ARTICLE

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Genome editing of donor-derived T cells to generate allogeneic chimeric antigen receptor-modified T cells: optimizing αβ T-cell-depleted haploidentical hematopoietic stem cell transplantation

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Haematologica 2021
Volume 106(3):847-858

ABSTRACT

Allogeneic hematopoietic stem cell transplantation is an effective therapy for high-risk leukemias. In children, graft manipulation based on the selective removal of αβ T cells and B cells has been shown to reduce the risk of acute and chronic graft-*versus*-host disease, thus allowing the use of haploidentical donors which expands the population of recipients in whom allogeneic hematopoietic stem cell transplantation can be used. Leukemic relapse, however, remains a challenge. T cells expressing chimeric antigen receptors can potently eliminate leukemia, including those in the central nervous system. We hypothesized that by engineering the donor αβ T cells that are removed from the graft by genome editing to express a CD19-specific chimeric antigen receptor, while simultaneously inactivating the T-cell receptor, we could create a therapy that enhances the anti-leukemic efficacy of the stem cell transplant without increasing the risk of graft-*versus*-host disease. Using genome editing with Cas9 ribonucleoprotein and adeno-associated virus serotype 6, we integrated a CD19-specific chimeric antigen receptor in-frame into the TRAC locus. More than 90% of cells lost T-cell receptor expression, while >75% expressed the chimeric antigen receptor. The initial product was further purified with less than 0.05% T-cell receptor-positive cells remaining. *In vitro*, the chimeric antigen receptor T cells efficiently eliminated target cells and produced high cytokine levels when challenged with $CD19^+$ leukemia cells. *In vivo*, the gene-modified T cells eliminated leukemia without causing graft-*versus*-host disease in a xenograft model. Gene editing was highly specific with no evidence of off-target effects. These data support the concept that the addition of αβ T-cell-derived, genome-edited T cells expressing CD19-specific chimeric antigen receptors could enhance the anti-leukemic efficacy of αβ T-cell-depleted haploidentical hematopoietic stem cell transplantation without increasing the risk of graft-*versus*-host disease.

Introduction

Despite improvements over the last decades, treatment outcomes remain unfavorable in pediatric patients with relapsed or refractory B-cell precursor acute lymphoblastic leukemia.[1,2] Allogeneic hematopoietic stem cell transplantation (HSCT) has been successfully employed to treat high-risk leukemias,[3] providing a graft-*versus*-leukemia[4] effect but also carrying the risk of graft-*versus*-host disease (GvHD).[5]

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Received: August 15, 2019.
Accepted: March 19, 2020.
Pre-published: April 2, 2020.

https://doi.org/10.3324/haematol.2019.233882

In order to overcome the challenge of limited donor availability,[6] innovative protocols have been developed that allow the use of grafts from haploidentical donors. A successful strategy for haploidentical transplantation is based on the selective elimination of αβ T cells and CD19$^+$ B cells (αβ haplo-HSCT),[7] which is associated with a very low incidence of transplantation-related mortality and GvHD (Figure 1A).[8-10] In contrast to CD34$^+$-cell selection, this method of manipulation allows the transfer not only of donor hematopoietic stem cells to the recipient, but also committed hematopoietic progenitors as well as mature natural killer and γδ T cells,[11,12] which may provide a protective effect against leukemia relapse and reduce the risk of infectious complications.[13] With significant improvement in non-relapse mortality, disease relapse has become the most important cause of treatment failure in patients with malignancies undergoing αβ haplo-HSCT.[8] In particular, the outcome of αβ haplo-HSCT in children with leukemia not in complete remission or beyond second complete remission has been poor.[9,14] For this reason, it is necessary to develop novel strategies to reduce leukemic relapse after haplo-HSCT, without increasing the incidence of GvHD or transplant-related mortality.

An intriguing approach to reducing leukemic relapse is to follow haplo-HSCT with subsequent anti-leukemic cell therapy[15] derived from the stem cell donor (Figure 1A-D), since these cells are from healthy immune systems and are also syngeneic (functionally autologous) to the donor graft, rendering them resistant to immune rejection after transplantation. While the infusion of donor-derived T cells (donor lymphocyte infusion) has been used in various contexts to enhance antileukemic efficacy (Figure 1B), it is accompanied by a high risk of severe GvHD.[16-22] An improvement over donor lymphocyte infusion is to genetically engineer the donor T cells with a safety switch (suicide gene) such that the cells can be quickly eliminated if severe GvHD occurs (Figure 1C). Early trials have suggested that this strategy does help to prevent relapse and the suicide switch (inducible caspase 9) is effective at eliminating alloreactive cells if GvHD occurs.[23-25] Although this strategy allows GvHD to be controlled after it occurs, the benefit of the graft-*versus*-leukemia effect and the risk of GvHD remain linked to each other. It would be an improvement, therefore, to establish an approach that provides anti-leukemic activity without GvHD.

Chimeric antigen receptors (CAR) can redirect T-cell cytotoxicity towards cancer-related antigens and achieve remissions in otherwise refractory hematologic malignancies expressing these targets.[26,27] Currently, the most commonly used CAR T-cell products are manufactured from patient-derived autologous T cells that are harvested and transduced with a semi-randomly integrating viral vector for delivery and expression of the CAR gene, and then infused back into the patient after lymphodepleting therapy.[28] This is associated with high variability in the CAR T-cell product and manufacturing failures. Furthermore, contaminations of the autologous cells with leukemic cells[29] and the risk of insertional mutagenesis associated with randomly integrating viral vectors[30] are challenges associated with the established approach.

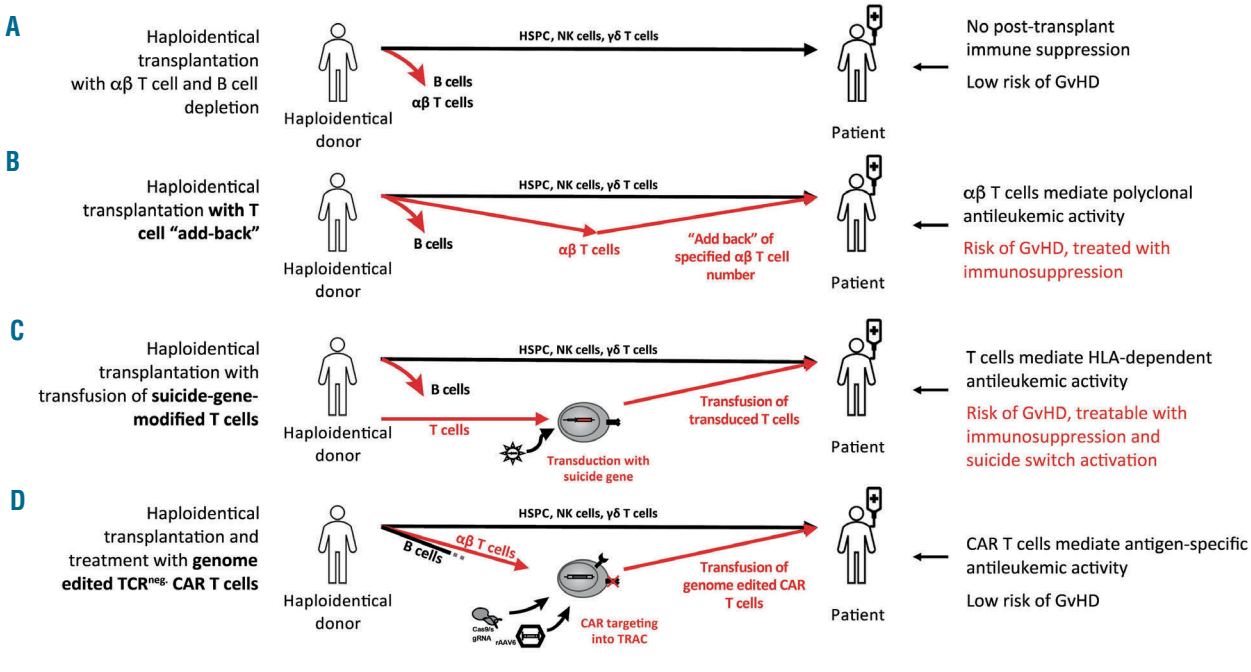

Figure 1. T-cell therapy approaches in combination with T-cell receptor αβ$^+$/CD19$^+$-depleted haploidentical stem cell transplantation aiming to decrease relapse rates. (A) The protocol for haploidentical hematopoietic stem cell transplantation (HSCT) with TCRαβ$^+$/CD19$^+$-depletion, which establishes a backbone for additional cellular immunotherapies. (B) In order to improve immune reconstitution and enhance antileukemic activity, a specified number of T cells is transfused to the patient separate from the graft. (C) In order to retain control over the T cells and be able to intervene in the case of severe graft-versus-host disease (GvHD), the T cells can be transduced with a safeguard system such as herpes simplex virus-derived thymidine kinase or inducible caspase 9. (D) The αβ T cells are removed from the graft before transplantation and can be used as starting material to create genome-edited chimeric antigen receptor (CAR) T cells by targeted integration of a CD19-CAR into the TRAC locus, in order to target residual leukemia after HSCT without causing GvHD. sgRNA: single guide RNA; CAR: chimeric antigen receptor; GvHD: graft-*versus*-host disease; HSPC: hematopoietic stem and progenitor cells; TCR: T-cell receptor; NK cells: natural killer cells: TRAC: T-cell receptor alpha chain; HLA: human leukocyte antigen.

We hypothesized that αβ haplo-HSCT in combination with CAR T cells generated through genome editing of donor-derived T cells could provide the foundation for an optimal approach that addresses these challenges (Figure 1D). We here show that the T-cell receptor (TCR) αβ+/CD19+ cell fraction that is removed from the graft can be used to engineer non-alloreactive CAR T cells through homologous recombination-mediated genome editing by targeted integration of a CD19-specific CAR in-frame into the TCR alpha chain (TRAC) locus ("αβTCR-CD19 CAR-T"), and demonstrate the antileukemic efficacy of this product *in vitro* and *in vivo*. This novel and innovative approach allows for the creation of two different cellular immunotherapy products, with complementary antileukemic mechanisms, from a single apheresis: the αβ haplo-HSCT which provides donor-derived natural killer cells, γδ T cells and the human leukocyte antigen (HLA)-dependent activity of polyclonal T cells, while the "left-over" cell fraction is salvaged to become a therapeutic CAR T-cell product with an HLA-independent mechanism and potential to improve cure rates without causing GvHD.

Methods

Plasmid cloning and adeno-associated virus production

Transfer plasmids were cloned between the inverted terminal repeat sequences in pAAV-MCS (Agilent Technologies). The CAR comprises a GM-CSFRα leader sequence, the FMC63 scFv,[31] CD28 hinge, transmembrane and intracellular sequences and the CD3ζ intracellular domain. Recombinant adeno-associated virus serotype 6 (rAAV6) production and titration are described in the *Online Supplementary Methods*.

Apheresis and cell processing

αβ haplo-HSCT donors received granulocyte-colony stimulating factor for 4 days at the total dose of 16 μg/kg body weight and apheresis was performed on the fifth day. If the CD34+ cell count was <40/μL on day 4, a CXCR4 antagonist (plerixafor, Mozobil) was given. Manipulations were performed in a closed system according to Good Manufacturing Practice (GMP) standards with clinical grade reagents and instruments from Miltenyi Biotec (Bergisch Gladbach, Germany).

T-cell culture and genome editing

All human cells were handled according to a protocol approved by the Institutional Review Board at Stanford University. The TCRαβ+/CD19+ cell fraction (non-target fraction from the graft manipulation procedure) was used Fresh or cryopreserved. T cells were activated for 3 days and beads removed before electroporation. Electroporation and gene targeting were performed as previously described.[32]

Depletion by magnetic bead activated cell sorting

Depletion of TCRαβ+ cells was performed using reagents from Miltenyi according to the manufacturer's instructions, except after coating with the Streptavidin-microbeads, when the cells were diluted and passed through the column without the washing step. For details see the *Online Supplementary Methods*.

In vitro cytokine measurement and cytotoxicity assay

CD19+ Nalm6-GL cells or CD19+ Raji cells (GFP-Luc+) were used in co-culture assays with the CAR T cells or control T cells to determine interleukin-2 and interferon-γ production of the CAR T cells and cytotoxicity. For details see the *Online Supplementary Methods*.

In vivo xenograft assay

All experiments involving mice were performed according to a protocol approved by the Administrative Panel on Laboratory Animal Care at Stanford University. CD19+ Nalm6-GL cells ($5x10^5$) were transplanted intravenously (i.v.) into 6- to 12-week old male NSG mice. Four days later, tumor burden was evaluated by IVIS bioluminescence imaging (PerkinElmer) and the indicated numbers of CAR T cells or control cells injected i.v.. Tumor burden was followed weekly by IVIS imaging.

Antibodies used for flow cytometry

NGFR-APC, NGFR-PE, TCRαβ-FITC, CD19-A488, CD19-A700, CD62L-BV421, CD45RA-PE, CD4-PerCP-Cy5.5, CD8a-APC-Cy7 (all from Biolegend) were used. For CD45RA/CD62L staining, isotype controls as recommended by the manufacturer were used to determine positive and negative populations. The APC-conjugated CD19-CAR idiotype antibody was a gift from Crystal Mackall.

Off-target analysis

sgRNA target sites were identified and their specificity score calculated by bioinformatics (*crispor.tefor.net*[33]). COSMID (*crispr.bme.gatech.edu*[34]) was used to identify potential off-target sites in the human genome. For analysis of predicted off-targets, gene editing or mock treatment was performed on T cells from six different donors and predicted off-target sites sequenced using an Illumina MiSeq as described previously.[35] For details see the *Online Supplementary Methods*.

Statistics

Plots show means with error bars representing either the standard deviation or 95% confidence interval (95% CI), as indicated. Groups were compared by statistical tests as described in the figure legends using Prism 7 (GraphPad). Asterisks indicate statistical significance: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. All *t* tests are two-tailed.

Results

Genome editing on TCRαβ+ cells depleted from the graft during αβ haploidentical hematopoietic stem cell transplanation to create chimeric antigen receptor T cells

We prospectively collected the TCRαβ+/CD19+ cell fraction (non-target fraction) removed from grafts during αβ haplo-HSCT procedures. It has recently been shown that homologous recombination-mediated genome editing using Cas9 ribonucleoprotein (RNP) and AAV6 can mediate targeted integration of a CAR into the TRAC locus,[36,37] with up to 50% of cells expressing the CAR. This approach offers the advantages that it establishes TCR knockout in the majority of CAR+ cells, avoids the risk of insertional mutagenesis of randomly-integrating viral vectors, and allows the cells to modulate CAR expression if the CAR is integrated in-frame into the endogenous locus.[37]

We used homologous recombination-based genome editing[32] to integrate a CD19.28.ζ-CAR in-frame into the open-reading frame of the TRAC locus (Figures 1D and 2A), similarly to a recently described approach.[37] Disruption of TRAC leads to loss of expression of the

TCR complex on the cell surface.[38] TRAC is advantageous over TRBC because it only exists once per haploid genome. Although 4-1BB co-stimulation has been shown to lead to enhanced persistence of CAR T cells due to decreased exhaustion,[39] we chose CD28 as the co-stimulatory domain in the CAR in order to account for the expected low number of target cells in the setting after HSCT. In the presence of minimal disease burden and absent or low numbers of B cells immediately after HSCT, the stronger effector signaling from CD28 co-stimulation could lead to enhanced activation and proliferation of the CAR T cells. It was recently shown that the method of targeted integration of a CAR into the TRAC locus with expression from the endogenous promotor can preserve functionality of cells with CD28 co-stimulation,[37] which are otherwise prone to exhaustion.

We used an sgRNA (termed TRAC-1) that had previously been shown to have high on-target activity and no detectable off-target activity.[40] Potential off-target sites across the human genome were predicted by the COSMID algorithm[34] and comparison to other possible sgRNA (*Online Supplementary Figure S1A, B*) confirmed that this sgRNA was among the most specific in exon 1 of TRAC with no highly similar off-targets. The most similar predicted off-target site had three mismatched nucleotides, suggesting a low probability of Cas9 cleavage activity (see below).

To investigate whether genome editing with TCR knockout and targeted integration of a CAR is feasible in TCRαβ$^+$ T cells removed from the graft during αβ haplo-HSCT, we cultured and stimulated these cells and electroporated them with an RNP complex consisting of a high-fidelity version of the Cas9 protein[41] complexed with chemically-modified sgRNA,[42] immediately followed by transduction of a DNA repair template by a non-integrating rAAV6. Following this process, on average 95.7% of the cells lost TCR expression (95% CI: 94.2-97.3) and 79.4% (95% CI: 73.5-85.3) of bulk cells and 81.4% (95% CI: 75.7-87.1) of TCR$^-$ cells expressed tNGFR (a truncated non-signaling cell surface form of the nerve growth factor receptor which has been used safely in clinical immunotherapy trials[24]) (Figure 2B and C). These unprecedented efficiencies of targeted integration of a large gene expression cassette (2.7 kb) in primary T cells was reproduced in cells from 11 different donors with similar efficiencies (Figure 2C). Importantly, this proves that cellular double-strand break repair can efficiently be skewed toward homologous recombination to the level at which it constitutes the predominant repair pathway and targeted integration becomes more frequent than insertion/deletion formation by non-homologous end-joining. Notably, the starting cells had been processed at two different GMP facilities (5 at the University of California, San Francisco and 6 at the Stanford University Laboratory for Cell and Gene Medicine), but the outcome after gene editing was highly reproducible (Figure 2C). To confirm co-expression of the CAR in the NGFR$^+$ cells, we stained the cells with an antibody that detects the CAR, which confirmed that both genes of the bicistronic expression cassette are translated (Figure 2D).

Efficient depletion of potentially alloreactive TCR$^+$ cells and optimization of editing methods

Despite the efficiency of the genome editing process, a small fraction of cells (<8%) retained expression of their TCR. Prior studies have suggested that the frequency of GvHD occurrence for allogeneic CAR T cells with CD28 co-stimulation is low,[43] supposedly due to exhaustion and clonal deletion of alloreactive cells[44] stimulated through both the CAR and their TCR. Despite these promising results, this is not guaranteed to be universally true, especially since our method creates CAR expression levels different from virally transduced CAR T cells and could lead to different biological properties. The residual TCR$^+$ cells – being HLA-haploidentical to the recipient - carry the potential for alloreactivity, and their further depletion from the cell product could decrease the probability of GvHD and allow higher doses of cells to be administered. We therefore evaluated the depletion of residual TCRαβ$^+$ cells from the expanded cell population by magnetic bead activated cell sorting using reagents for which GMP-compatible counterparts are available. We were able to achieve efficient depletion with a maximum of 0.03% TCRαβ$^+$ cells remaining in the resulting cell product (a depletion efficiency of 2-3 orders of magnitude) (Figure 2E and F), a higher efficiency than in prior studies that created TCR$^-$ CAR T cells.[45,46] We termed the resulting cell product after genome editing, expansion and TCRαβ$^+$ depletion "αβTCR-CD19 CAR-T".

The cells rapidly expanded following genome editing (over 60-fold in 7 days), with no negative effect of RNP electroporation on cell yields, an 11% decrease in expansion after AAV transduction, and a decrease of 27% for cells electroporated with RNP and transduced with AAV (*Online Supplementary Figure S2A*). This suggested that AAV transduction is the main factor affecting cell expansion, which led us to determine the optimal AAV dose for maximal gene targeting efficiency. Interestingly, we found that a change in the multiplicity of infection beyond 2,500 vector genomes (vg)/cell only led to a minor change in targeting outcomes with saturation at 5,000 vg/cell (*Online Supplementary Figure S2B*). Instead, the duration of time during which the cells were kept at a high concentration for AAV transduction (>5x10^6 cells/mL) directly after electroporation and before dilution to the target cell density influenced gene targeting outcomes to a greater extent (*Online Supplementary Figure S2C*). Using a multiplicity of infection of 5,000 vg/cell and a prolonged transduction time at high density (>12 h), we observed that the cells expanded on average 103-fold within the 7 days following gene editing (Figure 2G). With these conditions for gene editing, the expansion rate of the cells was primarily dependent on the culture density, reaching the threshold of >100-fold expansion in 7 days if cultured at 0.125x10^6 cells/mL or within 10 days if cultured at 0.5x10^6 cells/mL (Figure 2H). This confirms that the CAR T cells are able to expand rapidly without further TCR stimulation after gene editing despite the manipulation during the gene editing process. This will aid in the development of a cell product at clinically relevant scale.

To summarize, we achieved efficient disruption of the TCR and high frequencies of CAR expression in T cells derived from the otherwise discarded TCRαβ$^+$ T cells, while allowing for rapid expansion of the resulting cells after the editing process when using an optimized protocol.

Phenotype and *in vitro* efficacy

To measure *in vitro* cytokine production and cytotoxic activity, we used the CD19$^+$ lymphoblastic cell lines

Nalm6-GL and Raji (GFP-Luciferase transduced) as target cells. After co-culture for 20 h, we were able to measure production of interleukin-2 and interferon-γ in the cell culture supernatant specifically for cells with integration of the CAR (Figure 3A). To estimate the fraction of target cells that was killed, we determined the counts of target cells (identified by their GFP expression) after 20 h of co-culture with either CAR T cells or control cells relative to samples cultured without effector cells, which showed cytotoxic activity even at low effector-to-target (E:T) ratios (Figure 3B).

To determine the fate of the B cells, which are part of

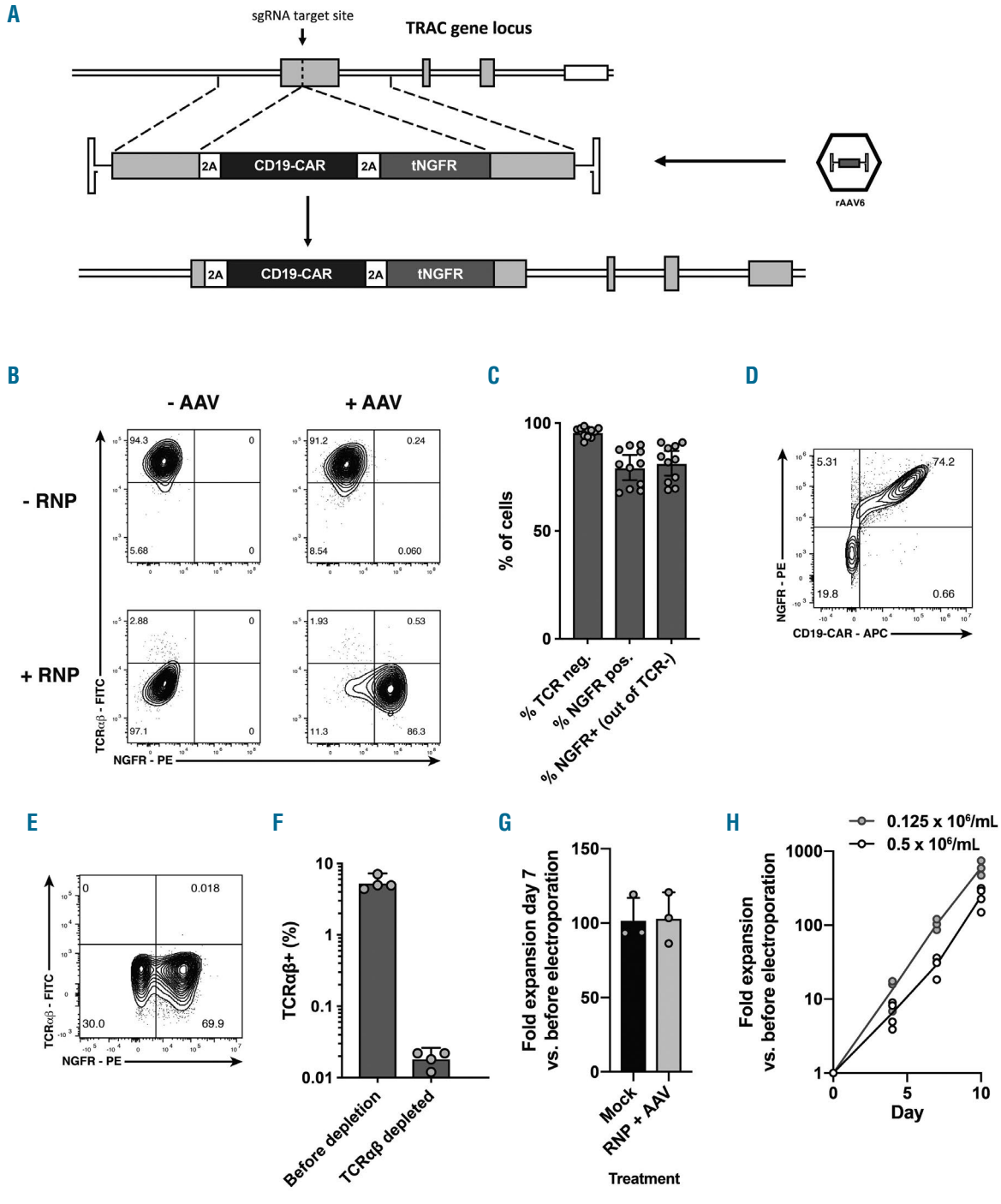

Figure 2. Targeted integration of a CD19-specific chimeric antigen receptor into the TRAC locus. (A) Targeting strategy using Cas9 ribonucleoprotein and recombinant adeno-associated virus serotype 6. (B) Representative fluorescence activated cell sorting (FACS) plots for cells treated as indicated 4 days after targeting to evaluate the efficiency of T-cell receptor (TCR)αβ knockout and nerve growth factor receptor (NGFR) expression. (C) Quantification of the populations after targeting of T cells from 11 different donors. (D) Representative FACS plot of the cells stained for NGFR and a CD19-CAR idiotype-specific antibody. (E) FACS plot showing NGFR and TCRαβ expression after depletion of cells expressing the αβ T-cell receptor. (F) Quantification of αβ TCR depletion efficiency for four different replicates, plotted as mean ± standard deviation. (G) Expansion of T cells during the 7 days after gene editing compared to numbers before electroporation using optimized conditions for adeno-associated virus transduction. (H) Expansion of T cells (compared to numbers before gene editing) cultured at different densities after electroporation. RNP: ribonucleoprotein; rAAV6: recombinant adeno-associated virus serotype 6; NGFR: nerve growth factor receptor; pA: poly-adenylation signal; 2A: 2A peptide.

the source cell population besides the αβ T cells, we followed the CD19⁺ cell population by phenotyping and were able to detect their disappearance as early as 24 h after gene targeting (2.53%, *vs.* 7.9-11.8% in the control condition in which the T cells did not express CAR), suggesting early cytotoxic activity of CD19-specific CAR T cells (Figure 3C, *Online Supplementary Figure S2D*). Follow-up showed that the cell product continued to self-deplete from the residual B cells over time (Figure 3C). The presence of CD19⁺ cells in the manufacturing process may

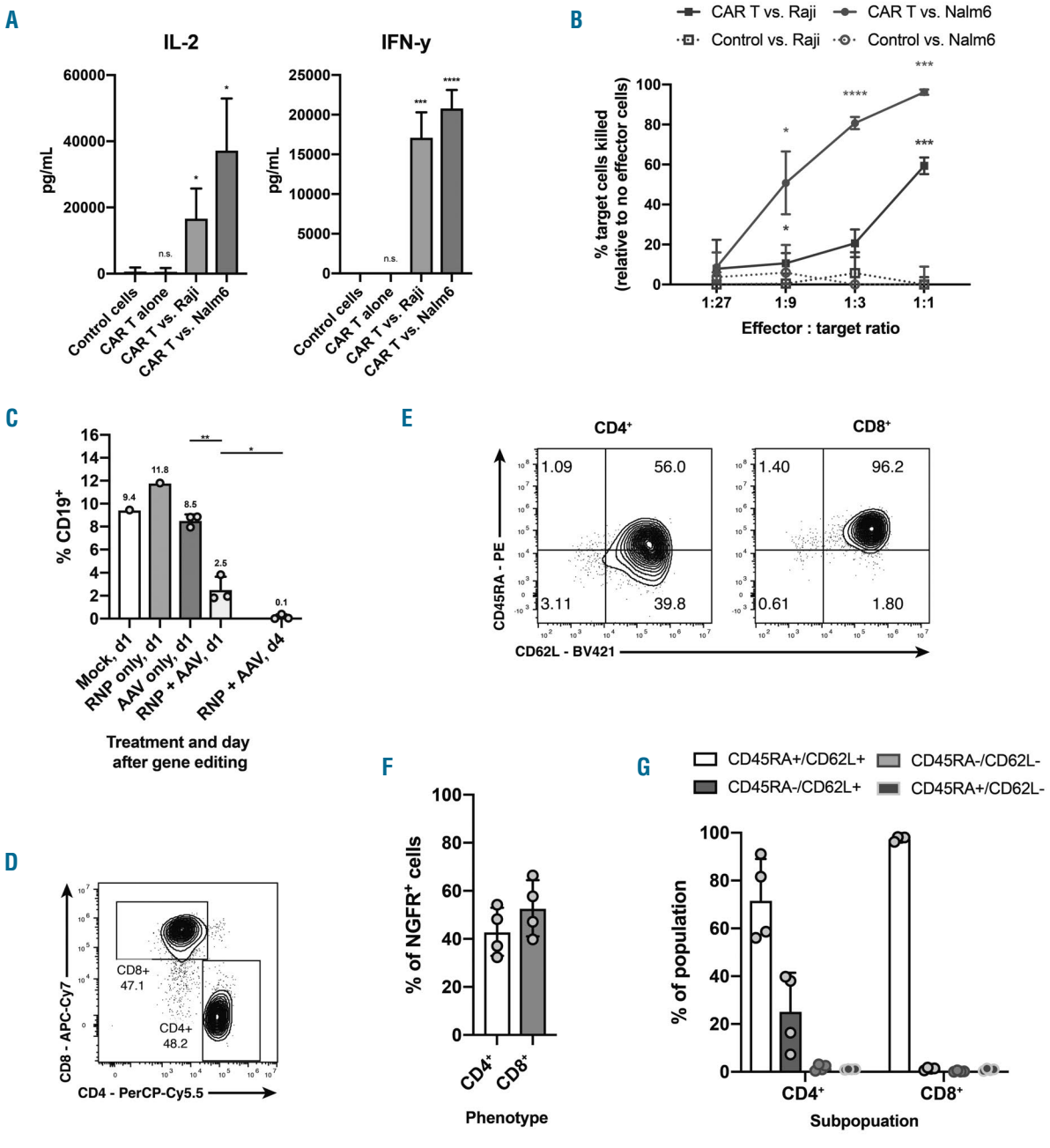

Figure 3. *In vitro* functionality of chimeric antigen receptor T cells engineered from αβ⁺ T cells. (A) Interleukin-2 and interferon-γ concentrations in cell culture supernatant after culture of control cells or chimeric antigen receptor (CAR) T cells alone, or co-cultures of CAR T cells with Nalm6 or Raji cells. Control cells were treated with ribonucleoprotein only (TRAC knockout without CAR expression). Bars and error bars represent mean ± standard deviation (SD) from three biological replicates. Asterisks depict levels of significance compared to control cells as analyzed by *t* tests. (B) *In vitro* cytotoxicity assay of CAR T cells co-cultured for 20 h with Nalm6 cells or Raji cells (both CD19⁺ and GFP⁺) at different effector-to-target ratios. Counts of viable cells were assessed for target cells co-cultured with control cells or CAR T cells and the fraction of target cells killed was calculated using samples without effector cells as reference. Bars and error bars represent means ± SD from three biological replicates and asterisks depict levels of significance (*t* tests). (C) Quantification of B cells for differentially treated cell populations on day 1 and day 4 after gene editing, for cell populations that had undergone gene targeting (RNP + AAV) or control treatments. Groups were compared by *t* tests and levels of significance are indicated by asterisks. (D-G) Phenotyping of the CAR T-cell product, gated on NGFR⁺ cells. (D) Distribution of CD4⁺ and CD8⁺ cells. (E) Expression of memory and effector T-cell markers among CD4⁺ and CD8⁺ cells. (F) Quantification of CD4/CD8 distribution from four biological replicates. Bars and error bars represent mean ± SD. (G) Quantification of the memory/effector populations on cells from four different donors. Bars represent mean ± SD. CAR: chimeric antigen receptor; IFN: interferon; IL: interleukin; RNP: ribonucleoprotein; AAV: adeno-associated virus, NGFR: nerve growth factor receptor.

have been the stimulus for the excellent T-cell expansion we observed in the TCR- cells during the manufacturing process.

We next phenotyped the resulting CAR T cells after the editing process and found that the ratio of CD4 to CD8 cells in the resulting CAR T cell product was about 0.8:1 (Figure 3D and F). The majority of CD4+ cells were of naïve and central memory phenotype, while in the CD8+ subpopulation the majority of cells showed a naïve phenotype (Figure 3E and G). This confirms that the CAR T cells had a balanced CD4:CD8 ratio, and that despite the TCR stimulation before genome editing and the transient activation through the CAR mediated by B-cell cytotoxicity, both subpopulations had a high fraction of naïve and CM cells.

Antileukemic efficacy *in vivo*

We determined the activity of the αβTCR-CD19 CAR T-cell product *in vivo* using a standard Nalm6 xenograft model.[47] We transplanted $5x10^5$ $CD19^+$ Nalm6 cells i.v. into NSG mice to create the $CD19^+$ leukemia model. Four days later, we i.v. infused $1x10^6$ or $5x10^6$ αβTCR-CD19 CAR T or control cells and followed leukemia burden by bioluminescence imaging. The higher dose of CAR T cells led to rapid and complete eradication of leukemia which lasted for at least 3 months, while the lower dose led to a transient decrease in leukemia burden and improved survival although the mice eventually relapsed (Figure 4A). While control mice became moribund and died from disease within 4 weeks, life was significantly extended at both doses of CAR T cells (Figure 4B) (log-rank test: $P<0.01$ for $5x10^6$ cells, $P<0.05$ for $1x10^6$ cells). No xenogeneic GvHD was observed in any of the mice, demonstrating the low GvHD potential of the T-cell products. We repeated the experiment at the CAR T-cell dose level of $5x10^6$ cells per mouse using two different control groups, either mock-treated T cells (expressing their endogenous TCR) or RNP-treated T cells (TCR knockout), which confirmed comparable CAR T-cell efficacy and showed no difference between those control groups (*Online Supplementary Figure S3A, B*).

Off-target evaluation

While the sgRNA specificity has previously been evaluated in an integration-deficient lentivirus capture assay,[40] we extended the specificity analysis to measure the off-target activity of the TRAC-targeting RNP using targeted next-generation sequencing. We created a list of predicted off-target sites determined by the COSMID online tool (Figure 5A) and performed targeted deep sequencing of the sites in T cells, from six different donors, electroporated with the RNP (or mock electroporated to determine background). Sequencing confirmed the specificity of the endonuclease with high activity at the on-target site, but no detectable insertions/deletions at off-target sites above the detection limit of 0.1% in any of the samples (Figure 5B).

Discussion

In the case of persistent minimal residual disease after HSCT, relapse risk is high, but treatment options are limited during the time of engraftment and immune recovery. Many immune-based therapies are futile in this period as the immune system is only slowly developing, and transplant protocols typically include immune suppression which would inhibit any adoptive cell-based therapy suchs as CAR T cells. αβ haplo-HSCT represents an excellent platform for adoptive immunotherapy not only because it helps to overcome the limited availability of HLA-matched donors, but also because post-HSCT immunosuppression is not required. It has shown robust clinical results in pediatric patients,[8] but some patients still relapse.

We here hypothesize that a donor-derived CAR T-cell product with TCR knockout after haplo-HSCT has the potential to dissociate the beneficial anti-leukemic activity from the harmful GvHD, two phenomena that are inherently connected to each other when infusing unmanipulated donor-derived lymphocytes (donor lymphocyte infusion). This would take advantage of both the graft-*versus*-leukemia effect of allogeneic HSCT and the antileukemic activity of CAR T cells. It will also supplement the polyclonal, HLA-dependent immune response that the transplanted immune system elicits after HSCT with the antigen-specific, HLA-independent cytotoxicity of CAR T cells, in order to address relapses after allogeneic HSCT that occur due to downregulation of HLA molecules.[48-50] Moreover, manufacturing CAR T cells from the donor would maintain immune tolerance between the CAR T-cell product and the donor immune system (which are HLA identical) while taking advantage of the beneficial features of healthy donor T cells.

Our innovative approach avoids the risk of manufacturing failures that comes with the use of autologous T cells, but is distinct from allogeneic, "off-the-shelf" CAR T cells, as it creates a personalized CAR T-cell product for every patient from the respective haploidentical donor. It will therefore not benefit from the same cost-effectiveness that "off-the-shelf" CAR T cells promise, which aim to reduce prices by manufacturing doses for multiple patients during a single run. On the other hand, creating αβTCR-CD19 CAR T cells from the left-over cell fraction and administering them after HSCT will be more economic than the common practice of following the administration of autologous CAR T cells with allogeneic HSCT,[51] which carries the high price tag of current CAR T-cell products but then results in their eradication by the donor immune system. Our proposed protocol, in contrast, allows for increased CAR T-cell persistence, since the cells are HLA-identical to the immune system after HSCT. This could create prolonged antileukemic surveillance from a single cell dose, or alternatively enable administration of multiple CAR T-cell doses for the same patient created from one manufacturing run. Moreover, the cells would benefit from the lymphopenia after HSCT, enabling their engraftment and prolonged activity without additional lymphodepleting therapy. Importantly, our approach allows for the creation of both the product containing the hematopoietic stem cells and the gene edited CAR T cells from a single apheresis, as the CAR T cells are made from the otherwise discarded cell fraction. This avoids an additional procedure and thereby leads to cost reduction and mitigates the risks and discomfort for the donor. This will be of particular importance when very young persons are the HSCT donors, e.g., younger siblings of pediatric patients, or the children of adult patients.

Clinical trials have shown that CAR T cells lead to

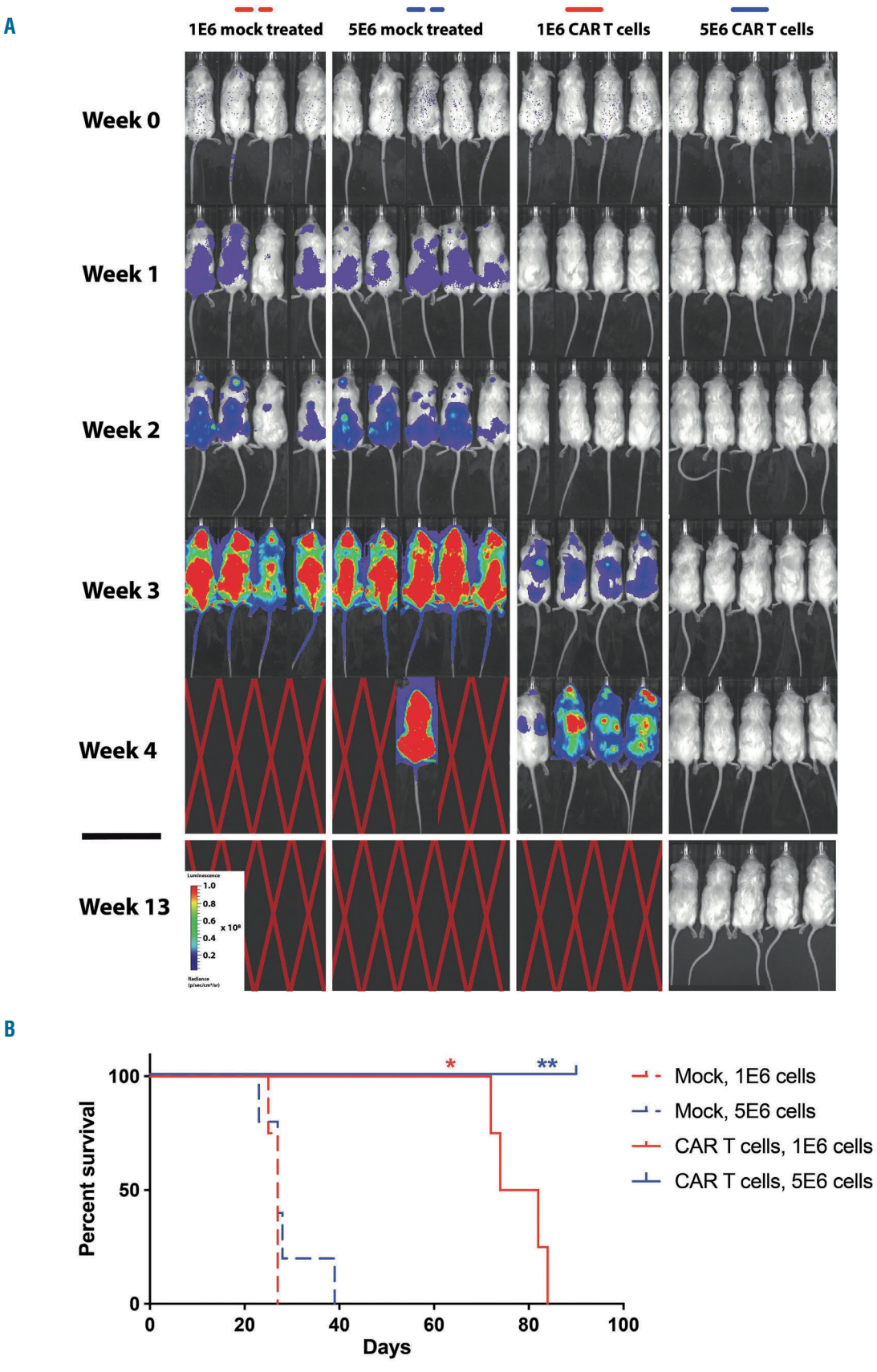

Figure 4. Antileukemic activity of genome-edited chimeric antigen receptor T cells *in vivo*. (A) Bioluminescence imaging of Nalm6 xenografts in NSG mice treated with genome-edited CD19-specific chimeric antigen receptor (CAR) T cells ($1x10^6$) that were manufactured from αβ-TCR$^+$ T cells. The experiment was repeated at the dose level of $5x10^6$ cells per mouse with comparable outcome. (B) Kaplan-Meier survival plot of mice treated with control T cells or CAR T cells. Asterisks indicate levels of significance of the CAR T-cell group compared to the respective control group (mock) of the same cell dose using log-rank tests. CAR: chimeric antigen receptor.

A

Label	sgRNA target sequence + PAM	Gene name	Annotation
On-target	GAGAATCAAAATCGGTGAATAGG	TRAC	exon (exon 2 of 5)
OT1	TAGAGTTGAAATCGGTGAATAGG	PUDP	Intergenic
OT2	ATCAATCAAATTCGGTGAATTGG	LOC105755953	Intergenic
OT3	ACGAATTAAAATTGGTGAATTGG	LINC01090	Intergenic
OT4	GAAAAGCAAAATAGGTGAATGGG	AJAP1	Intergenic
OT5	AAGAAAAAAAAATAGGTGAATTGG	VPS13A-AS1	intron (NM_001018037, intron 1 of 70)
OT6	AAAAAACAAAATCAGTGAATTGG	PIK3R1	Intergenic
OT7	CAGCTTCAAAATCTGTGAATGGG	LINC01854	intron (NR_122040, intron 1 of 4)
OT8	GAGAAAAAAAAATCAGTGAATTGG	CYP1B1-AS1	Intergenic
OT9	GAGAAGAAAAAATCAGTGAATTGG	NKX2-2	Intergenic
OT10	AAAAATCAATATCTGTGAATGGG	GALC	Intergenic
OT11	TAAAATCAAAAGGGGTGAATTGG	SYPL1	intron (NM_006754, intron 2 of 5)
OT12	GAGTATGAAAATCGCTGAATAGG	NEBL	intron (NM_213569, intron 2 of 6)
OT13	CAGAAGAAAAATCGATGAATTGG	AHNAK	intron (NM_024060, intron 5 of 5)
OT14	CAGAATCATAATACGTGAATTGG	LINC02027	Intergenic
OT15	AAGAATAAAAATTGATGAATTGG	FLJ43315	Intergenic
OT16	AAGAATAAAAATTGATGAATTGG	TEKT4P2	Intergenic
OT17	AAGAATAAAAATTGATGAATTGG	LOC442028	intron (NR_037597, intron 7 of 9)
OT18	AAGAATAAAAATTGATGAATTGG	FRG1BP	Intergenic
OT19	TATAATCAAAATTGGAGAATAGG	LRRK2	intron (NM_198578, intron 21 of 50)
OT20	TAGAATCAGAATAGGAGAATGGG	LINC01361	promoter-TSS (NR_110633)
OT21	AAGAATCATTATCGGTAAATTGG	GSE1	Intergenic
OT22	AAAAACCAAAATCGGTGTATCGG	GATA3	intron (NM_002051, intron 3 of 5)
OT23	AAGAAACAAAAACGGTGAGTTGG	RP1L1	exon (NM_178857, exon 3 of 4)
OT24	TAGAATCTAAATTGGTGACTCGG	CADPS	intron (NM_183394, intron 3 of 27)
OT25	AAGTATAAAAATCGGTGAAAGGG	LOC100506403	Intergenic
OT26	TAGAATCAGAATTGGTGACTAGG	LOC101929452	Intergenic
OT27	TAGAATCAGAATTGGTGATTTGG	LINC01029	Intergenic
OT28	CAGAATAAAACTCGGTGAACAGG	IMMP2L	intron (NM_032549, intron 5 of 5)
OT29	CTGAATCAAAATCAGTGAAGTGG	ADD2	intron (NM_017482, intron 1 of 12)
OT30	AATAATCAAAATCCGTGAAAAGG	DIO2-AS1	intron (NR_038355, intron 8 of 8)
OT31	TAGAATCAGAATCAGTGAAAGGG	MIR1269A	Intergenic
OT32	AAGAATCAATATCAGTGAAATGG	EBF2	Intergenic
OT33	AAGAATCAACATCGTTGAAAGGG	ZNF292	Intergenic
OT34	AAGAATCAATATCGTTGAAATGG	SLITRK6	Intergenic
OT35	TAGAATCAAAATAGCTGAACTGG	FAM41AY1	Intergenic
OT36	GAGCATCAAAATCGGTAAAGAGG	GABRA3	intron (NM_000808, intron 3 of 9)
OT37	AAGAATCAAAATCCTTGAAATGG	PABPC5-AS1	Intergenic
OT38	AGGAATCAAAATGGGTGAATGAG	DNAH14	intron (NM_001373, intron 62 of 83)
OT39	AAGAATGAAAATTGGTGAATTAG	LEKR1	intron (NM_001004316, intron 5 of 12)
OT40	CAGAATGAAAATCAGTGAATGAG	TMEM38B	Intergenic

B

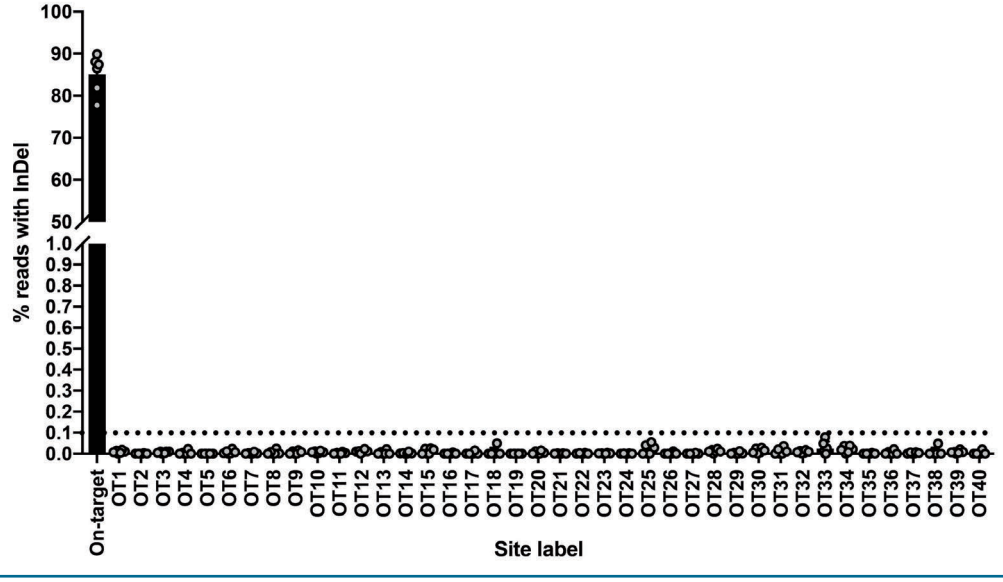

Figure 5. Evaluation of endonuclease specificity. (A) Putative off-target (OT) sites in the human genome (hg38) determined by COSMID and sorted by predicted relevance in descending order. Mismatches to the target site are marked red. OT 1-37 have three relevant mismatches in the protospacer region without insertions/deòetopms and OT sites 38-40 have two relevant mismatches and a protospacer-adjacent motif (PAM) mismatch. The nucleotide furthest from the PAM was ignored for sorting due to mismatch tolerance at this location by Cas9. (B) Human T cells from six different donors were electroporated with the high-fidelity Cas9 protein complexed with the sgRNA targeting the TRAC locus (or mock electroporated to determine background). Next-generation sequencing was performed on all predicted OT sites. The dotted line depicts the sensitivity limit attributed to this method of 0.1%. sgRNA: single guide RNA, PAM: protospacer-adjacent motif; OT: off-target.

increased survival if disease burden is low before administration of the cells,[52] and that high disease burden[53] is associated with an increased risk of side effects such as cytokine release syndrome. A potential advantage of using CAR T cells after HSCT is therefore the diminished leukemia burden due to the conditioning regimen and the transplant, which might translate into improved outcomes of the CAR T treatment and a lower incidence of cytokine release syndrome, but in the absence of informative animal models this needs to be tested in a clinical trial.

An alternative haplo-HSCT approach using post-transplant cyclophosphamide for *in-vivo* T-cell depletion has shown promising results, although published work, to date, has focused primarily on adults.[54,55] Although the use of αβ haplo-HSCT requires specific manufacturing expertise and upfront costs to establish the graft processing, we believe that the absence of post-HSCT pharmacological GvHD prophylaxis, the very low rate of severe GvHD and the low infection rate render this approach ideal for combination with post-HSCT adoptive immunotherapy. Eventually, it will need to be determined in prospective trials comparing αβ haplo-HSCT and post-transplant cyclophosphamide in children which alternative is the optimal treatment under what circumstances.

CAR T cells from healthy, allogeneic donors - which have preserved T-cell numbers and functionality and promise to overcome the manufacturing challenges and product variability of autologous CAR T cells[29,56-59] – carry the potential to mediate GvHD if they still carry their endogenous TCR.[60] Only a limited number of patients have been treated with allogeneic CAR T cells but the frequency of GvHD was surprisingly low when a co-stimulatory domain derived from the CD28 molecule was used in the CAR construct,[43] which raises the question of whether TCR deletion is necessary. Mechanistic studies suggest that the simultaneous activation of both the CD28-co-stimulated CAR and the TCR can lead to exhaustion and clonal deletion of alloreactive cells.[44] The selective deletion of alloreactive T cells in this mode occurred, however, only at certain ratios between CAR T cells and target cells, and an excess of CAR T cells was able to induce GvHD.[44] Furthermore, most CAR T-cell products are transfused without selection of the transduced cells and therefore contain untransduced cells not expressing a CAR which retain their alloreactive potential. Removal of the TCR from the cell surface, e.g., by genome editing approaches,[37,38,46] is the best approach to reduce the risk of GvHD of allogeneic cells and additionally might prevent the induction of the T-cell dysfunction that can develop if the CAR and TCR are engaged on the same cell.[61] CAR T cells with genome editing-based disruption of the TCR are currently being explored in clinical trials.[62]

Although CAR T cells with TCR knockout are often referred to as "universal" cells, they can still be rejected by the host immune system as it recovers from the immunodepletion given prior to CAR T-cell infusion.[63] Additional genetic engineering has been proposed to prevent recognition by the host immune system, e.g., the use of genome editing to remove HLA class 1 expression[64,65] and the expression of molecules that suppress natural killer cell activity.[66] These strategies raise the issue that, if they succeed, cells completely avoid recognition and clearance by the host immune system, but also escape immune surveillance in the case they become infected with viruses or turn malignant. Therefore, engineering an allogeneic CAR T-cell graft that achieves bidirectional immune tolerance with a host immune system including satisfactory immune surveillance remains an unresolved challenge. The use of donor-derived T cells to create a TCR^- CAR T-cell product that is administered after allogeneic HSCT enables HLA compatibility of the CAR T cells with the donor-derived host immune system after immune reconstitution. A remaining limitation to full immune compatibility is the nature or the CAR that we used, which is a synthetic protein with non-human parts and potential immunogenicity. Fully humanized CAR are currently in early stages of clinical trials.[67] It is possible that in the post-transplant setting the development of an immune response to the CAR will not occur but that can only be tested in a human clinical trial.

It has previously been shown that transfusion of 10^4 T cells/kg can mediate rapid and protective immune reconstitution,[68] while among a cohort of 98 patients undergoing αβ haplo-HSCT who received a median of $4x10^4$ $TCR\alpha\beta^+$ cells, no patient developed high-grade acute GvHD and only one patient developed extensive chronic GvHD.[8] With the $TCR\alpha\beta^+$ depletion efficiency that we demonstrated, we estimate that therapeutically relevant doses of the cell product (theoretically up to $33x10^6$ cells/kg) could be infused without administering more than 10^4 TCR^+ cells/kg. It remains to be evaluated in a clinical trial whether the small number of αβ T cells that are transfused with the hematopoietic stem cell fraction, together with the residual TCR^+ cells in the CAR T-cell product, substantially increase the GvHD risk.

In conclusion, we here establish a preclinical proof-of-concept for using the non-target fraction that is normally discarded during $\alpha\beta^+$ T cell/$CD19^+$ B-cell depletion to engineer a CD19-specific CAR T-cell product with a low risk of causing GvHD. αβ haplo-HSCT combined with graft-derived αβTCR-CD19 CAR T cells represents an appealing combination that: (i) allows a donor to be identified for virtually every patient in need; (ii) overcomes the issues related to manufacturing autologous CAR T cells; (iii) abrogates the risk of GvHD through genome editing of the TRAC locus; and (iv) provides persistent targeted immune surveillance after HSCT. Furthermore, the use of adoptive post-HSCT immunotherapy can potentially translate in the future into desirable conditioning regimens with lower toxicity and better preservation of fertility.

Disclosures

Patent submissions are planned. MHP serves on the scientific advisory boards of and has equity in CRISPR Tx and Allogene Therapeutics. Neither companies had any input into the design, execution, interpretation, or publication of this research. All the other authors declare that they have no competing financial conflicts.

Contributions

Conception and design: VW, RB, AB and MHP; in vitro studies: VW and PL; in vivo studies: VW and NM; off-target analysis: CML and GB; data collection: VW, CML; analysis and interpretation of data: VW, MHP and AB; supervision: AB, MHP and MGR; writing of the manuscript: VW and MHP. All authors reviewed the manuscript.

Acknowledgments

The authors thank Crystal Mackall, Rachel Lynn and Louai

Labanieh (Stanford University) for providing the CAR construct, the CD19-CAR idiotype-specific antibody and the Nalm6-GL and Raji-GL cell lines. The authors are also grateful to the Stanford Small Animal Imaging Facility, in which bioluminescence imaging was performed, for providing training and access to equipment. The authors thank the Veterinary Service Center at Stanford Medicine for providing training and animal care and Synthego for providing chemically modified sgRNA. VW was supported by fellowships from Deutsche Forschungsgemeinschaft (DFG) and Care-for-Rare Foundation (Germany). MHP thanks the Amon Carter Foundation, the Sutardja Foundation and the Laurie Kraus Lacob Faculty Scholar Award in Translational Research for support. MHP also thanks the Chan-Zuckerberg Biohub for support.

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