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

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Running head: Genome editing of donor-derived αβ⁺ T cells

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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 that allogeneic hematopoietic stem cell transplantation can be used in. Leukemic relapse, however, remains a challenge. T cells expressing chimeric antigen receptors can potently eliminate leukemia, including 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 integrate a CD19-specific chimeric antigen receptor in-frame into the TRAC locus. Greater than 90% of cells lost TCR expression, while >75% expressed the CAR. The initial product was further purified with less than 0.05% TCR+ cells remaining. In vitro, the CAR 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 (r/r BCP-ALL)\(^1,2\). Allogeneic HSCT (allo-HSCT) has been successfully employed to treat high-risk leukemias\(^3\), which provides a graft-versus-leukemia (GvL)\(^4\) effect but carries the risk for graft-versus-host disease (GvHD)\(^5\). 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 \(\alpha\beta\) T cells and CD19\(^+\) B-cells (\(\alpha\beta\) haplo-HSCT)\(^7\), which is associated with a very low incidence of transplantation-related mortality (TRM) and GvHD (figure 1A)\(^8-10\). In contrast to CD34\(^+\) selection, this manipulation method allows the transfer not only of donor HSCs to the recipient, but also committed hematopoietic progenitors as well as mature natural killer (NK) and \(\gamma\delta\) 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 (NRM), disease relapse has become the most important cause of treatment failure in patients with malignancies undergoing \(\alpha\beta\) haplo-HSCT\(^9\). In particular, the outcome of \(\alpha\beta\) haplo-HSCT in children with leukemia not in complete remission (CR) or beyond second CR 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 TRM.

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 leukocyte infusion, DLI) has been used in various contexts to enhance antileukemic efficacy (figure 1B), it is accompanied by a high risk of severe GvHD\textsuperscript{16–22}. An improvement over DLI 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 prevent relapse and the suicide switch (inducible caspase 9) is effective at eliminating allo-reactive cells if GvHD occurs\textsuperscript{23–25}. Although this strategy allows to control GvHD after it occurs, the beneficial effect of GvL and the risk of GvHD remain linked to each other. It would be an improvement, therefore, to establish an approach that provides antileukemic activity without GvHD.

Chimeric antigen receptors (CAR) can redirect T cell cytotoxicity towards cancer-related antigens and achieve remissions in otherwise refractory hematological malignancies expressing these targets\textsuperscript{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\textsuperscript{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\textsuperscript{29} and the risk of insertional mutagenesis associated with randomly integrating viral vectors\textsuperscript{30} are challenges associated with the established approach.

We hypothesized that $\alpha\beta$ 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 TCR$\alpha\beta^+/CD19^+$ cell fraction that is removed from the graft can be used to engineer non-alloreactive CAR T cells through homologous recombination (HR)-mediated genome editing by targeted integration of a CD19-
specific CAR in-frame into the TRAC locus (“αβTCR CD19 CAR-T”), and demonstrate the anti-leukemic 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 NK cells, γδ T cells and the 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 AAV production

Transfer plasmids were cloned between the ITRs in pAAV-MCS (Agilent Technologies). The CAR comprises a GM-CSFRα leader sequence, the FMC63 scFv31, CD28 hinge, transmembrane and intracellular sequences and the CD3ζ intracellular domain. rAAV6 production and titration is described in suppl. methods.

Apheresis and cell processing

αβ haplo-HSCT donors received granulocyte-colony stimulating factor (G-CSF) for 4 days at the total dose of 16 µg/kg body weight and apheresis was performed on the 5th day. When on day 4 the CD34+ cell count was <40/µL, a CXCR4 antagonist (Plerixafor, Mozobil) was given. Manipulations were performed in a closed system according to GMP standards with clinical grade reagents and instrumentation from Miltenyi Biotec (Bergisch Gladbach, Germany).

T cell culture and genome editing

All human cells were handled according to a protocol approved by the IRB 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 described32.
MACS depletion

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 washing step. For details see suppl. 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 IL-2 and IFN-γ production of the CAR T cells and cytotoxicity. For details see suppl. 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. 5x10^5 CD19+ Nalm6-GL cells were transplanted i.v. into 6-12 week old male NSG mice. 4 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 up 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 Biolegend). 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

gRNA target sites were identified and their specificity score calculated by bioinformatics (crispor.tefor.net33). COSMIC (crispr.bme.gatech.edu34) 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 6 different donors and predicted off-target sites sequenced using an Illumina MiSeq as described previously35. For details see suppl. methods.

Statistics

Plots show means with error bars representing either standard deviation or 95% confidence interval (CI), as indicated. Groups were compared by statistical tests as indicated 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 haplo-HSCT to create CAR 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 HR-mediated genome editing using Cas9 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 for 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, 2A), similar 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 costimulatory 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 right 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 sgRNAs (suppl. figure 1A, 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 3 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 the TCR\(\alpha\beta^+\) T cells that were removed from the graft during \(\alpha\beta\) haplo-HSCT, we cultured and stimulated these cells and electroporated them with ribonucleoprotein (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 recombinant adeno-associated virus serotype 6 (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 NGFR which has been used safely in clinical immunotherapy trials\(^24\)) (figure 2B, C). These unprecedented efficiencies of targeted integration of a large gene expression cassette (2.7kb) 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 (DSB) repair can efficiently be skewed toward homologous recombination to the level where it constitutes the predominant repair pathway and targeted integration becomes more frequent than insertion/deletion (InDel)-formation by non-homologous end-joining (NHEJ). Notably, the starting cells had been processed at two different GMP facilities (five at UCSF and six 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 costimulation 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 TCR$αβ^+$ cells from the expanded cell population by magnetic bead activated cell sorting (MACS) using reagents for which GMP-compatible counterparts are available. We were able to achieve efficient depletion with a maximum of 0.03% TCR$αβ^+$ cells remaining in the resulting cell product (a depletion efficiency of 2-3 orders of magnitude, figure 2E, F), a higher efficiency than in prior studies that created TCR-negative CAR T cells$^{45,46}$. We termed the resulting cell product after genome editing, expansion and TCR$αβ^+$ depletion “$αβ$TCR 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
This suggested that AAV transduction is the main factor impacting cell expansion, which led us to determine the optimal AAV dose for optimal gene targeting efficiency. Interestingly, we found that a change in the MOI beyond 2500 vg/cell only led to a minor change in targeting outcomes with saturation at 5000 vg/cell (suppl. fig. 2B). Instead, the duration of time during which the cells are kept at a high concentration for AAV transduction (>5x10^6 cells per ml) directly after electroporation and before dilution to the target cell density influenced gene targeting outcomes to a greater extent (suppl. fig. 2C). Using an MOI of 5000 vg/cell and a prolonged transduction time at high density (>12h), 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 rapidly expand 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 hours, we were able to measure production of IL-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 hours 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 the source cell population besides the $\alpha\beta$ T cells, we followed the CD19$^+$ cell population by phenotyping and were able to detect their disappearance as early as 24 hours after gene targeting (2.53%, vs 7.9-11.8% in the control condition in which the T cells do not express CAR), suggesting early cytotoxic activity of CD19-specific CAR T cells (figure 3C, suppl. fig. 2D). Follow up showed that the cell product continues to self-deplete from the residual B cells over time (figure 3C). The presence of CD19$^+$ cells in the manufacturing process may be the stimulus for the excellent T cell expansion we observe in the TCR$^-$ cells during the manufacturing process.

We next performed phenotyping of 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 is about 0.8:1 (figure 3D, F). The majority of CD4$^+$ were of naïve and central memory (CM) phenotype, while in the CD8$^+$ subpopulation the majority of cells showed a naïve phenotype (figure 3E, G). This confirms that the CAR T cells have a balanced CD4:CD8 ratio, and that despite the TCR stimulation before genome editing and the transient activation through the CAR mediated by the B cell cytotoxicity, both subpopulations have a high fraction of naïve and CM cells.

Antileukemic efficacy in vivo

We determined the activity of the $\alpha\beta$ TCR CD19 CAR-T product in vivo using a standard Nalm6 xenograft model$^{47}$. We transplanted $5 \times 10^5$ CD19$^+$ Nalm6 cells i.v. into NSG mice to create the
CD19+ leukemia model. Four days later, we i.v. infused $1 \times 10^6$ or $5 \times 10^6$ αβ TCR-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 durable 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 $5 \times 10^6$ cells, $p<0.05$ for $1 \times 10^6$ cells). No xenogeneic GvHD was observed in any of the mice demonstrating low GvHD potential in the T cell products. We repeated the experiment at the CAR T cell dose level of $5 \times 10^6$ cell 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 (suppl. fig. 3a, b).

**Off-target evaluation**

While the gRNA specificity has previously been evaluated in an IDLV 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 6 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 INDELs at off-target sites above the detection limit of 0.1% in any of the samples (figure 5B).

**Discussion**
In the case of persistent MRD 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 like CAR T cells. \(\alpha\beta\) haplo-HSCT represents an excellent platform for adoptive immunotherapy because not only does it help overcome the limited availability of HLA-matched donors, but post-HSCT immunosuppression is not required. It has shown robust clinical results in pediatric patients\(^8\), but a fraction of patients still relapses.

We here hypothesize that a donor-derived CAR T cell product with TCR knockout after haplo-HSCT has the potential to dissociate the beneficial antileukemic activity from harmful GvHD, which are inherently connected to each other when infusing unmanipulated donor-derived lymphocytes (DLI). This would take advantage of both the GvL-effect of allo-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 product and 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 from the left-over cell fraction and administering them after HSCT will be more economical than the common practice of following the administration of autologous CAR T cells with allogeneic HSCT\textsuperscript{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 increased survival if disease burden is low before their administration\textsuperscript{52}, and that high disease burden\textsuperscript{53} is associated with an increased risk for side effects like cytokine release syndrome (CRS). 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 to improved outcomes of the CAR T treatment and a
lower CRS incidence, but in the absence of informative animal models this needs to be tested in a clinical trial.

An alternative haploidentical HSCT approach using post-transplant cyclophosphamide (pT-Cy) for *in-vivo* T cell depletion has shown promising results, though published work, to date, has focused primarily on adults. Although the use of αβ haplo-HSCT requires a 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 the combination with post-HSCT adoptive immunotherapy. Eventually, it will need to be determined in prospective trials comparing αβ haplo-HSCT and pT-Cy in children which alternative represents the optimal treatment under which 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 – carry the potential to mediate GVHD if they still carry their endogenous TCR. Only a limited number of patients have been treated with allogeneic CAR T cells but the frequency of GvHD has been surprisingly low when a co-stimulatory domain derived from the CD28 molecule was used in the CAR construct, which raises the question whether TCR deletion is necessary. Mechanistic studies suggest that the simultaneous activation of both the CD28-costimulated CAR and the TCR can lead to exhaustion and clonal deletion of alloreactive cells. 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. Furthermore, most CAR T cell products are transfused without selection of the transduced cells and therefore contain untransduced cells not expressing a CAR that retain
their alloreactive potential. Removal of the TCR from the cell surface, e.g. by genome editing approaches\textsuperscript{37,38,46}, is the best approach to reduce the risk of GvHD of allogeneic cells and additionally might prevent the induction of T cell dysfunction that can develop if the CAR and TCR are engaged on the same cell\textsuperscript{61}. CAR T cells with genome editing-based disruption of the TCR are currently being explored in clinical trials\textsuperscript{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 infusion\textsuperscript{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\textsuperscript{64,65} and the expression of molecules that suppress NK cell activity\textsuperscript{66}. These strategies raise the issue that if they succeed to completely avoid recognition and clearance by the host immune system, the cells also escape immune surveillance in case they become infected with viruses or turn malignant. Therefore, engineering an allogeneic CAR T cell graft that achieves bi-directional 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 CARs are currently in early stages of clinical trials\textsuperscript{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 per kg can mediate rapid and protective immune reconstitution\textsuperscript{68}, while among a cohort of 98 patients undergoing $\alpha\beta$ haplo-HSCT that received a median of $4\times 10^4$ TCR$\alpha\beta^+$ cells, no patient developed high-grade acute and only 1 patient developed extensive chronic GvHD\textsuperscript{8}. With the TCR$\alpha\beta^+$ depletion efficiency that we demonstrated, we estimate that therapeutically relevant doses of the cell product (theoretically up to $33\times 10^6$ cells/kg) can 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 $\alpha\beta$ T cells that are transfused with the HSC fraction, together with the residual TCR$^+$ cells in the CAR T cell product, substantially increase the GvHD risk.

In conclusion, we here establish 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 low risk of causing GvHD. $\alpha\beta$ haplo-HSCT combined with graft-derived $\alpha\beta$TCR$^+$CD19 CAR-T cells represents an appealing combination that allows to 1) identify a donor virtually for every patient in the need, 2) overcome the issues related to manufacturing autologous CAR T cells, 3) abrogate the risk of GvHD through genome editing of the TRAC locus and 4) provide a 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.
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<td>CI</td>
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<td>Magnetic bead activated cell sorting</td>
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<td>MOI</td>
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<td>MRD</td>
<td>Minimal residual disease</td>
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<tr>
<td>NHEJ</td>
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NRM Non-relapse mortality
ORF Open reading frame
pT-Cy post-transplantation cyclophosphamide
rAAV6 Recombinant adeno-associated virus serotype 6
RNP Ribonucleoprotein
TCR T cell receptor
tNGFR Truncated version of the nerve growth factor receptor
vg Vector genomes

Author contributions

Conception and design: Volker Wiebking, Rasmus Bak, Alice Bertaina and Matthew Porteus
In vitro studies: Volker Wiebking, Premanjali Lahiri
In vivo studies: Volker Wiebking, Nathalie Mostrel
Off-target analysis: Ciaran M. Lee and Gang Bao
Data collection: Volker Wiebking, Ciaran M. Lee
Analysis and interpretation of data: Volker Wiebking, Matthew Porteus, Alice Bertaina
Supervision: Alice Bertaina, Matthew Porteus, Maria Grazia Roncarolo
Writing of manuscript: Volker Wiebking, Matthew Porteus
Review of the manuscript: all co-authors

Disclosure of Conflicts of Interest

Matthew Porteus serves on the scientific advisory boards of CRISPR Tx and Allogene Therapeutics. Both companies had not input into the design, execution, interpretation, or publication of this research.

All other authors declare that no competing financial conflict exists.
References


Figure legends

Figure 1 – T cell therapy approaches in combination with TCRαβ+/CD19+-depleted haploidentical stem cell transplantation aiming to decrease relapse rates. A) The protocol for haploidentical HSCT with TCRαβ+/CD19+-depletion, which establishes a backbone for additional cellular immunotherapies. B) In order to improve immune reconstitution and enhance anti-leukemic 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 GvHD, the T cells can be transduced with a safeguard system like herpes simplex virus-derived thymidine kinase (HSV-TK) or inducible Caspase 9 (iCasp9). D) The αβ T cells are removed from the graft before transplantation and can be used as starting material to create genome edited 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. CAR = chimeric antigen receptor, GvHD = graft-versus-host disease, HSPCs = hematopoietic stem and progenitor cells, TCR = T cell receptor, NK cells = natural killer cells.

Figure 2 – Targeted integration of a CD19-specific CAR into the TRAC locus. A) Targeting strategy using Cas9 RNP and rAAV6. B) Representative FACS plots for cells treated as indicated 4 days after targeting to evaluate efficiency of TCRαβ knockout and 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 idiootype-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 4 different replicates, plotted as mean +/- SD. G) Expansion of T cells during the 7 days after gene editing compared to numbers before electroporation using optimized conditions for AAV 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.
**Figure 3 – In vitro functionality of CAR T cells engineered from αβ+ T cells.** A) IL-2 and IFNγ concentrations in cell culture supernatant after culture of control cells or CAR T cells alone, or co-cultures of CAR T cells with Nalm6 or Raji cells. Control cells were treated with RNP only (TRAC knockout without CAR expression). Bars and error bars represent mean +/- SD from 3 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 20h with Nalm6 cells or Raji cells (both CD19+ and GFP+) at different effector-to-target (E:T) 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 3 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 have undergone gene targeting (RNP + AAV) or control treatments. Groups were compared by t tests and levels of significance 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 4 biological replicates. Bars and error bars represent mean +/- SD. G) Quantification of the memory/effector populations on cells from 4 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.

**Figure 4 – Antileukemic activity of genome edited CAR T cells in vivo.** A) Bioluminescence imaging of Nalm6 xenografts in NSG mice treated with genome edited CD19-specific CAR T cells that were manufactured from αβ-TCR+ T cells. The experiment was repeated at the dose level of 5E6 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.
Figure 5 – Evaluation of endonuclease specificity. A) Putative off-target 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 3 relevant mismatches in the protospacer region without InDels and OT sites 38-40 have 2 relevant mismatches and a PAM mismatch. The nucleotide furthest from the PAM was ignored for sorting due to of mismatch tolerance at this location by Cas9. B) Human T cells from 6 different donors were electroporated with the high-fidelity Cas9 protein complexed with the sgRNA targeting the TRAC locus (or mock electroporated to determine background). NGS 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, InDel = insertion or deletion.
**Figure 1**

**A**
- Haploidentical transplantation with αβ T cell and B cell depletion
- Haploidentical donor → αβ T cells → B cells → HSPCs, NK cells, γδ T cells → Patient
- No post-transplant immune suppression
- Low risk of GvHD

**B**
- Haploidentical transplantation with T cell “add-back”
- Haploidentical donor → αβ T cells → B cells → HSPCs, NK cells, γδ T cells → Add back of specified αβ T cell number → Patient
- αβ T cells mediate polyclonal antileukemic activity
- Risk of GvHD, treated with immunosuppression

**C**
- Haploidentical transplantation with transfusion of suicide-gene-modified T cells
- Haploidentical donor → T cells → Transfusion of transduced T cells → B cells → HSPCs, NK cells, γδ T cells → Patient
- T cells mediate HLA-dependent antileukemic activity
- Risk of GvHD, treatable with immunosuppression and suicide switch activation

**D**
- Haploidentical transplantation and treatment with genome edited TCRαβ CAR T cells
- Haploidentical donor → αβ T cells → B cells → CAR T cells → Transfusion of genome edited CAR T cells → Patient
- CAR T cells mediate antigen-specific antileukemic activity
- Low risk of GvHD
### A

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### B

**% reads with InDel**

![Bar graph showing percentage of reads with InDel for each site label from OT1 to OT40.](image-url)
Supplemental file for

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

Volker Wiebking, Ciaran M. Lee, Nathalie Mostrel, Premanjali Lahiri, Rasmus Bak, Gang Bao, Maria Grazia Roncarolo, Alice Bertaina and Matthew H. Porteus

Contents:

- Suppl. Figures 1-3
- Suppl. Methods
Suppl. Figures

A) Counts of COSMID-predicted off-target sites for sgRNAs in exon 1 of the TRAC locus, ranked from left to right by increasing counts of predicted sites, prioritizing sgRNAs with low numbers of highly similar off-target sites (low COSMID scores).

B) Overview over sgRNAs in exon 1 of TRAC with specificity scores calculated by CRISPOR (MIT algorithm) and COSMID. sgRNA = single guide RNA, PAM = protospacer-adjacent motif.

Suppl. figure 1 – Off-target prediction for sgRNAs at exon 1 of the TRAC locus.
Suppl. figure 2 – Optimization of T cell targeting conditions and B cell detection.

A) Expansion after electroporation of CAR T cells or control cells treated with the indicated conditions, relative to their count before electroporation. Cells originated from 2 different donors. B) Titration of different rAAV6 MOIs shows a slight increase in the frequency of targeted cells between 2500 and 5000 vg/cell but no further increase beyond that. C) A strong determinant of targeting frequencies is the duration of transduction at high density (>5x10⁶ cells per ml) before dilution with medium to the target density for expansion (5x10⁵ cells per ml). Transduction was performed at the MOI of 5000 vg/cell. D) Detection of B cells 24h after gene targeting shows early disappearance of CD19⁺ cells for the condition that leads to CAR expression but not in the control conditions (mock electroporation, RNP electroporation only, AAV transduction only). AAV = adeno-associated virus, RNP = ribonucleoprotein, MOI = multiplicity of infection, NGFR = nerve-growth-factor receptor.
Suppl. figure 3 – CAR T cell efficacy in vivo using two different control groups.

A) Survival graph for Nalm6 xenografts in NSG mice after transplantation of mock treated T cells (Mock), TRAC RNP treated T cells (RNP) or RNP + AAV treated T cells (CAR T) at 5 million cells per mouse. Groups were compared by log-rank test and statistical significance indicated in the graph. B) BLI imaging result on day 15 after injection comparing the 3 groups.
Suppl. methods

AAV production

rAAV6 was produced as previously described\textsuperscript{1} or acquired from Vigene Biosciences Inc. The absolute concentration of ITR copy numbers was determined by Droplet Digital PCR (Bio-rad) using previously reported primer and probe sets\textsuperscript{2}.

T cell culture and genome editing

The TCRαβ\textsuperscript{+}/CD19\textsuperscript{+} cell fraction (non-target fraction from the graft manipulation procedure) was used fresh or cryopreserved. Cells were cultured in X-VIVO 15 (Lonza) supplemented with 5% human AB serum (Sigma) and 100 IU/ml recombinant human IL-2 (Peprotech). Medium changes were performed every 2-4 days and T cells maintained at a target density of 5x10\textsuperscript{5} cells/ml unless otherwise indicated.

T cells were activated with Dynabeads Human T cell Activator (Gibco) for 3 days and beads removed before electroporation. For gene editing, electroporation was performed as previously described\textsuperscript{1}. HPLC-purified sgRNA with 2’-O-methyl-3’-phosphorothioate modifications at the three terminal nucleotides on both ends\textsuperscript{3} (Synthego) was complexed with high-fidelity spCas9 protein\textsuperscript{4} (IDT) at a molar ration of 2.5:1 (sgRNA : protein) and electroporated in buffer P3 (Lonza) into activated T cells using a 4D-Nucleofector (Lonza) in 16-cuvette strips. 1E6 activated T cells were used per electroporation using program EO-115. The cells were resuspended directly after electroporation in 80μl of complete T cell medium and then diluted to the target density. For gene targeting, cells were incubated within 15 minutes after electroporation with rAAV6 for transduction at a multiplicity of infection (MOI) of >5000 vg/cell unless otherwise stated. After
the specified transduction time, the suspension was diluted with complete medium to reach the target cell concentration as indicated.

*In vitro cytokine measurement and cytotoxicity assay*

CD19⁺ Nalm6-GL cells stably expressing GFP and Firefly Luciferase (FLuc)⁵ and CD19⁺ Raji cells, also stably expressing GFP and Firefly Luciferase, were gifts from Crystal Mackall (Stanford) and used in co-culture assays with the CAR T cells or control T cells at different effector:target ratios for 20 hours. Concentrations of IL-2 and IFN-γ in supernatant were measured with the respective ELISA kits (Biolegend). For cytotoxicity assays, the cells were co-cultured and the absolute number of GFP⁺ cells in culture determined by high-throughput flow cytometry on a CytoFLEX (Beckman Coulter) after adding a specified number of CountBright Absolute Counting Beads (Thermo Fisher Scientific) to the cell suspension. The number of GFP⁺ cells killed were estimated relative to control samples in which target cells were cultured without effector cells with the formula \(100 - \left(\frac{\text{GFP}^+\text{count}_{\text{Control}} - \text{GFP}^+\text{count}_{\text{sample}}}{\text{GFP}^+\text{count}_{\text{Control}}}\right) \times 100\).

*Off-target analysis*

COSMID (crispr.bme.gatech.edu⁶) was used to identify potential off-target sites in the human genome (hg38) allowing up to 3 mismatches or 1 bp deletion/insertion and 1 mismatch in the 19 PAM-proximal bases.

For empirical analysis of predicted off-targets, T cells from 6 different donors were electroporated with Cas9 RNP targeting TRAC or mock electroporated and genomic DNA extracted using the Qiagen Blood and Tissue kit. Primers for amplification of all predicted sites were designed by the COSMID program. All genomic loci were amplified by specific PCR, barcoded in a second round
of PCR, pooled at equimolar ratios and sequenced using an Illumina MiSeq with 250bp paired end reads as described previously\(^7\). The resulting data was analyzed with the script indelQuantificationFromFastqPaired-1.0.1.pl\(^8\).

References used in supplement:


