


# Combining CRISPR-Cas9 and TCR exchange to generate a safe and efficient cord blood-derived T cell product for pediatric relapsed AML

Vania Lo Presti,<sup>1,2</sup> Angelo Meringa,<sup>2</sup> Ester Dunnebach,<sup>1</sup> Alice van Velzen ,<sup>1</sup> Aida Valera Moreira,<sup>1</sup> Ronald W Stam,<sup>1</sup> Rishi S Kotecha,<sup>3,4</sup> Anja Krippner-Heidenreich,<sup>1</sup> Olaf T Heidenreich,<sup>1</sup> Maud Plantinga,<sup>2</sup> Annelisa Cornel,<sup>1,2</sup> Zsolt Sebestyen,<sup>2</sup> Jurgen Kuball,<sup>2</sup> Niek P van Til,<sup>2,5,6</sup> S Nierkens<sup>1,2</sup>

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NPvT and SN contributed equally.

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For numbered affiliations see end of article.

## Correspondence to

Dr S Nierkens;  
S.Nierkens-2@prinsesmaximacentrum.nl

## ABSTRACT

**Background** Hematopoietic cell transplantation (HCT) is an effective treatment for pediatric patients with high-risk, refractory, or relapsed acute myeloid leukemia (AML). However, a large proportion of transplanted patients eventually die due to relapse. To improve overall survival, we propose a combined strategy based on cord blood (CB)-HCT with the application of AML-specific T cell receptor (TCR)-engineered T cell therapy derived from the same CB graft.

**Methods** We produced CB-CD8<sup>+</sup> T cells expressing a recombinant TCR (rTCR) against Wilms tumor 1 (WT1) while lacking endogenous TCR (eTCR) expression to avoid mispairing and competition. CRISPR-Cas9 multiplexing was used to target the constant region of the endogenous TCR $\alpha$  (*TRAC*) and TCR $\beta$  (*TRBC*) chains. Next, an optimized method for lentiviral transduction was used to introduce recombinant WT1-TCR. The cytotoxic and migration capacity of the product was evaluated in coculture assays for both cell lines and primary pediatric AML blasts.

**Results** The gene editing and transduction procedures achieved high efficiency, with up to 95% of cells lacking eTCR and over 70% of T cells expressing rWT1-TCR. WT1-TCR-engineered T cells lacking the expression of their eTCR (eTCR<sup>-/-</sup> WT1-TCR) showed increased cell surface expression of the rTCR and production of cytotoxic cytokines, such as granzyme A and B, perforin, interferon- $\gamma$  (IFN $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), on antigen recognition when compared with WT1-TCR-engineered T cells still expressing their eTCR (eTCR<sup>+/+</sup> WT1-TCR). CRISPR-Cas9 editing did not affect immunophenotypic characteristics or T cell activation and did not induce increased expression of inhibitory molecules. eTCR<sup>-/-</sup> WT1-TCR CD8<sup>+</sup> CB-T cells showed effective migratory and killing capacity in cocultures with neoplastic cell lines and primary AML blasts, but did not show toxicity toward healthy cells.

**Conclusions** In summary, we show the feasibility of developing a potent CB-derived CD8<sup>+</sup> T cell product targeting WT1, providing an option for post-transplant allogeneic immune cell therapy or as an off-the-shelf

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Relapses still occur in significant numbers after hematopoietic cell transplantation in children with high-risk acute myeloid leukemia (AML), necessitating the development of add-on immune therapies to protect against residual disease.

## WHAT THIS STUDY ADDS

⇒ This study shows that elimination of both alpha and beta chains of endogenous T cell receptor (eTCR), by targeting *TRAC* and *TRBC* genes using CRISPR/Cas9, combined with simultaneous genetic modification to express a recombinant Wilms tumor 1 (WT1)-TCR, is feasible and highly successful in cord blood T cells.

⇒ Cord blood-derived T cells lacking eTCR expression and expressing WT1-TCR are efficient in killing primary AML blasts in a *in vitro* three-dimensional model system.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Applying the multiplex CRISPR/Cas9 technology, to eliminate the eTCR expression, T cells in cord blood-derived T cells expressing WT1-TCR may provide an efficient and safe approach to reduce AML relapses after cord blood hematopoietic cell transplantation.

product, to prevent relapse and improve the clinical outcome of children with AML.

## INTRODUCTION

Relapse after hematopoietic cell transplantation (HCT) in children with acute myeloid leukemia (AML) is still a leading cause of mortality, accounting for approximately 30% of deaths in patients transplanted for AML.<sup>1</sup> Additional therapeutic approaches are therefore essential to increase overall survival. Wilms tumor 1 (WT1) overexpression is

reported in 75%–90% of all patients with AML<sup>2</sup> and has been proposed as a potential marker of minimal residual disease.<sup>3</sup> The use of allogeneic T cell receptor (TCR) T cells targeting WT1, post-HCT, recently showed promising results in terms of safety and prevention of relapse in adults,<sup>4</sup> prompting the question of whether WT1-TCR-mediated therapy could also be implemented in pediatric AML HCT patients.

Cord blood (CB)-HCT has been shown to be an effective treatment for relapsed or refractory AML, especially in children who lack a matched bone marrow donor.<sup>5</sup> CB-T cells have an intrinsic capacity to stimulate graft versus leukemia responses with a low probability of developing graft versus host disease (GvHD), compared with adult peripheral blood mononuclear cells (PBMCs).<sup>6</sup> The transfer of a product bearing tumor-specific recombinant TCR (rTCR) post-CB-HCT may further enhance the efficacy of the antileukemia response. Generating tailor-made TCR-T cell products from immune cells of children with leukemia is complex and often not feasible due to lymphocytopenia, inherent to the disease condition and/or cancer treatment (eg, chemotherapy).<sup>7</sup> In addition, the time between immune cell harvest and availability of the cellular drug product remains a limiting factor, especially in the late stage of the disease, which leads to rapid deterioration in patients' health.<sup>8</sup> Instead of using allogeneic T cells,<sup>4</sup> we propose the generation of a graft-derived rTCR-engineered T cell product. Around 20% of the available CB units are stored separately as a frozen product and predominantly serve for quality control or research purposes, but can also be used for generating complementary cellular immunotherapeutic products. The use of CB-derived graft T cells for adoptive T cell therapy post-HCT may be a promising option.<sup>9</sup>

To limit the risk of mispairing TCRs and to reduce coreceptor binding competition due to the simultaneous presence of rTCR and endogenous TCR (eTCR) alpha and beta chains, recently developed gene editing techniques can be exploited to eliminate eTCR expression and further improve efficacy and safety. Here we show an efficient and relatively fast strategy for gene editing in CB-CD8<sup>+</sup> T cells using CRISPR-Cas9, graphically illustrated in online supplemental figure 1, that eliminates the expression of both the alpha and beta chains of the eTCR, coupled with the use of lentiviral vectors coding for a WT1-specific TCR (WT1-TCR) to produce eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells. As previously described in PBMCs, eliminating both chains of the eTCR abolishes the possibility of generating TCR mispairing and increases the potential of the final retrovirally engineered rTCR CB-derived T cell product.<sup>10 11</sup> eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells are characterized by high proliferation rate and viability, and display high killing capacity for both immortalized and primary AML cells naturally expressing WT1.

## MATERIALS AND METHODS

### CB-derived CD8<sup>+</sup> T cell isolation and expansion

Fresh umbilical CB was collected after informed consent was obtained according to the Declaration of Helsinki. CB was processed to isolate CD8<sup>+</sup> T cells using Ficoll (GE Healthcare Bio-Sciences AB) separation and CD8-positive magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotec). CD8<sup>+</sup> T cells were subsequently cultured in cytotoxic T lymphocyte (CTL) media (consisting of RPMI (Fisher Scientific) supplemented with 10% human serum, 1% penicillin/streptomycin (P/S), 50 U of IL (Interleukin)-2/mL (Proleukin, UMCU Pharmacy), 5 ng/mL IL-7, and 5 ng/mL IL-15 (Miltenyi Biotec)) and activated for 3 days with anti CD3/CD28 Dynabeads (GIBCO, Thermo Fisher Scientific) at a 1:3 ratio (beads:T cells).

### CRISPR-Cas9-mediated gene editing of CB-CD8<sup>+</sup> T cells

Ribonucleoprotein (RNP) complexes were generated using an equal concentration (45 μM) of Alt-R S.p. Cas9 Nuclease V3 (1081059; IDT) and Alt-R CRISPR-Cas9 crRNA:Alt-R CRISPR-Cas9 tracrRNA (online supplemental figure 2) at a 1:1 volume ratio and incubated for 15 min at RT (Room Temperature). Magnetic separation was applied to expanded CB-CD8<sup>+</sup> T cells to remove CD3/CD28 beads. 5×10<sup>5</sup> cells per condition were resuspended in T buffer (Neon Transfection System 10 μL Kit, ThermoFisher). Electroporation was performed following the producer-suggested settings for Jurkat cells (1600 V/10 ms/3 pulses). After electroporation, cells were transferred into a 96-well plate with CTL media supplemented with 200 U of IL-2/mL, 20 ng/mL IL-7, and 20 ng/mL IL-15 (supplemented CTL media) and incubated at 37°C and 5% CO<sub>2</sub>.

### Lentiviral vector production and titration

HIV-derived self-inactivating third-generation lentiviral vectors were constructed using the CMV (Cytomegalovirus) enhancer and promoter driving expression of the viral transcript (pCCL plasmid backbone).<sup>9 12–14</sup> A WT1-specific codon-optimized TCR sequence<sup>15</sup> was cloned into the lentiviral transfer vector plasmid under control of the myeloproliferative sarcoma virus enhancer (MND) promoter.<sup>16</sup> Lentiviral vectors were produced by transient cotransfection of HEK293T cells with the lentiviral transfer vector plasmid (LV.WT1-TCR) and respective packaging plasmids (pRSV-Rev, pMDLg/pRRE, and pMD2-VSV-G) using CalPhos Mammalian Transfection Kit (Clontech), as previously described.<sup>9</sup> Viral supernatants were filtered through 0.45 μm low-protein binding filters, concentrated by ultracentrifugation at 20 000 rpm for 2 hours, resuspended in StemMACS HSC Expansion Media (Miltenyi Biotec) and stored at –80°C. Vesicular stomatitis virus (VSV)-G protein pseudotyped lentiviral particles were titrated on Jurkat cells. Concentrated viral vector supernatants were serially diluted on Jurkat cells. At 72 hours post-transduction, cells were harvested and analyzed by flow cytometry for expression of TCRVβ21.3.

recognizing the recombinant WT1-TCR, to determine viral titer.

### CB-CD8<sup>+</sup> T cell transduction

Expanded and stimulated CB-CD8<sup>+</sup> T cells were transduced overnight (14–16 hours) in StemMACS HSC Expansion Media XF (Miltenyi Biotec) supplemented with 1% P/S at multiplicity of infection (MOI) 10 using LV.WT1-TCR. After 14–16 hours, transduction media was replaced with complete CTL media. Transduction was performed in the presence of the transduction adjuvant LentiBOOST (1:100 of the total volume; SIRION Biotech).

### Flow cytometry

Cells were stained with fluorescently labeled antibodies against CD3-Pacific Blue (clone UCHT1, ref. 558117, BD), CD8-PE (clone RPA-T8, ref. 301008, Biolegend), Fixable Viability Dye eFluor 780 (ref. 65-0865-14, ThermoFisher), CD45-PeCy7 (clone HI30, ref. 557748, BD), and TCR-pan- $\alpha\beta$  (clone IP6, ref. 17-9986-42, eBioscience) to evaluate knockout (KO) efficiency, and TCRV $\beta$ 21.3 (clone IG125, ref. PN IM1483, Beckman Coulter) to evaluate transduction efficiency. Antigen recognition was confirmed using tetramer staining (PE-WT1-126/APC-WT1-126; peptide sequence: RMFPNAPYL), generated as previously described.<sup>9,17</sup> To determine the immunophenotype, transduced CD8<sup>+</sup> T cells were stained with fluorescently labeled antibodies against Dye eFluor 780 (ref. 65-0865-14, ThermoFisher), CD62L-BV650 (clone DREG-56, ref. 2124160, Sony Biotechnology), CD25-PerCP Cy5.5 (clone BC96, ref. 2113130, Sony Biotechnology), TIM3-APC (clone 34482, ref. FAB2365A, R&D), CD3-AF700 (clone UCHT1, ref. 300424, Sony Biotechnology), CD45RA-BV421 (clone HI100, ref. 304118, Biolegend), PD1-BV711 (clone EH12.2H7, ref. 329928, Biolegend), LAG3-PE (ref. FAB2319P, R&D), and CD8-PeCy7 (clone SK1, ref. 335822, BD). HLA-A2 and WT1 positivity was determined in target cells (K562, K562 HLA-A2<sup>+</sup>, PER-485, PER-703<sup>17</sup>) by flow cytometry (HLA-A2: clone BB7.2, ref. 558570, BD Pharmingen; clone WT1: F6-H2, Dako). For the expression of WT1, cells were fixed/permeabilized using the Foxp3/transcription factor staining buffer set (ref. 00-5523-00, eBioscience) according to the manufacturer's description. After fixation, cells were intracellularly stained with a primary antibody against WT1 (clone F6-H2, Dako) and a secondary polyclonal fluorescently labeled antibody against F(ab')<sub>2</sub> anti-mouse IgG-PE (ref. 12-4010-82, eBioscience). Flow cytometry analysis was performed on a BD Fortessa using FACSDiva, with acquisition of a fixed number of cells.

### ImageStream

The Amnis ImageStream X Mark II multispectral imaging system was used.  $1 \times 10^6$  unmanipulated CB-CD8<sup>+</sup> cells and eTCR<sup>-/-</sup> CD8<sup>+</sup> T cells were washed and stained with antibodies against CD3 (clone OKT3, ref. 317306, Biolegend), CD8-PE (clone RPA-T8, ref. 301008, Biolegend), and

TCR-pan- $\alpha\beta$  (clone IP6, ref. 17-9986-42, eBioscience) to detect membrane expression. Subsequently, cells were fixed, permeabilized, and stained with anti-CD3 (clone UCHT1, ref. 300420, Biolegend) to determine cytoplasmic expression of the coreceptor.

### Functional assays

T2 cells, K562, K562 HLA-A2<sup>+</sup>, PER-485, and PER-703 were kept in culture in complete RPMI (Glutamax, 1% P/S, 10% FCS) at a concentration of  $0.3 \times 10^6$ /mL.

For the activation assay, eTCR<sup>+/+</sup> WT1-TCR and eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells were incubated overnight in four conditions: (1) T cells only, (2) in the presence of PMA (phorbol myristate acetate)-ionomycin, (3) with T2 cells, and (4) with T2 cells loaded with WT1-p126. Effector cells and target cells were maintained at a ratio of 1:1. After 14–16 hours, supernatant was collected to measure the concentration of secreted cytokines. Cells were washed and stained with CD3, CD8, CD137, and TCRV $\beta$ 21.3. The percentage of activated T cells in coculture with T2s was calculated proportionally to the activation after PMA-ionomycin treatment. The LEGENDplex CD8/NK cells kit (Biolegend) was used to quantify the cytokine concentrations released in the supernatant. The experiment was performed following the manufacturer's protocol and analyzed using a BD Fortessa. Data analysis was performed using the Qognit software supplied by Biolegend.

For the killing assay, control untransduced (CTRL-untransduced), eTCR<sup>+/+</sup> WT1-TCR, and eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells were incubated overnight with tumor cell lines HLA-A2<sup>+</sup> naturally expressing WT1. K562 HLA-A2<sup>-</sup> cells were used as a negative CTRL to determine bystander killing. K562 HLA-A2<sup>+</sup> was generated via overnight lentiviral transduction using a lentivirus encoding for the HLA-A2 gene (LV.HLA-A2). Effector cells and target cells were maintained at a ratio of 5:1 (K562 and K562 HLA-A2<sup>+</sup>) or 10:1 (PER-485 and PER-703). Target cells were labeled with CellTrace Violet before coculture. After 12–14 hours, the cells were washed, stained with Fixable Viability Dye eFluor 780 (ref. 65-0865-14, ThermoFisher), and analyzed for cell death with the BD Fortessa.

For the safety evaluation, eTCR<sup>+/+</sup> WT1-TCR and eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells were incubated overnight with HLA-A2<sup>+</sup> CB-derived stem cells (CD34<sup>+</sup>). Effector cells were labeled with CellTrace Violet before coculture. After 16 hours, cells were washed, stained with Fixable Viability Dye eFluor 780 (ref. 65-0865-14, ThermoFisher), and analyzed for cell death with the BD Fortessa.

### 3D model

The three-dimensional (3D) bone marrow model experiments were performed based on previously described protocol with the alterations mentioned in the following paragraph.<sup>18</sup> In short, primary pediatric AML blasts (AML8-9-10/SKION Biobank) were selected at relapse

and for HLA-A2 and WT1 positivity. The WT1<sup>+</sup> tumor cells were dyed with Vybrant DiO (ThermoFisher, USA) and 25 000 AML blasts were seeded in Matrigel (Corning, USA) combined with 25 000 endothelial progenitor cells (EPCs) and multipotent 70 000 mesenchymal stromal cells (MSCs). The EPCs and MSCs were dyed with Vybrant DiD (ThermoFisher, USA). After 4 days of incubation, 50 000 transduced T lymphocytes or WT1-TCR<sup>+</sup> T lymphocytes were dyed with Vybrant DiI (ThermoFisher, USA) and administered to the 3D models. Two days post-T cell administration, Matrigel was dissolved using Cell Recovery Solution (Corning) to retrieve a single cell suspension. Subsequently, tumor cells, T lymphocytes, and stromal cell numbers were quantified by flow cytometry using Flow-Count Fluorospheres (Beckman Coulter, USA).

### Statistical analysis

Error bars represent SD across at least three donors. Significance was calculated using Student's t-test or two-way analysis of variance in GraphPad Prism V.8.3. Statistical significance is presented as \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  and \*\*\*\* $p \leq 0.0001$ .

## RESULTS

### Highly efficient KO of TCR $\alpha\beta$ using a CRISPR-Cas9 multiplexing approach to target the *TRAC* and *TRBC* locus

eTCR<sup>-/-</sup> in CB-CD8<sup>+</sup> T cells was performed using RNP complex electroporation (gRNAs:Cas9), with gRNAs targeting both the constant region of the alpha chain (*TRAC*) and the constant region of the beta chain (*TRBC*). The use of single gRNAs showed a KO efficiency, measured as lack of TCR expression, of 82.4% $\pm$ 10.6 for the alpha chain and 96.8% $\pm$ 1.5 for the beta chain. Concomitant use of both gRNAs showed lack of TCR expression in 96.5% $\pm$ 1.5 of cells. As expected, eTCR<sup>-/-</sup> lacked membrane expression of both eTCR and the coreceptor CD3 (figure 1A,B). Single cell images produced using ImageStream analyses confirmed complete absence of the eTCR and CD3 on the cell membrane, but edited CB-CD8<sup>+</sup> T cells maintained CD8 membrane presence and showed intracellular presence of CD3 (figure 1C).

### eTCR<sup>-/-</sup> CB-CD8<sup>+</sup> T cells show similar transduction efficiency compared with eTCR<sup>+/+</sup> CB-CD8<sup>+</sup> T cells

eTCR<sup>-/-</sup> CB-CD8<sup>+</sup> T cells and paired unedited eTCR<sup>+/+</sup> CB-CD8<sup>+</sup> T cells were transduced with a lentiviral vector coding for WT1-TCR, with an MOI of 10. Transduction efficiency, defined by expression of the specific V $\beta$  chain characterizing the WT1-TCR (V $\beta$ 21.3) and by tetramer reactivity, was high in both eTCR gene edited and unedited cells. However, eTCR<sup>-/-</sup> cells showed a slight increase in the percentage of cells expressing the WT1-TCR, with an average of 79.8% $\pm$ 9.1 compared with an average of 73.8% $\pm$ 17.2 for eTCR<sup>+/+</sup> CB-CD8<sup>+</sup> T cells. In addition, eTCR<sup>-/-</sup> WT1-TCR compared with eTCR<sup>+/+</sup> WT1-TCR CB-CD8<sup>+</sup> T cells showed a higher expression of WT1-TCR, calculated as mean fluorescent intensity, of 2151 $\pm$ 700 and

1775 $\pm$ 731, respectively (figure 2A,B). In both cases, the difference was not statistically significant. In both T cell products, reactivity to the target antigen was confirmed by staining with fluorescent tetramer molecules, confirming that the WT1-TCR-expressing cells were fully recognizing the cognate peptide (figure 2C,D).

### eTCR<sup>-/-</sup> does not alter CB-CD8<sup>+</sup> T cell phenotype but increases activation and cytotoxic cytokine production compared with eTCR<sup>+/+</sup> WT1-TCR CB-CD8<sup>+</sup> T cells

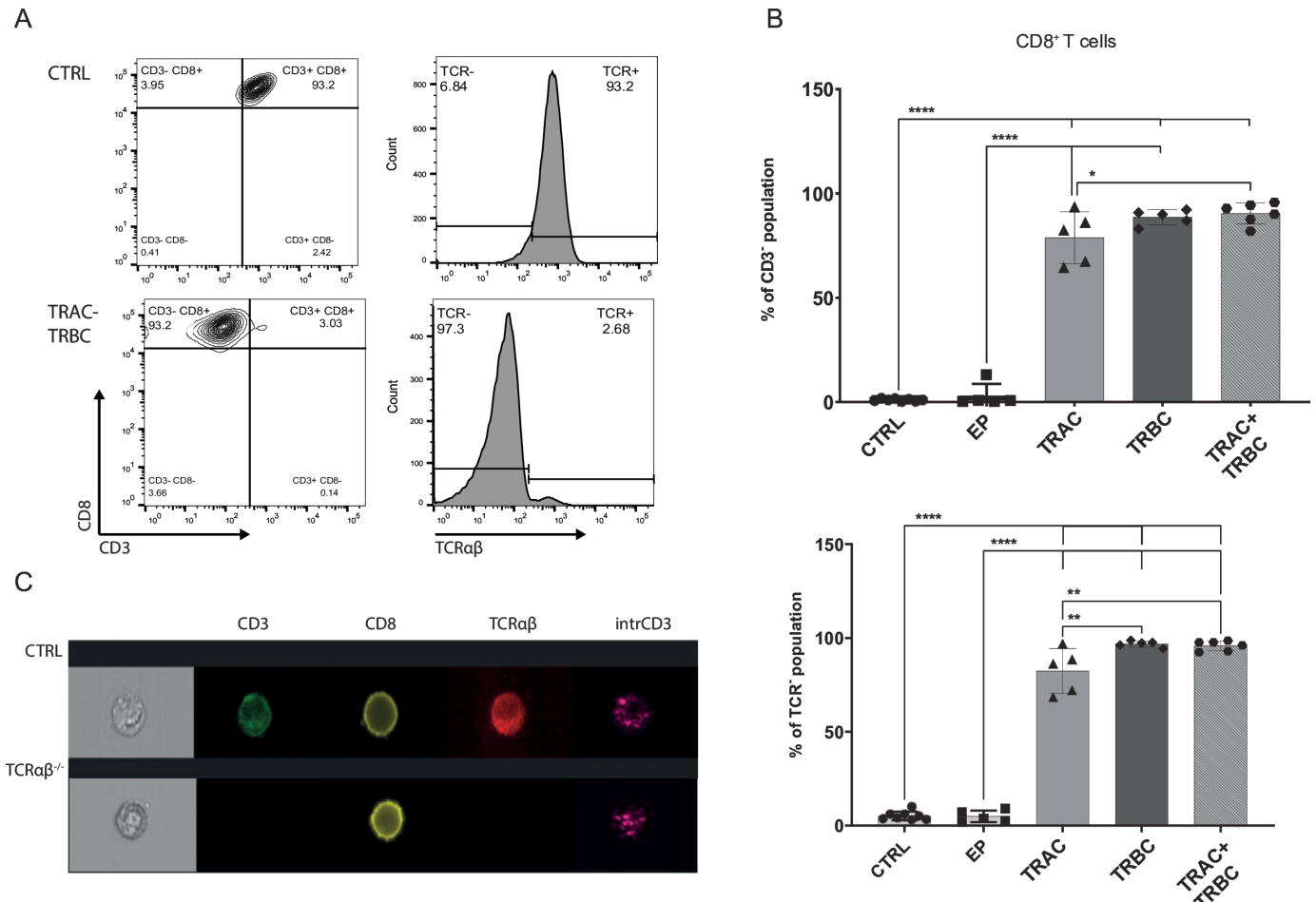
Immunophenotyping of the transduced cells showed that CRISPR-Cas9 editing did not affect the phenotype of the cells. Both eTCR<sup>-/-</sup> WT1-TCR and eTCR<sup>+/+</sup> WT1-TCR T cells expressed similar levels of CD45RA and CD62L and were predominantly characterized by two populations, namely T1 (CD45RA<sup>+</sup>, CD62L<sup>-</sup>) and T2 (CD45RA<sup>+</sup>, CD62L<sup>+</sup>), with a small percentage of double-negative cells, T4 (CD45RA<sup>-</sup>, CD62L<sup>-</sup>). Moreover, the expression of activation and inhibitory markers remained unaltered between the two products, with the only exception being an increased expression of CD127 (IL-7Ra) in eTCR<sup>-/-</sup> WT1-TCR T cells (figure 3A,B). Nevertheless, when cultured with T2 cells loaded with the cognate WT1 peptide, eTCR<sup>-/-</sup> WT1-TCR T cells showed a higher level of activation, defined by CD137 expression (figure 3C), compared with eTCR<sup>+/+</sup> WT1-TCR T cells. Both cell products were able to upregulate CD25 and PD1 on antigen recognition (online supplemental figure 3). Moreover, a trend of higher cytotoxicity, measured by the ability of eTCR<sup>-/-</sup> WT1-TCR T cells to release cytotoxic cytokines, was observed especially for Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), Interferon- $\gamma$  (IFN $\gamma$ ), and granzyme A and B (figure 3D).

### eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells show an increased killing capacity toward cell lines naturally expressing WT1

K562 cells have been reported to lack expression of human leukocyte antigen (HLA) class I and II proteins,<sup>19</sup> but these cells are WT1 positive. Hence, these cells were considered as a negative control for HLA-independent reactivity of the T cells. In addition, K562 cells were modified to express HLA-A2 (K562 HLA-A2<sup>+</sup>) to monitor reactivity toward WT1 peptide presented in HLA-A2 (figure 4A). To assess the direct cytotoxic capacity of the aforementioned cell products, a preliminary screening on AML patient-derived cell lines was performed to define the expression of HLA-A2 and WT1. Two lines were selected for further experiments, further referred to as PER-485 and PER-703 (figure 4A). Overnight coculture experiments with different ratios of effector and target cells were performed to define the best ratio (5:1 for K562s and 10:1 for PER-485/PER-703) (data not shown). In line with the increased CD137 and cytokine expression, in all cases, the cytotoxicity of eTCR<sup>-/-</sup> WT1-TCR T cells was more pronounced than of eTCR<sup>+/+</sup> WT1-TCR T cells (figure 4B,C).

### eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells show cytotoxic capacity in a 3D model resembling the bone marrow niche of patients

A 3D bone marrow niche model was used to determine the cytotoxic capacity toward primary AML blasts



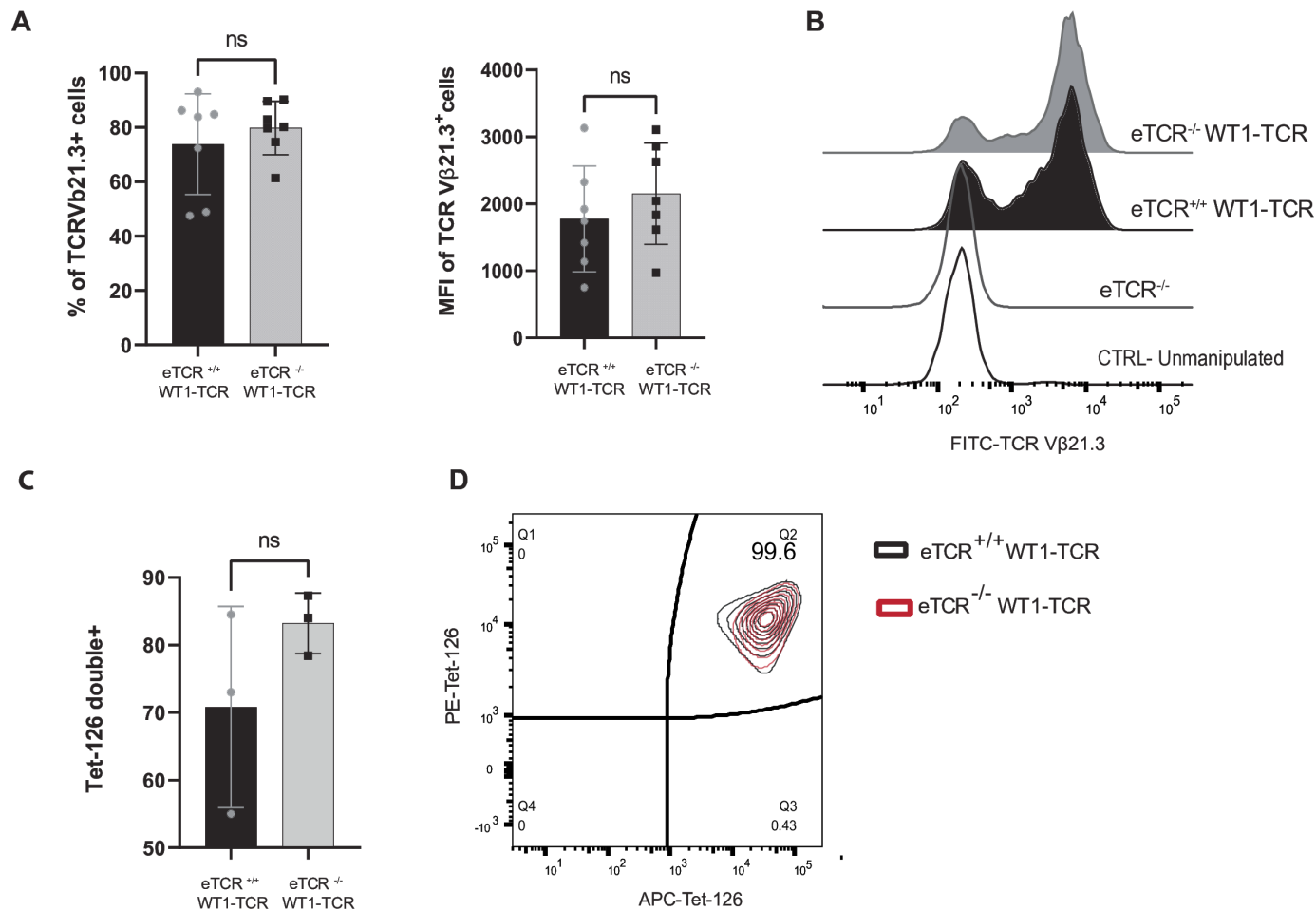
**Figure 1** CRISPR-Cas9-mediated TCR $\alpha\beta$  gene editing targeting the alpha (*TRAC*) and beta (*TRBC*) chains in CB-CD8<sup>+</sup> T cells. (A) Representative flow cytometry plot of TCR $\alpha\beta$  membrane complexes in expanded and unedited CB-CD8<sup>+</sup> T cells (CTRL) and CB-CD8<sup>+</sup> transfected with ribonucleoprotein complex targeting the *TRAC* and *TRBC* locus separately or simultaneously. Flow cytometry analysis was performed 4 days after electroporation. (B) Percentage of CD3-negative population (top panel) and TCR $\alpha\beta$ -negative population (bottom panel) comparing single targeting of *TRAC* (n=5), *TRBC* (n=5), and simultaneous *TRAC+TRBC* (n=6) with CTRL cells. (C) Representative images of single cells using ImageStream. CTRL is shown in the top panel and gene-edited CB-CD8<sup>+</sup> T cells with both *TRAC+TRBC* gRNAs in the bottom panel. Data points refer to biological replicates and independent CB samples. Data are shown as mean $\pm$ SD; \*p $\leq$ 0.05, \*\*\*p $\leq$ 0.001, \*\*\*\*p $\leq$ 0.0001. CB, cord blood; TCR, T cell receptor; CTRL, unedited CB-CD8<sup>+</sup> T cells; EP, sham electroporated.

from children at time of relapse. Three AML samples were tested for HLA typing and WT1 expression (data not shown). **Figure 5A** visually summarizes the components of the 3D model and the timeline of the experiment. eTCR<sup>-/-</sup> WT1-TCR T cells were tested for killing capacity alongside untransduced and unedited CTRL CD8<sup>+</sup> T cells. As shown in **figure 5B**, eTCR<sup>-/-</sup> WT1-TCR T cells had a killing capacity that ranged from 20% to 70% of the primary AML cells, when normalized to the killing capacity of untransduced CTRL cells. Moreover, eTCR<sup>-/-</sup> WT1-TCR T cells showed a trend of increased migration potential toward the target cells and stroma (**figure 5C**). To determine the safety of the T cell products and non-specific killing, the number of stromal cells left in the 3D system was calculated. No differences were detected between untransduced CTRL T cells and eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells (**figure 5D**). Moreover, the safety profile of eTCR<sup>-/-</sup> WT1-TCR T cells and

eTCR<sup>+/+</sup> WT1-TCR T cells was also tested in a coculture experiment with CB-derived stem cells (CD34<sup>+</sup>). No major impact on viability was detected for both products (**figure 5E**).

## DISCUSSION

CB-HCT is a commonly used treatment option in pediatric hematological malignancies, such as AML, when a matched bone marrow donor is not available. Transplant practice has generally improved over the last 20 years, decreasing the chance of graft failure and fatal GvHD, but the relapse rate for children with AML remains around 30%.<sup>1</sup> The AML blasts of most patients, both adult and pediatric, exhibit overexpression of WT1, a zinc finger motif nuclear protein.<sup>20</sup> WT1 has been considered a relevant target for the application of adoptive cell therapy and TCR-T cells.<sup>21</sup> Previous in vitro and in vivo studies



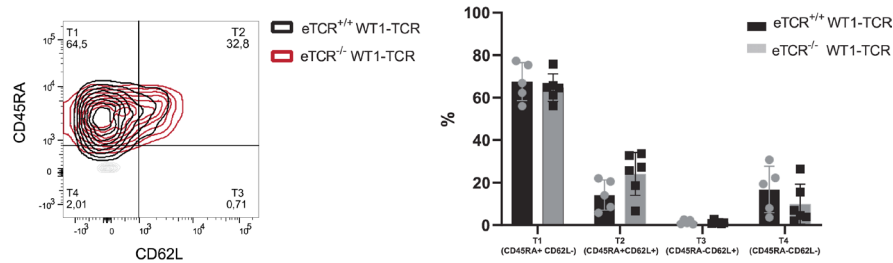
**Figure 2** Transduction efficiency and expression of recombinant TCR after lentiviral transduction in eTCR<sup>+/+</sup> and eTCR<sup>-/-</sup> CB-CD8<sup>+</sup> T cells. (A) Percentage and median fluorescence intensity of TCRVb21.3<sup>+</sup> cells determined by flow cytometry 6 days after lentiviral transduction of eTCR<sup>+/+</sup> (black) and eTCR<sup>-/-</sup> (gray) CB-CD8<sup>+</sup> T cells. (B) Representative comparative flow cytometry plot of TCRVb21.3 (WT1-TCR) expression in transduced eTCR<sup>+/+</sup> (black; n=7) and eTCR<sup>-/-</sup> (gray; n=7) CB-CD8<sup>+</sup> T cells compared with untransduced eTCR<sup>+/+</sup> and eTCR<sup>-/-</sup> CB-CD8<sup>+</sup> T cells (white lines). (C) Tetramer reactivity of eTCR<sup>+/+</sup> WT1-TCR (black; n=3) and eTCR<sup>-/-</sup> WT1-TCR (gray; n=3) CB-CD8<sup>+</sup> T cells. (D) Representative flow cytometry plot of tetramer binding in eTCR<sup>+/+</sup> WT1-TCR (black; n=3) and eTCR<sup>-/-</sup> WT1-TCR (red; n=3) CB-CD8<sup>+</sup> T cells. Data points refer to biological replicates and independent CB samples. Data are shown as mean±SD. CB, cord blood; CTRL, control; eTCR, endogenous T cell receptor; FITC, fluorescein isothiocyanate; MFI, mean fluorescent intensity; TCR, T cell receptor; WT1, Wilms tumor 1.

have successfully demonstrated the effectiveness of WT1-TCR-specific T cells in eliminating leukemic blasts expressing WT1 protein.<sup>22–24</sup> Moreover, the safety profile of the product was tested in a xenograft model, in which normal hematopoietic CD34<sup>+</sup> cells were not killed by T cells expressing the WT1-TCR, as opposed to leukemic CD34<sup>+</sup> cells that were effectively eliminated.<sup>23</sup> Likewise, in a WT1 peptide vaccination study, no hematotoxicity or renal toxicity was observed in patients with AML or myelodysplastic syndrome, but a proportion of the patients had clinical benefit, including one with complete remission or stable disease, and a threefold reduction of WT1 expression was observed in 35% of patients.<sup>25</sup> These studies show favorably that WT1 is specifically targeted in tumor blasts, and not in normal cells expressing physiological WT1 protein.

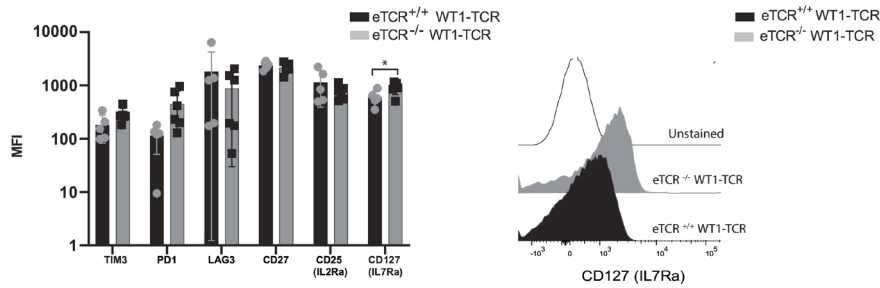
In addition, the expansion of newly identified WT1 peptides and TCR clones for generating rTCRs provides

more opportunities for T cell gene therapy,<sup>26</sup> but effectiveness requires high-affinity TCRs to mediate robust antitumor function.<sup>27</sup> A recent clinical trial corroborated preclinical studies by preventing relapse of AML in adults post-hematopoietic stem cell transplant by infusing high-affinity WT1-TCR expressed in Epstein-Barr virus donor CD8<sup>+</sup> T cells to minimize GvHD risk and enhance transferred T cell survival.<sup>4</sup> However, in polyclonal T cells, the efficacy of the rTCR-T cell therapy approach can be hampered by the presence of the eTCR due to the possibility of mispairing with the rTCR and competition for coreceptors.<sup>28</sup> The rTCR that was used in our studies incorporated cysteines in the constant domains of the alpha and beta chains to enhance preferential pairing with each other, increase total surface expression of the introduced TCR chains, and reduce mismatching with eTCR chains.<sup>15</sup> Furthermore, mispairing also affects safety due to the creation of new TCR alpha and beta

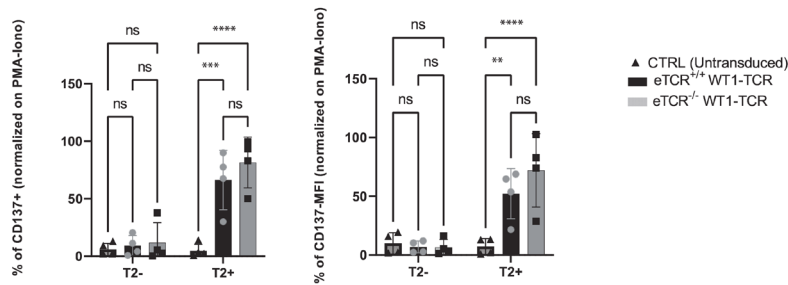
## A Memory Markers



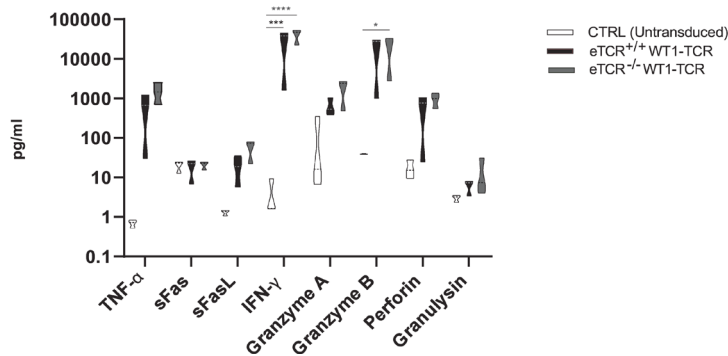
## B Activation-Inhibitory Markers



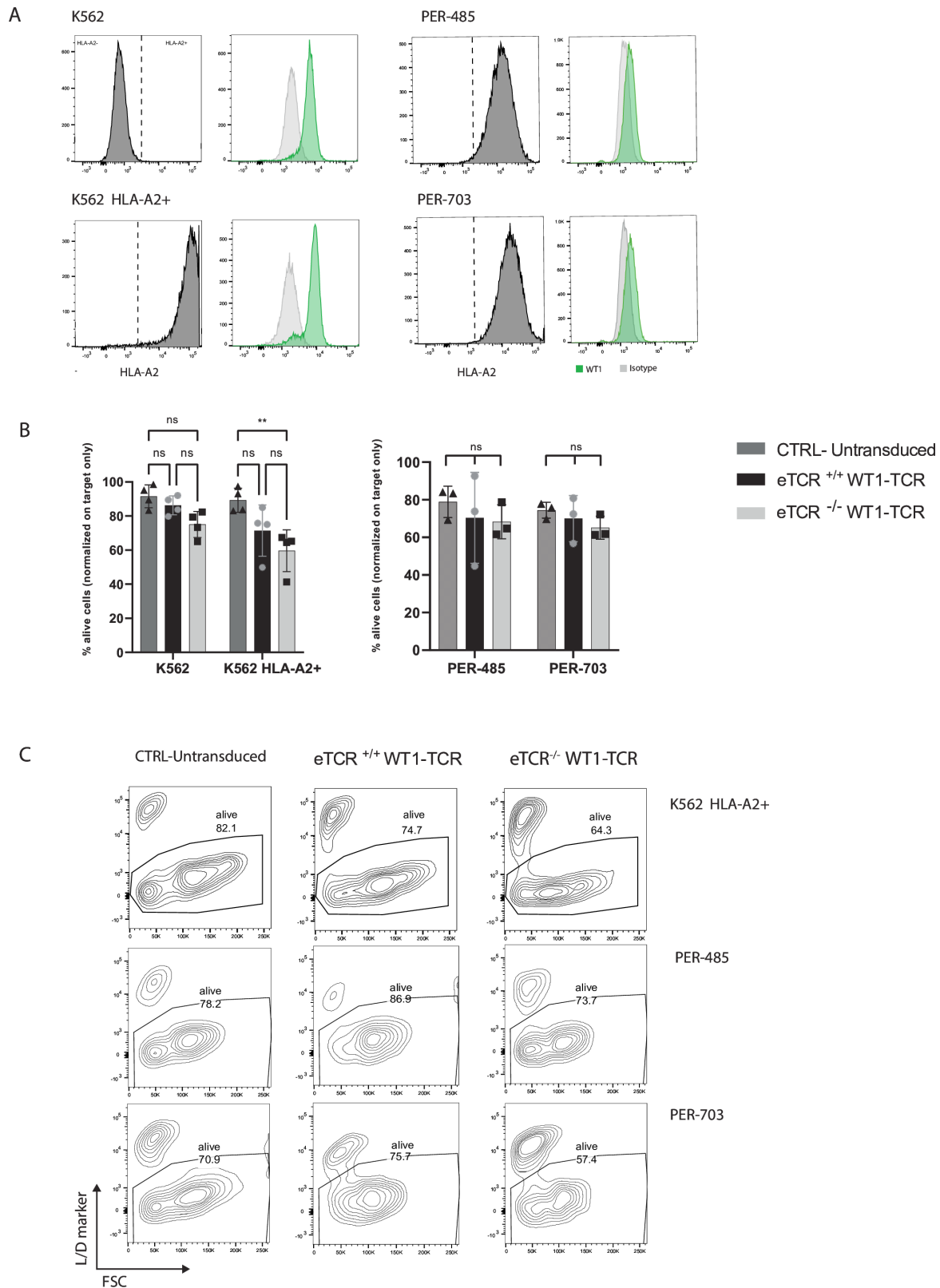
## C



## D

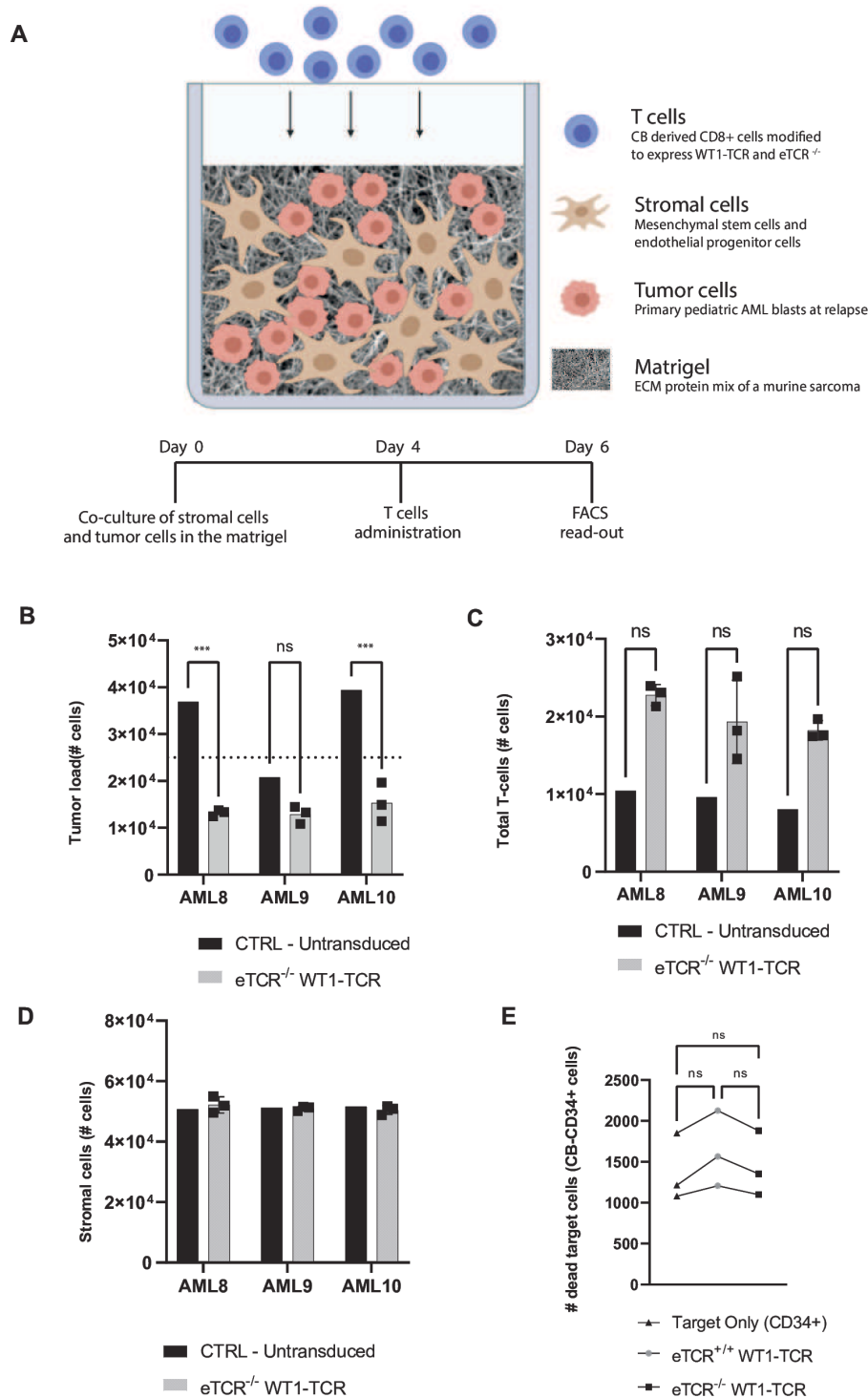


**Figure 3** Immune phenotype and activation capacity of eTCR<sup>+/+</sup> WT1-TCR and eTCR<sup>-/-</sup> WT1-TCR. (A) The left panel shows a representative flow cytometry plot of CD45RA and CD62L expression in eTCR<sup>+/+</sup> WT1-TCR (black) and eTCR<sup>-/-</sup> WT1-TCR (red); unstained cells are depicted in light gray. The right panel shows the percentage of cells characterized by several maturation markers in eTCR<sup>+/+</sup> WT1-TCR (black; n=5) and eTCR<sup>-/-</sup> WT1-TCR (gray; n=5) CB-CD8<sup>+</sup> T cells. Subpopulations were defined as T1 (CD45RA<sup>+</sup>, CD62L<sup>-</sup>), T2 (CD45<sup>+</sup>, CD62L<sup>+</sup>), T3 (CD45RA<sup>-</sup>, CD62L<sup>+</sup>), and T4 (CD45RA<sup>-</sup>, CD62L<sup>-</sup>). (B) The left panel shows the expression of multiple receptors on the surface of eTCR<sup>+/+</sup> WT1-TCR (black) and eTCR<sup>-/-</sup> WT1-TCR (gray) calculated as MFI. The right panel shows a representative flow cytometry plot of CD127(IL7RA) expression in eTCR<sup>+/+</sup> WT1-TCR (black) and eTCR<sup>-/-</sup> WT1-TCR (gray). Data points refer to biological replicates; >5 independent CB samples (n >5). Data are shown as mean±SD. (C) Percentage (left panel) and MFI (right panel) of CD137<sup>+</sup> T cells in untransduced CTRL (black triangle), eTCR<sup>+/+</sup> WT1-TCR (black), and eTCR<sup>-/-</sup> WT1-TCR (gray) after 24 hours of coculture with T2 cells (T2-) or T2 cells loaded with the specific WT1 peptide (T2+). (D) Cytokine production after 24 hours of coculture with T2+ cells of CTRL-untransduced (white), eTCR<sup>+/+</sup> WT1-TCR (black), and eTCR<sup>-/-</sup> WT1-TCR (gray) CB-CD8<sup>+</sup> T cells. Data points refer to biological replicates; four independent CB samples. Data are shown as mean±SD; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001. CB, cord blood; CTRL, control; eTCR, endogenous T cell receptor; IL7RA, interleukin-7 receptor alpha; MFI, mean fluorescent intensity; PMA, phorbol myristate acetate; sFas, soluble Fas; sFasL, soluble Fas Ligand; TCR, T cell receptor; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN $\gamma$ , interferon- $\gamma$ ; WT1, Wilms tumor 1.



**Figure 4** Cytotoxic capacity of eTCR<sup>+/+</sup> WT1-TCR and eTCR<sup>-/-</sup> WT1-TCR. (A) HLA-A2 expression (gray) and WT1 intracellular expression (green) in target tumor cell lines (K562, K562 HLA-A2<sup>+</sup>, PER-485, PER-703). (B) Percentage of viable target cells after 16 hours of coculture with CTRL -untransduced (dark gray), eTCR<sup>+/+</sup> WT1-TCR (black), and eTCR<sup>-/-</sup> WT1-TCR (gray). The percentage of viable target cells in coculture was normalized to the percentage of viable target cells in standard culture without the presence of CB-CD8<sup>+</sup> T cells. (C) Representative flow cytometry plot depicts target cell viability after 16 hours of coculture with CB-CD8<sup>+</sup> T cells. Data points refer to biological replicates; four independent CB samples for K562 and K562 HLA-A2<sup>+</sup>, and three independent CB samples for PER-485 and PER-703. Data are shown as mean $\pm$ SD; \*\*p $\leq$ 0.01. CB, cord blood; CTRL, control; eTCR, endogenous T cell receptor; TCR, T cell receptor; WT1, Wilms tumor 1.





**Figure 5** Cytotoxic capacity of eTCR<sup>-/-</sup> WT1-TCR in a 3D bone marrow niche model of pediatric AML. (A) Representative illustration of the main components of the 3D model and timeline of cell administration. (B) Cytotoxic capacity of eTCR<sup>-/-</sup> WT1-TCR (gray) and CTRL-untransduced (black) CB-CD8<sup>+</sup> T cells after 4 days of coculture in the 3D model, based on the number of primary AML cells left in the model. Cell number was measured via flow cytometry and compared with the initial number of tumor cells used on day 0 (dotted line). (C) Proliferation capacity on antigen recognition of eTCR<sup>-/-</sup> WT1-TCR (gray) and untransduced CTRL (black) CB-CD8<sup>+</sup> T cells after 4 days of coculture in the 3D model. (D) Viability of stromal cells after 4 days of coculture in the 3D model in the presence of eTCR<sup>-/-</sup> WT1-TCR (gray) and CTRL untransduced (black) CB-CD8<sup>+</sup> T cells. Cell number is measured via flow cytometry. No statistically significant difference was detected. Data are shown as mean±SD from three biological replicates for eTCR<sup>-/-</sup> WT1-TCR and one for untransduced CTRL cells; \*\*\*p≤0.001. (E) Safety profile of eTCR<sup>-/-</sup> WT1-TCR (black square) and eTCR<sup>+/+</sup> WT1-TCR (gray circle) in coculture (16 hours) with CB-derived CD34<sup>+</sup> cells (black triangle). Data are shown from three CB-CD34<sup>+</sup> donors in coculture with one CB donor for eTCR<sup>-/-</sup> WT1-TCR and eTCR<sup>+/+</sup> WT1-TCR. 3D, three-dimensional; AML, acute myeloid leukemia; CB, cord blood; CTRL, control, ECM, extracellular matrix, eTCR, endogenous T cell receptor; TCR, T cell receptor; WT1, Wilms tumor 1.

combinations that can recognize novel HLA-presented peptides, risking autoimmunity. Gene editing techniques, such as zinc finger nucleases and transcription activator-like effector nucleases (TALENs), have successfully been shown to eliminate the expression of the  $\epsilon$ TCR<sup>29 30</sup> and have completed clinical testing in both children (NCT02808442) and adults (NCT02746952).<sup>31</sup> Removal of the  $\epsilon$ TCR should reduce the risk of GvHD. One study in mice reported that pairing of introduced and  $\epsilon$ TCR chains in TCR gene-modified T cells led to the formation of self-reactive TCRs that were responsible for lethal autoimmunity.<sup>32</sup> However, data in human studies are still unclear. In fact, in a study where 106 patients were treated with autologous T cells transduced with seven different gammaretroviral vectors encoding anti-tumor TCRs, there was no evidence found of GvHD in any patient.<sup>33</sup> On the other hand, in a CD19-CAR trial, in which donor PBMC T cells gene-edited with TALEN to remove  $\epsilon$ TCR alpha and beta for a universal T cell product, followed by a stringent procedure to remove residual non-edited TCR alpha/beta T cells, two infants developed skin GvHD.<sup>31</sup> This shows that a small risk exists that residual TCR alpha/beta T cells cause GvHD, possibly also when using CB-T cells as a source. The recent advent of an easy-to-access and clinically translatable gene editing technique, known as CRISPR/Cas9, has promoted the fast application of multiplexing genome-edited T cells to improve cellular immunotherapeutic products.<sup>34</sup>

In this study, we generated an efficient and safe T cell product originating from CB, characterized by the elimination of both alpha and beta chains of the  $\epsilon$ TCR and simultaneous genetic modification to express a recombinant WT1-TCR, which could potentially be used as an adjuvant therapy after CB-HCT in children with AML to boost antitumor specific responses. It has been shown that the elimination of either the alpha and beta chains of the  $\epsilon$ TCR can increase the efficacy of the final T cell product, measured as the percentage of transduced cells, lytic capacity, and IFN $\gamma$  production, when in culture with target cells.<sup>35 36</sup> Eliminating only one of the two chains (alpha or beta) has been described to improve the functionality of edited T cells; however, this procedure can increase the probability of mispairing between the unedited chain and the rTCR.<sup>11</sup> Using PBMCs as a T cell source, elimination of the  $\epsilon$ TCR, via editing of both the constant regions of alpha and beta chains, in combination with gammaretroviral vector introduction of the rTCR was shown as a viable approach for incorporation into current T cell production protocols.<sup>10</sup>

In this study, we confirmed the high efficiency of eliminating both alpha and beta chains in WT1-TCR CB-CD8<sup>+</sup> T cells by targeting different regions of the *TRAC* and *TRBC* genes of the  $\epsilon$ TCR and by using electroporation of the CRISPR-Cas9 RNP complex, without affecting the viability and proliferation capacity of CB-CD8<sup>+</sup> T cells. Lentiviral transduction efficiency was very high and comparable between  $\epsilon$ TCR<sup>-/-</sup> cells and  $\epsilon$ TCR<sup>+/+</sup> CB-CD8<sup>+</sup> cells (with an average of 79.8% $\pm$ 9.1 for  $\epsilon$ TCR<sup>-/-</sup> and

73.8% $\pm$ 17.2 for  $\epsilon$ TCR<sup>+/+</sup> T cells). It has been shown that lentiviral vectors can resist proviral methylation, conferring long-term expression, but expression is affected by vector variegation.<sup>37</sup> In a clinical trial of hematopoietic stem and progenitor cell gene therapy for cerebral adrenoleukodystrophy (ALD), a lentiviral vector containing the MND promoter was used, and this provided long-term expression in vivo, as ALD protein expression in myeloid and lymphoid cells was still present 12 years after transplantation, hence the decision to use the MND promoter in this study.<sup>38</sup>

To further investigate the differences between the two T cell products, immunophenotypic analysis was performed, and generally the  $\epsilon$ TCR<sup>+/+</sup> WT1-TCR CD8<sup>+</sup> T cells and  $\epsilon$ TCR<sup>-/-</sup> WT1-TCR CD8<sup>+</sup> T cells did not differ in the phenotypic markers expressed. Based on the expression of CD45RA and CD62L, three populations were observed in both groups, containing mainly a population with central memory phenotype (CD62L<sup>-</sup>CD45RA<sup>+</sup>), and two smaller populations of potentially naïve (CD62L<sup>+</sup>CD45RA<sup>+</sup>) and an effector memory (CD62L<sup>-</sup>CD45RA<sup>-</sup>) phenotype, without the presence of a noticeable population of effector (CD62L<sup>+</sup>CD45RA<sup>-</sup>) CD8<sup>+</sup> T cells. In both products, the expression of activation and exhaustion markers remained comparable throughout culture, except for CD127 (IL-7Ra), which appeared to be slightly elevated in  $\epsilon$ TCR<sup>-/-</sup> WT1-TCR CD8<sup>+</sup> T cells. Interestingly, both T cell products maintained a high expression of the costimulatory receptor CD27, indicating no progression toward terminally differentiated effector function. These phenotypic features in CB-derived T cell products suggest the potential presence of a subpopulation with similar features to the previously described T stem cell memory (Tscm), with the CCR7<sup>+</sup>CD62L<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD27<sup>+</sup>CD28<sup>+</sup>CD127<sup>+</sup> phenotype.<sup>39</sup> In vivo studies have shown that cellular products derived from the T(n) fraction of peripheral blood CD8<sup>+</sup> T cells expanded with IL-7 and IL-15 have shown an increased capacity to persist and proliferate and have been categorized as Tscm.<sup>40</sup> Additional phenotypic markers, such as the presence of CD95 and CCR7, and more in-depth genome expression analysis, using RNAseq, will be necessary to better characterize the phenotypic properties of the CB-derived final CD8<sup>+</sup> T cell product; however, we speculate that its homogenous initial naïve composition is a good indicator of maintaining the favorable antitumor phenotype.<sup>41</sup> Moreover, maintenance of CD27 and CCR7 expression, together with the presence of longer telomerase, positively correlates with the likelihood of achieving an objective clinical response after adoptive cell therapy.<sup>42 43</sup> New approaches are being investigated to further select and instruct the next generation of Tscm cell products, such as the use of protein kinase B (AKT) inhibitors in culture to enhance antitumor potency.<sup>44 45</sup>

Another concern regarding the clinical relevance of those products may be T cell exhaustion. Among many other features, upregulation of PD1, TIM3, and LAG3 is considered a hallmark of T cell exhaustion.<sup>46</sup> In our

production protocol, no chronic upregulation of these markers was detected in both eTCR<sup>-/-</sup> WT1-TCR and eTCR<sup>+/+</sup> WT1-TCR. Several of these inhibitory receptors are similarly upregulated in T cell activation, as seen for PD1 in our coculture with cells loaded with WT1 cognate peptide. To generate an effective T cell product, it will be important to maximize dosing and maybe perform repeat administrations, which reduces the risk of T exhaustion, resulting in an ineffective T cell product,<sup>47</sup> while the ability to persist and its functionality will be essential features to evaluate clinical efficacy.

eTCR<sup>+/+</sup> WT1-TCR CB-CD8<sup>+</sup> T cells and eTCR<sup>-/-</sup> WT1-TCR CB-CD8<sup>+</sup> T cells were further compared for their ability to be activated and produce cytokines on antigen recognition. eTCR<sup>-/-</sup> WT1-TCR T cells showed an increased population expressing CD137, both in terms of percentage of cells expressing the marker as well as intensity of signal, suggesting an enhanced response on cognate peptide recognition. This result was also corroborated by the overall higher production of cytotoxic cytokines by eTCR<sup>-/-</sup> WT1-TCR T cells, confirming earlier findings in peripheral blood mononuclear-edited cells regarding higher production of IFN $\gamma$  by eTCR<sup>-/-</sup> rTCR T cells.<sup>10 11</sup> In our study, eTCR<sup>-/-</sup> WT1-TCR T cells were also compared with eTCR<sup>+/+</sup> WT1-TCR T cells in more robust killing assays with endogenously expressing WT1 positive cell lines and with primary AML blast cells derived from children with relapsed disease, grown in a 3D model. Overall, eTCR<sup>-/-</sup> WT1-TCR T cells performed better than eTCR<sup>+/+</sup> WT1-TCR T cells.

Due to the high cell death rate in primary AML samples in vitro conditions and the limited availability of relevant in vivo models using primary AML cells, we decided to test the efficacy of our proposed eTCR<sup>-/-</sup> WT1-TCR T cell product in a 3D model of the bone marrow niche.<sup>18</sup> The presence of a supportive microenvironment improves the viability of primary AML cells, which were obtained from three children at the time of relapse. Using this method, we were able to define both the migration capacity and specific killing capacity of the eTCR<sup>-/-</sup> WT1-TCR T cell product. eTCR<sup>-/-</sup> WT1-TCR T cells showed sustained antileukemic capacity, achieving up to 65% active killing of primary tumor cells, which is similar to or higher than the results achieved by previous studies that used primary AML cells.<sup>4 31</sup>

In this study, we propose one specific application involving gene editing to improve CB-derived T cell therapeutic products, which is translatable to the field of TCR-T therapy. The application might not be as suitable for other T cell products, as it was recently shown that elimination of the eTCR reduced alloreactivity; however, this was at the expense of functionality and expansion capacity of chimeric antigen receptor (CAR)-T cells in vivo.<sup>48</sup> In addition, the use of gene editing techniques, especially CRISPR/Cas9, is relatively straightforward in terms of feasibility and translational capacity and so can be implemented to target different molecules to enhance the efficacy of final products, such as checkpoint

inhibitors,<sup>49 50</sup> in a multiplex approach.<sup>51 52</sup> Multiplex techniques, such as specifically targeting the three genes eTCR, CD52, and HLA class I (*via* B2M) simultaneously, have been tested using allogeneic CB CAR-T cells as a bridge to HCT in infants with B-cell acute lymphoblastic leukemia (B-ALL).<sup>31</sup> Using CB-T cells as a source for the development of “off-the-shelf” cellular immunotherapy products raises the potential to make cells readily available for quick implementation.<sup>29 53 54</sup> However, when using CRISPR/Cas9 for single or multiple targets, it is important to take the probability of off-target effects, such as translocations and off-target insertion/deletions, into consideration. The probability of off-target effects appears to be low<sup>55</sup>; however, further investigation with improved methods for quantitative evaluation is required.<sup>56</sup> The complexity of CRISPR/Cas9 in combination with lentiviral transduction provides opportunities to optimize the application and to minimize the probability for off-target effects to occur, for example using more specific high-fidelity nuclease proteins, minimizing exposure to gRNAs, and controlling dosage.<sup>57</sup> In addition, no detrimental treatment-related side effects have been reported in patients participating in clinical trials using gene-edited T cells to date; however, follow-up remains relatively short.<sup>51 58</sup>

In conclusion, we demonstrate the use of a multiplex CRISPR/Cas9 approach to eliminate the expression of the eTCR in CB-CD8<sup>+</sup> T cells, replacing it with a recombinant antitumor TCR against WT1 to improve its efficacy and safety against AML blasts. Altogether, a short practical and feasible protocol was created for the production of combined gene-edited and tumor antigen-redirection CB-CD8<sup>+</sup> T cells, which showed improved in vitro functionality, providing support for further development and translation of this strategy to prevent relapse and improve the outcome of children receiving HCT for AML.

#### Author affiliations

<sup>1</sup>Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands

<sup>2</sup>Center for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

<sup>3</sup>Department of Clinical Haematology, Oncology, Blood and Marrow Transplantation, Perth Children's Hospital, Perth, Western Australia, Australia

<sup>4</sup>University of Western Australia, Perth, Western Australia, Australia

<sup>5</sup>Department of Child Neurology, Amsterdam Leukodystrophy Center, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam and Amsterdam Neuroscience, Cellular & Molecular Mechanisms, Amsterdam, The Netherlands

<sup>6</sup>Department of Integrative Neurophysiology, Center for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

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**Contributors** Conceptualization: VLP, SN, NPvT, AC. Providing materials and methodology: AVM, RWS, AK-H, OTH, ZS, JK. Conducting experiments: VLP, ED, AC, AM, ZS. Data analysis: VLP, ED, AM. Writing—original draft preparation: VLP. Writing—review and editing: SN, NPvT, AVM, AvV, RWS, RSK, AK-H, OTH, MP, AC, ZS, JK. Supervision: NPvT, SN. Funding acquisition: SN, NPvT. Guarantor: SN.

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**Competing interests** JK reports grants from Gadeta, Novartis, and Miltenyi Biotec and is the inventor of patents dealing with  $\gamma\delta$ T cell-related aspects, as well as the cofounder and shareholder of Gadeta. ZS is an inventor of patents dealing with  $\gamma\delta$ T cell-related aspects.

**Patient consent for publication** Not required.

**Ethics approval** This study involves human participants. Fresh umbilical CB was collected after informed consent was obtained according to the Declaration of Helsinki. The Ethics Committee of the University Medical Center Utrecht approved the cord blood collections' protocol (TC-bio 15-345). Participants gave informed consent to participate in the study before taking part.

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#### ORCID iD

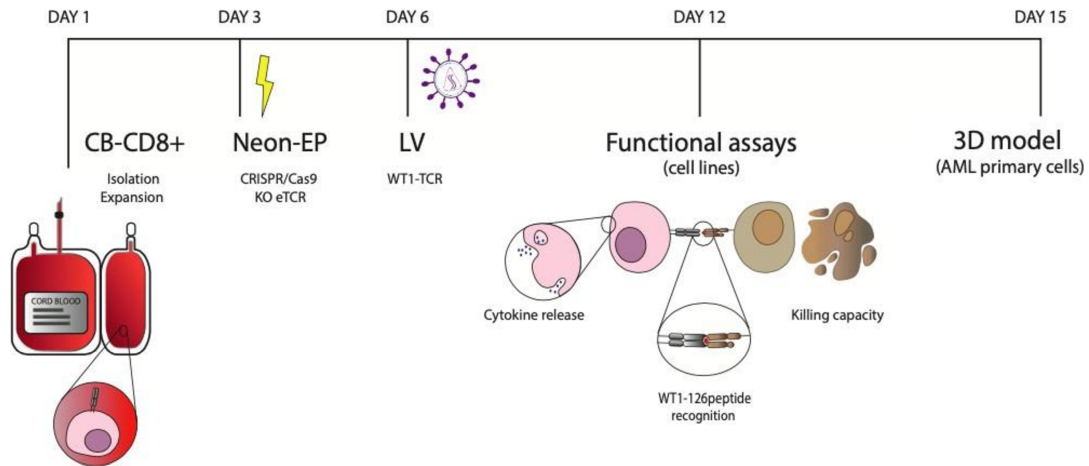
Alice van Velzen <http://orcid.org/0000-0002-8768-215X>

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Supplementary Figure 1 Graphical illustration of the processes and timeline to generate the final cell therapy product from the cord blood unit.

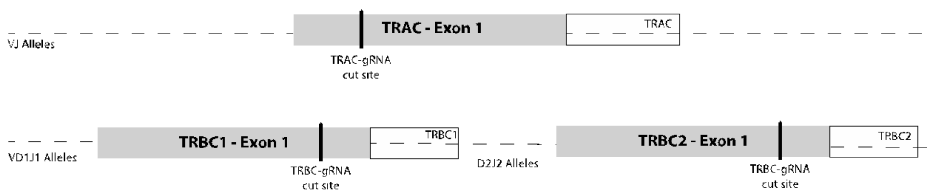


Supplementary Figure 2 Table with gRNA sequences targeting TRAC and TRBC regions. B) Schematic illustration of cut sites.

A

ID	Sequence
TRAC	GAGAAATCAAATCGGTGAAT
TRBC	CACCCAGATCGTCAGCGCGG

B



Supplementary Figure 3. Representative FACS plot of the increased expression of CD25 and PD1 upon recognition of WT1 peptide in eTCR  $-/-$  WT1-TCR and eTCR  $+/+$  WT1-TCR

