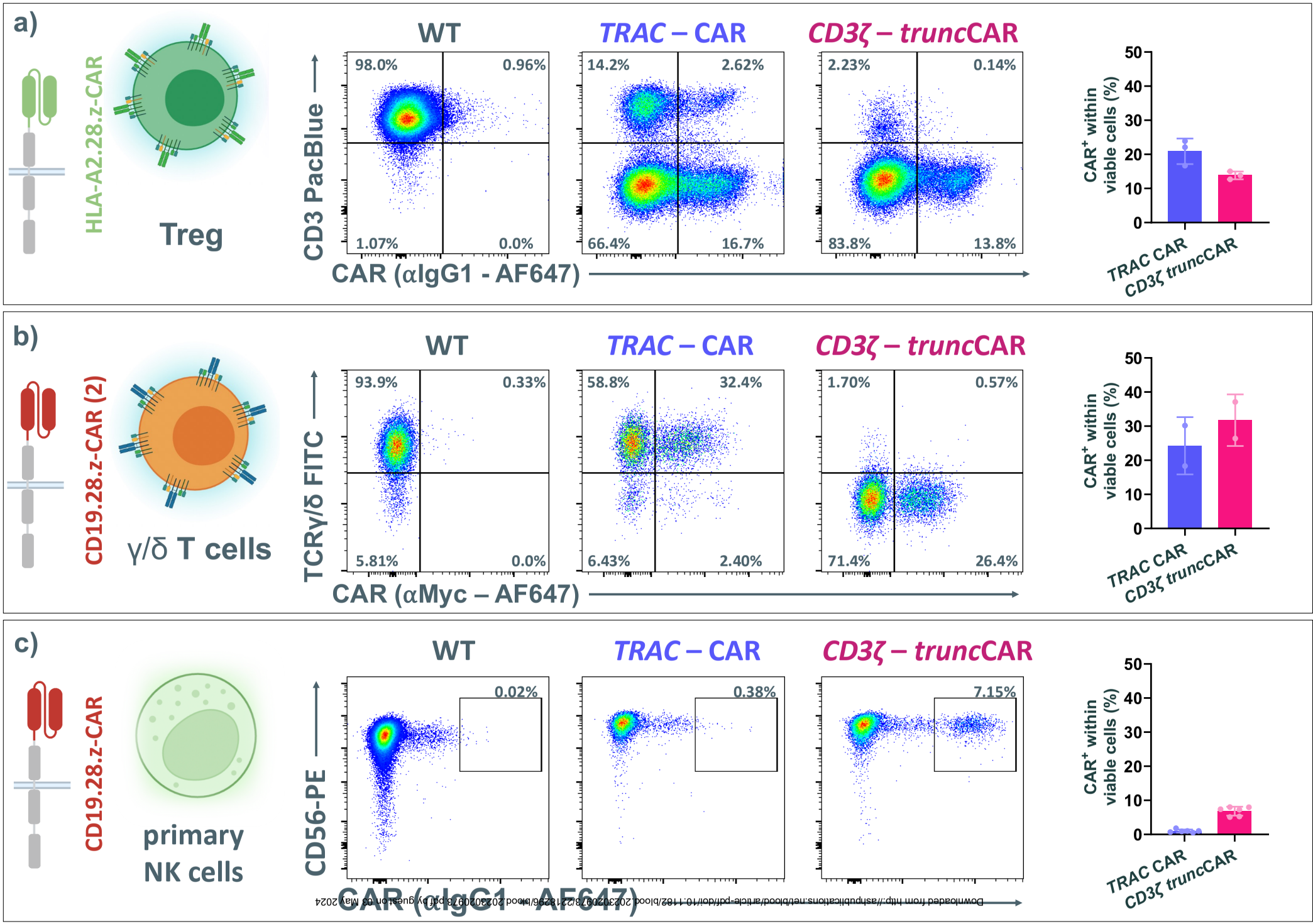


Figure 4

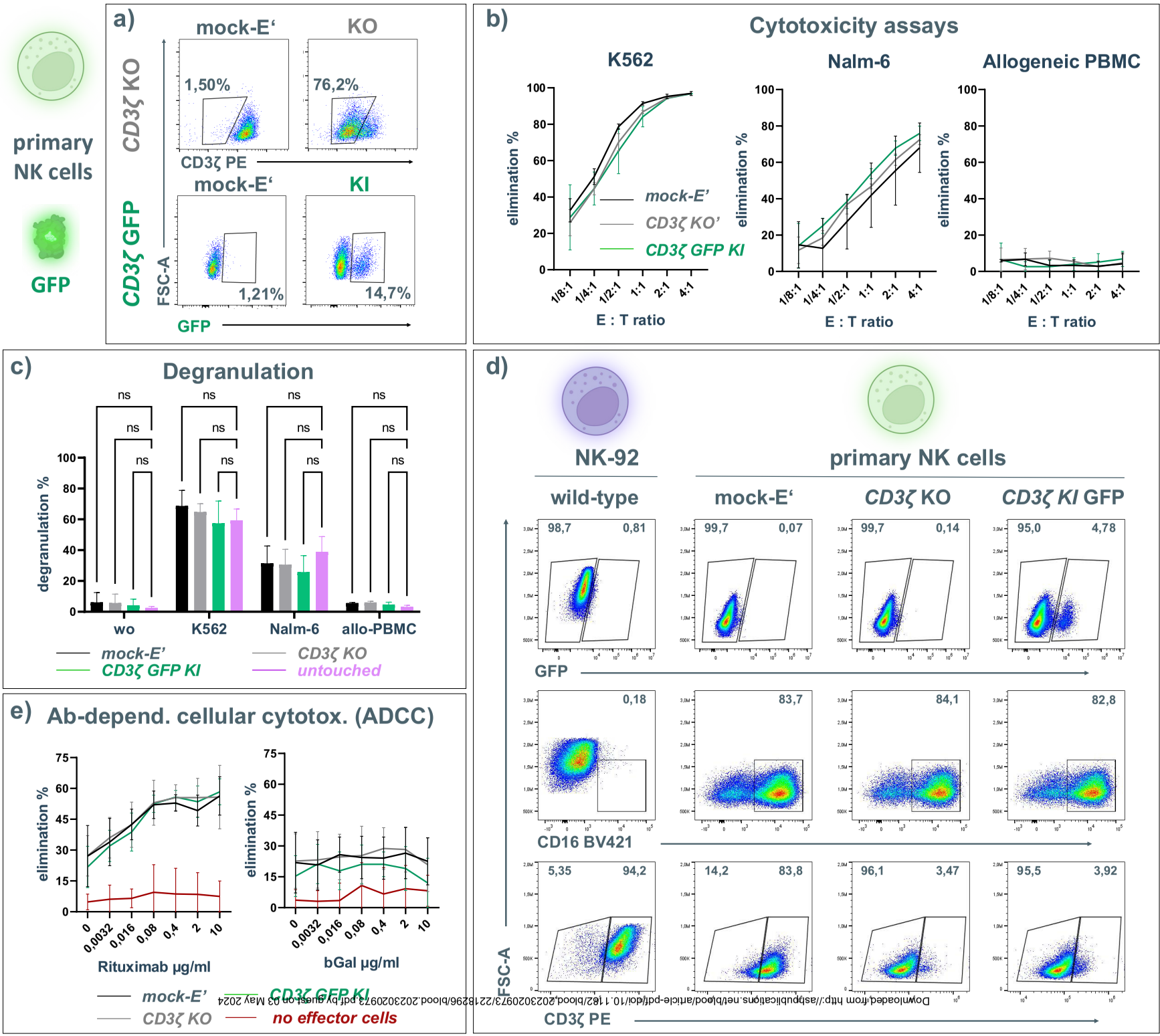


Figure 5