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# Simultaneous Deletion of Endogenous TCRαβ for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic

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**Generation of an optimal T cell therapeutic expressing high frequencies of transgenic T cell receptor (tgTCR) is essential for improving TCR gene therapy. Upon TCR gene transfer, presence of endogenous TCRαβ reduces expression of tgTCR due to TCR mixed-dimer formation and competition for binding CD3. Knockout (KO) of endogenous TCRαβ was recently achieved using CRISPR/Cas9 editing of the *TRAC* or *TRBC* loci, resulting in increased expression and function of tgTCR. Here, we adopt this approach into current protocols for generating T cell populations expressing tgTCR to validate this strategy in the context of four clinically relevant TCRs. First, simultaneous editing of *TRAC* and *TRBC* loci was reproducible and resulted in high double KO efficiencies in bulk CD8 T cells. Next, tgTCR expression was significantly higher in double *TRAC/BC* KO conditions for all TCRs tested, including those that contained structural modifications to encourage preferential pairing. Finally, increased expression of tgTCR in edited T cell populations allowed for increased recognition of antigen expressing tumor targets and prolonged control of tumor outgrowth in a preclinical model of multiple myeloma. In conclusion, CRISPR/Cas9-mediated KO of both endogenous TCRαβ chains can be incorporated in current T cell production protocols and is preferential to ensure an improved and safe clinical therapeutic.**

## INTRODUCTION

Adoptive T cell therapy is a promising approach for cancer immunotherapy that is supported by the initial successes of chimeric antigen receptor T cell (CART) therapy against hematological malignancies.[1] Despite this, antigen loss on targeted tumor cells causes relapse in treated patients, demonstrating the importance of antigenic selection for the success of T cell therapies. Unlike CARs, T cell receptors (TCRs) can recognize both extracellular- and intracellular-derived antigen in the context of human leukocyte antigen (HLA). This extends the range of antigenic options and increases tumor specificity via targeting of neo-antigens[2,3] or proteins promoting oncogenesis, such as transcription factors.[4] To date, the transfer of TCR genes specific for tumor antigens into T cells has demonstrated efficacy in clinical studies but has yet to show complete responses in patients.[5,6] Improvement of TCR gene therapy is currently focused toward two approaches. First, discovery of novel high-affinity tumor-reactive TCR aims to advance the therapeutic scope of TCR gene transfer and interesting candidates are being discovered.[3,4,7–12] Second, generating an optimal cellular product will benefit therapeutic effect, and identification of cell populations with increased proliferative capacity, persistence, and efficacy has been well researched.[13–15] Ideally, a cellular product bearing these qualities would contain a high frequency of T cells expressing transgenic TCR (tgTCR) to be effective, and currently, due to their high efficiencies and safety, retroviral or lentiviral systems are the preferred delivery methods for clinical applications. However, the presence of endogenous TCRαβ chains in bulk T cell populations hampers tgTCR expression due to mixed TCR dimer formation, as well as competition for binding to the CD3 signaling complex. Mixed TCR dimers have demonstrated their potential to cause unpredictable neo-reactivities,[16,17] and strategies to encourage preferential pairing, such as murinization or cystinylation of TCR constant domains, are often included in tgTCR design. Furthermore, structural modifications can increase the overall tgTCR expression frequencies in edited T cells, but competition for CD3 binding is forever present.[18] Competitive TCR environments are challenging for TCR gene therapy, particularly if the tgTCR is a weak competitor.[19] Without structural modification, these TCRs readily mispair, resulting in low-frequency TCR expression in edited T cells, as demonstrated previously.[19,20] Weak competitor TCRs are difficult to predict prior to TCR gene transfer, and eliminating the competition all together is an attractive strategy. Several studies have highlighted the benefit of endogenous TCRαβ knockout (KO) in primary CD8 T cells for TCR gene transfer using different laborious gene-editing approaches.[21,22] Recently, the feasibility of genome editing using CRISPR/Cas9 targeting the *TRAC* and *TRBC* loci in primary T cells was demonstrated.[23–25] Here, we adopt this approach into current protocols for generating CD8 T cell populations

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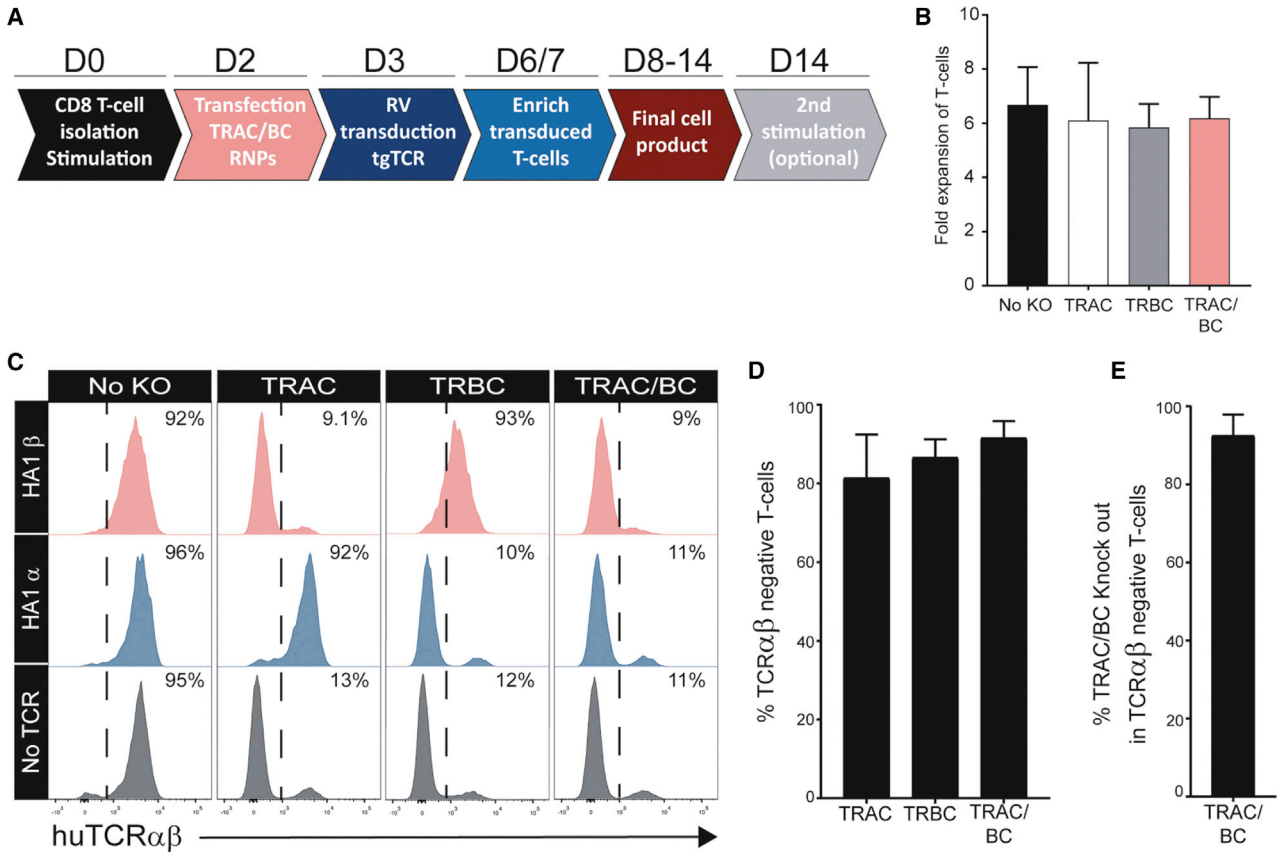


**Figure 1. Simultaneous CRISPR/Cas9 Targeting of *TRAC* and *TRBC* Loci Resulted in Reproducible High Double KO Efficiencies in Primary CD8 T Cells**

Primary CD8 T cells were transfected on day 2 (D2) post-stimulation with crRNA-trRNA Cas9 ribonuclear proteins (RNPs) targeting *TRAC*, *TRBC*, or simultaneous *TRAC/BC* loci. On day 3 (D3), HA1 TCRα or β chain was retrovirally transduced individually into the CD8 T cells. Expression of TCRαβ was measured by flow cytometry 4 days after transduction (D7). (A) Schematic of T cell production protocol used to create T cells depleted of endogenous TCRαβ. (B) D3–D8 fold expansion of T cells transfected without RNP (no KO) or with *TRAC*-RNP, *TRBC*-RNP, or *TRAC/BC*-RNP. Data combined from three separate donors (n = 3). Error bars show mean and SD. (C) TCRαβ expression of CD8 T cells. Values represent the percentage of TCRαβ expressed on live cells. T cells transduced with HA1α or HA1β were first gated on marker gene expression. (D) % TCRαβ-negative cells resulting from CRISPR/Cas9 targeting of *TRAC* (n = 7), *TRBC* (n = 7), and simultaneous *TRAC/BC* (n = 9) loci. (E) % *TRAC/BC* simultaneous knockout in TCRαβ-negative T cells (n = 9). Error bars represent mean with SD.

retrovirally transduced to express tgTCR. Within this study, we further validate the beneficial effect of endogenous TCRαβ KO in the context of four clinically relevant TCRs targeting hematological malignancies or cytomegalovirus (CMV) infections. We demonstrate that the creation of a non-competitive TCR environment following simultaneous KO of both endogenous TCRαβ chains results in an improved cellular therapeutic ultimately leading to increased performance in a preclinical murine model for human multiple myeloma.

## RESULTS

### Simultaneous KO of *TRAC* and *TRBC* Is Highly Efficient in Primary CD8 T Cells Using CRISPR/Cas9 Ribonucleoproteins (RNPs)

To create a non-competitive TCR environment in primary CD8 T cells, it is essential both endogenous TCRαβ genomic sequences are targeted by CRISPR/Cas9. CRISPR/Cas9 RNPs can be electroporated into CD8 T cells on day 2 post-stimulation without prolonging T cell production time (Figure 1A). Furthermore, CRISPR/Cas9 RNPs have limited impact on T cell expansion (Figure 1B). To determine the efficiency of double *TRAC/BC* KO, activated primary CD8 T cells were transfected separately with *TRAC* targeting RNP (*TRAC*-RNP), *TRBC* targeting RNP (*TRBC*-RNP), or simultaneously (*TRAC/BC*-RNP). Analysis of TCR expression on the cell surface of edited CD8 T cells revealed reproducible high-KO efficiencies when *TRAC*-RNP (81% ± 11%), *TRBC*-RNP (87% ± 4.5%), or *TRAC/BC*-RNP (91.6% ± 4.2%) were transfected (Figures 1C and 1D). To determine if *TRAC/BC*-RNP KOs elicited simultaneous editing of both the *TRAC* and *TRBC* loci, edited cells were retrovirally transduced to express the α chain or β-chain of HA1 TCR.[20] In single *TRAC*-RNP and *TRBC*-RNP transfected cells, the remaining endogenous α chain or β chain resulted in re-expression of TCR at the cell surface due to mixed dimer formation with HA1 TCRαβ chains (Figure 1C). In *TRAC/BC*-RNP transfected cells, little to no re-expression of TCR was observed, therefore demonstrating an absence of both endogenous α chain and

β chains (Figure 1C). Using this technique, the double KO efficiency within TCRαβ-negative T cell populations was calculated to be 93% ± 5.2% and thus demonstrated a simple, reproducible method for highly efficient KO of both endogenous TCRαβ chains (Figure 1E).

### Expression and Function of Weak Competitor HA1 TCR Is Optimal Only in *TRAC/BC* Double KO Primary CD8 T Cells

HA1 TCR specifically recognizes a peptide derived from HA1 minor histocompatibility antigen (miHA) in HLA-A*02:01.[26] In previous studies, retroviral transduction of unedited CD8 T cells (no KO) with unmodified, weak competitor HA1 TCR resulted in low-frequency expression in transduced cells,[20,26] further validated by low-frequency of tetramer-positive cells (median no KO, 12%) seen in this study (Figures 2A and 2B). Single KO of *TRAC* or *TRBC* genes was sufficient to improve frequencies of tetramer-positive cells (median *TRAC*, 31%; and *TRBC*, 31%), indicating both the HA1 TCR α and β chain contributed to HA1 TCR weak competitor phenotype (Figures 2A and 2B). However, simultaneous deletion of both *TRAC* and *TRBC* was essential for enhanced expression frequencies (median *TRAC/BC* KO, 67%) as well as increased cell-surface expression of HA1 TCR in transduced CD8 T cells (Figures 2A–2C). Consequently, single KO of *TRAC* or *TRBC* did not remove competition completely for cell-surface expression and may encourage mixed dimerization of the unmodified, weak competitor HA1 TCR (Figure 1A). The increased frequencies of HA1 TCR also benefited efficacy *in vitro*, and single KO of *TRAC* and *TRBC* allowed for increased interferon (IFN)-γ production in response to peptide-loaded target cells (Figure 2D) and target cells endogenously expressing HA1 miHA compared to no KO (Figure 2E). However, the largest increase of IFN-γ production was consistently seen from *TRAC/BC* KO T cells (Figures 2D and 2E). Furthermore, compared to no KO, antigen-specific cytotoxicity of T cells was also improved by double *TRAC/BC* KO (Figure 2F). Interestingly, endogenous TCR reactivity and alloreactivity of untransduced CD8 T cells against tumor targets was also reduced in *TRAC*, *TRBC*, and *TRAC/BC* KO, demonstrating an increased safety profile of edited T cells (Figure S1). These data indicated simultaneous KO of *TRAC* and *TRBC* enables a non-competitive TCR environment, which is essential for high expression of weak competitor TCR in the absence of structural modifications.

### Tumor-Specific TCRs Structurally Modified to Enhance Preferential Pairing Also Benefit from *TRAC/BC* Double KO

Cysteine (cys) modification of TCRαβ constant domains to encourage preferential pairing was previously shown to improve the weak competitor phenotype of HA1 TCR.[20,27] Ultimately, this resulted in increased frequency of HA1 TCR expressing CD8 T cells, as confirmed in this study (Figure 3A). To determine the effect of a non-competitive TCR environment on expression of cys-modified TCR, *TRAC*-, *TRBC*-, and *TRAC/BC*-edited CD8 T cells were transduced with HA1(cys) TCR alongside three clinically designed TCRs incorporating cys-modification, CMV(cys) TCR, preferentially expressed antigen on melanoma (PRAME)(cys) TCR, and BOB1(cys) TCR (Figure 3). TCR expression was then determined by the frequency of tetramer-positive transduced T cells as before. Cys-modification remained sub-optimal for TCR expression, and the non-competitive TCR environment created by *TRAC/BC* KO allowed for increased frequencies of tetramer binding cells for HA1(cys) TCR (median no KO, 47%; *TRAC/BC*, 74%), CMV(cys) TCR (median no KO, 7%; *TRAC/BC*, 41%), PRAME(cys) TCR (median no KO, 9%; *TRAC/BC*, 26%), and BOB1(cys) TCR (median no KO, 13%; *TRAC/BC*, 36%) (Figures 3B–3D). Furthermore, all TCRs showed increased cell-surface expression in *TRAC/BC* KO cells (Figure 3). Single KO of *TRAC* or *TRBC* revealed the requirement for removal of both endogenous TCRαβ chains varied for each introduced TCR as well as each donor T cell population (Figure 3). HA1(cys) TCR, like its unmodified counterpart, benefited marginally from *TRAC* and *TRBC* single KO (Figure 3A). In contrast, CMV(cys) TCR demonstrated comparable frequencies of tetramer binding in *TRAC* KO and *TRAC/BC* KO cells (Figure 3B). PRAME(cys) TCR, like HA1 TCR, benefited the most from *TRAC/BC* KO (Figure 3C), while BOB1(cys) TCR benefited from *TRBC* single KO as much as *TRAC/BC* KO (Figure 3D). However, in order to consistently achieve optimum TCR expression frequencies, *TRAC/BC* double KO was universally required (Figure 3).

These results were also represented functionally, demonstrating single KO was sufficient for some donor and TCR combinations (Figure S2). However, *TRAC/BC* KO cells reliably outperformed their no KO counterpart, as demonstrated by increased IFN-γ production in response to endogenous antigen-expressing tumor targets for all TCRs tested (Figure 4). Increased cytotoxicity was also observed in *TRAC/BC* KO cells, as shown by PRAME(cys) TCR-expressing T cells (Figure S3). The benefit of double *TRAC/BC* KO was also seen with T cells transduced with murinized BOB1 TCR and therefore not restricted to cys-modified TCR (Figure S4). In these cells, the reduced competition for cell-surface expression was clearly demonstrated. In no KO T cells, the majority of cells co-expressed murine TCRβ and human TCRαβ, whereas in *TRAC*, *TRBC*, and *TRAC/BC* KO T cells, the majority of cells expressed murine TCRβ alone (Figure S4A). Increased cell-surface expression in *TRAC/BC* KO T cells also allowed improved cytokine production and cytotoxicity against antigen-expressing targets (Figures S4D–S4F). These data suggested structural modification did not completely overcome the challenges of TCR expression in a competitive TCR environment and should be combined with endogenous TCRαβ KO to gain optimal TCR expression frequencies and consequently improve efficacy.

### *TRAC/BC* Double KO CD8 T Cells Expressing tgTCR Outperform Unedited CD8 T Cells in an *In Vivo* Model for Multiple Myeloma

To further demonstrate the functional advantages of *TRAC/BC* KO CD8 T cells for TCR gene transfer therapy, an *in vivo* xenograft model for human multiple myeloma was used. NOD *scid* gamma (NSG) mice were injected with luciferase-expressing multiple myeloma cell line U266, which expresses HA1 miHA in HLA-A*02:01 recognized by the HA1(cys) TCR. It has already been established that higher doses of tumor-specific TCR-expressing T cells correlates with a decreased tumor burden in xenograft tumor models (data not shown). Therefore, increased frequencies of tetramer-positive cells

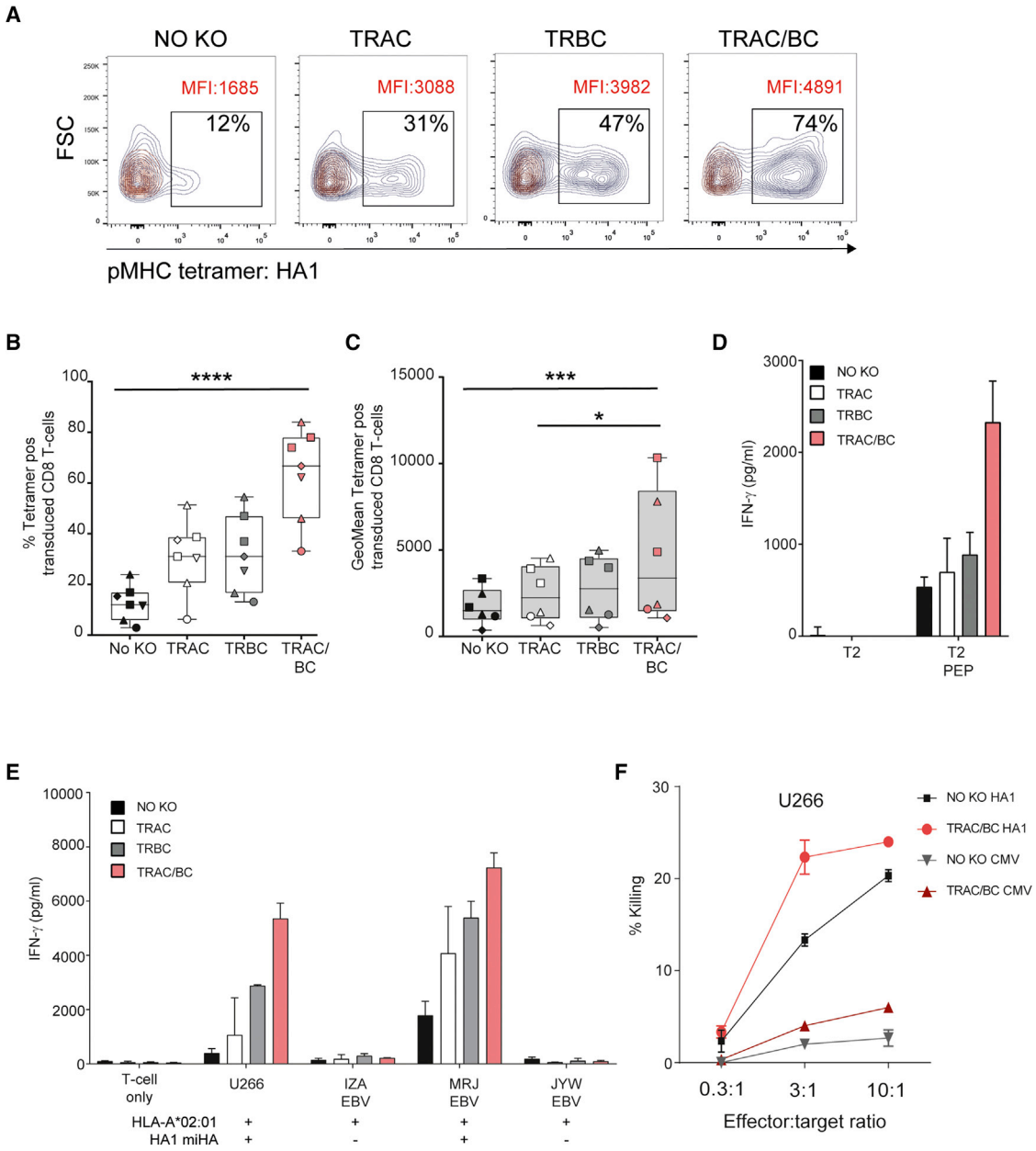


**Figure 2. Simultaneous CRISPR/Cas9 Targeting of *TRAC* and *TRBC* Loci Resulted in Increased Frequencies of Weak Competitor HA1 TCR-Expressing T Cells, Leading to Improved Function**

Primary CD8 T cells were transfected on day 2 post-stimulation with crRNA-trRNA RNPs targeting *TRAC*, *TRBC*, or simultaneous *TRAC/BC* loci or without RNP (no KO). On day 3, codon-optimized HA1 TCRαβ was retrovirally transduced into CD8 T cells. FACS analysis was performed on day 7. (A) Frequency of HA1 TCR expression was determined using HA1-specific tetramers and measured by FACS. Figure in bold represents frequency of tetramer-positive cells and red represents geometric mean of tetramer-positive cells. Where applicable, CD8 T cells were gated on transduced cells (blue) or untransduced cells (red). (B) Summary of tetramer expression in CD8 T cells expressing HA1 TCR (n = 7). (C) Summary of geometric mean of tetramer binding CD8 T cells expressing HA1 TCR (n = 7). *Statistical significance using paired Freidman's test. Each symbol represents a different donor from separate experiments, and error bars represent the median and the range. (D) IFN-γ production from unsorted CD8 T cells transduced with HA1 TCR against peptide-loaded T2 target cells. Data represents two separate donors from separate experiments (n = 2). (E) Representative experiment of IFN-γ production from sorted CD8 T cells transduced with HA1 TCR against tumor targets with (MRJ, U266) or without (IZA) endogenous HA1 miHA expression. (F) % killing of U266 by HA1 TCR or CMV TCR-bearing T cells, measured by chromium release after 6 h co-culture. Error bars represent mean and SD.

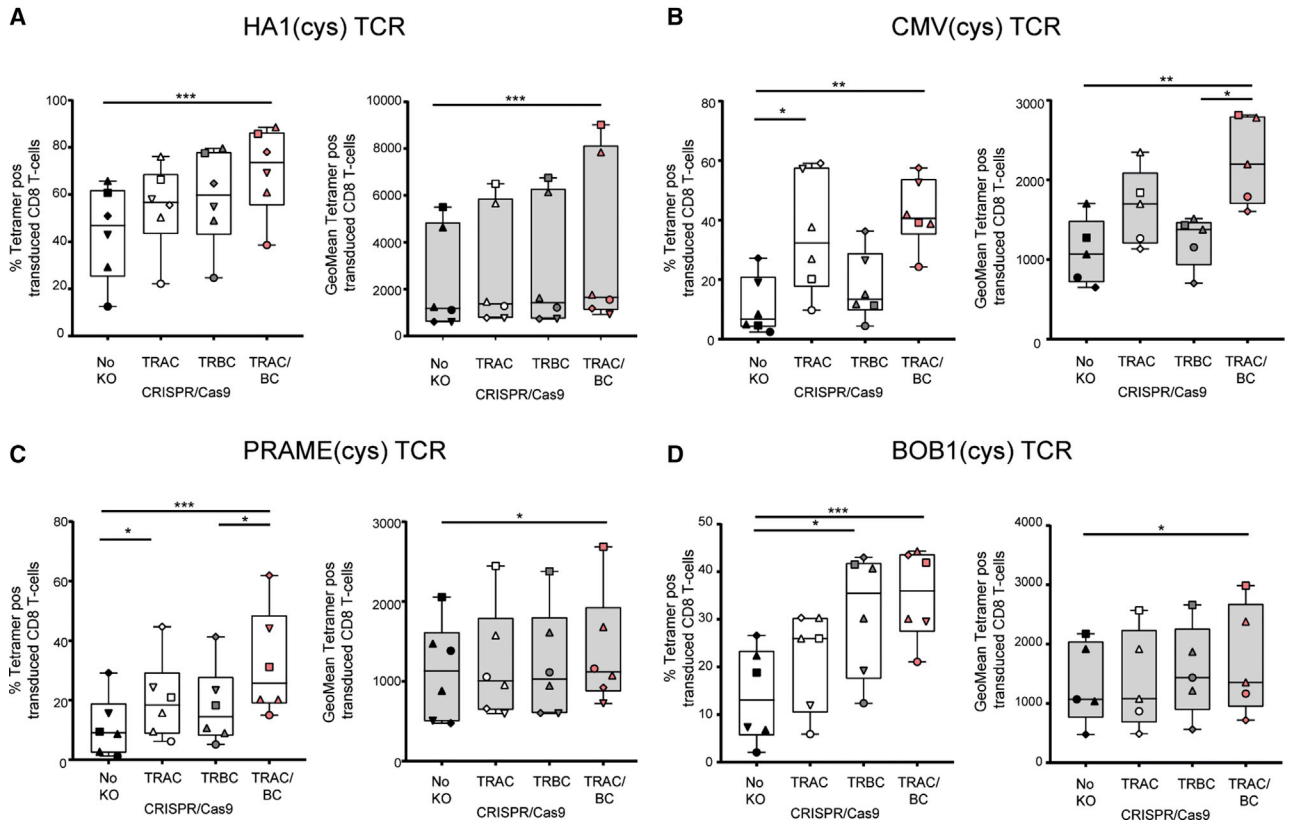


**Figure 3. Simultaneous CRISPR/Cas9 Targeting of *TRAC* and *TRBC* Loci Increased Expression Frequency of Clinically Designed Cysteine (cys)-Modified TCRs in Transduced CD8 T Cells**

Primary CD8 T cells were transfected on day 2 post-stimulation with crRNA-trRNA RNPs targeting *TRAC*, *TRBC*, or simultaneous TR-AC/BC loci or without RNP (no KO). On day 3, (A) HA1(cys) TCRαβ, (B) CMV(cys) TCRαβ, (C) PRAME(cys) TCRαβ, or (D) BOB1(cys) TCRαβ were retrovirally transduced into CD8 T cells. All TCRs were codon optimized and included a cys-modification in the constant domain to aid preferential pairing. 4 days after transduction, the frequency of TCR expression was determined by specific tetramer binding and measured by FACS. Transduced cells were first gated according to marker gene expression before subsequent analysis. Each symbol represents a different donor from separate experiments. White bars represent the frequency of tetramer-positive cells, and gray bars represent the geometric mean of tetramer positive cells. *Statistical significance calculated using paired Friedman multiple-comparison test. Error bars represent the median and the range.

resulting from *TRAC/BC* KO within an infused T cell product should elicit a similar effect when compared to no KO. Here, unedited CD8 T cells (no KO) or *TRAC/BC* KO CD8 T cells were transduced to express the HA1(cys) TCR or muCMV TCR as a negative control and enriched for marker-gene expression. Prior to injection, the frequency of tetramer binding cells was determined, and increased frequencies of HA1(cys) TCR-expressing cells were again demonstrated in the *TRAC/BC* KO setting compared to no KO (Figure 5A). These T cells were then infused into the mice at a low dose to investigate the effect on a high tumor burden. Following T cell infusion, U266 outgrowth was significantly reduced when treated with the HA1(cys) TCR compared to muCMV TCR in both the *TRAC/BC* KO and no KO conditions (Figure 5). However, *TRAC/BC* KO T cells expressing HA1(cys) TCR significantly outperformed their no KO counterpart in controlling U266 outgrowth (Figures 5B and 5C). Interestingly, HA1(cys) TCR-treated mice demonstrated prolonged control of U266 outgrowth for almost 3 weeks post-T-cell-injection in the *TRAC/BC* KO setting (Figure 5B). In a parallel experiment, BOB1(cys)TCR-expressing T cells were also infused into U266-bearing mice, and similarly, an enhanced effect on controlling tumor outgrowth was observed with *TRAC/BC* KO T cells compared to no KO (Figure S5). In conclusion, CRISPR/Cas9-mediated *TRAC/BC* KO creates a non-competitive TCR environment that allows optimal TCR expression in bulk-edited CD8 T cells.

## DISCUSSION

The transfer of TCR genes into bulk T cell populations potentiates the redirection of T cells toward chosen antigens, which is desirable for adoptive T cell therapies. It is known that the presence of endogenous TCRαβ interferes with cell-surface expression of the introduced TCR. This is due to mixed dimerization of introduced TCR with endogenous TCR but also from competition for CD3 binding. Here, we implemented CRISPR/Cas9 editing of the *TRAC* and *TRBC* loci to create a non-competitive TCR environment. Other approaches to induce simultaneous KO are often laborious, result in low KO efficiencies, and require multiple enrichment steps to achieve double KO T cell

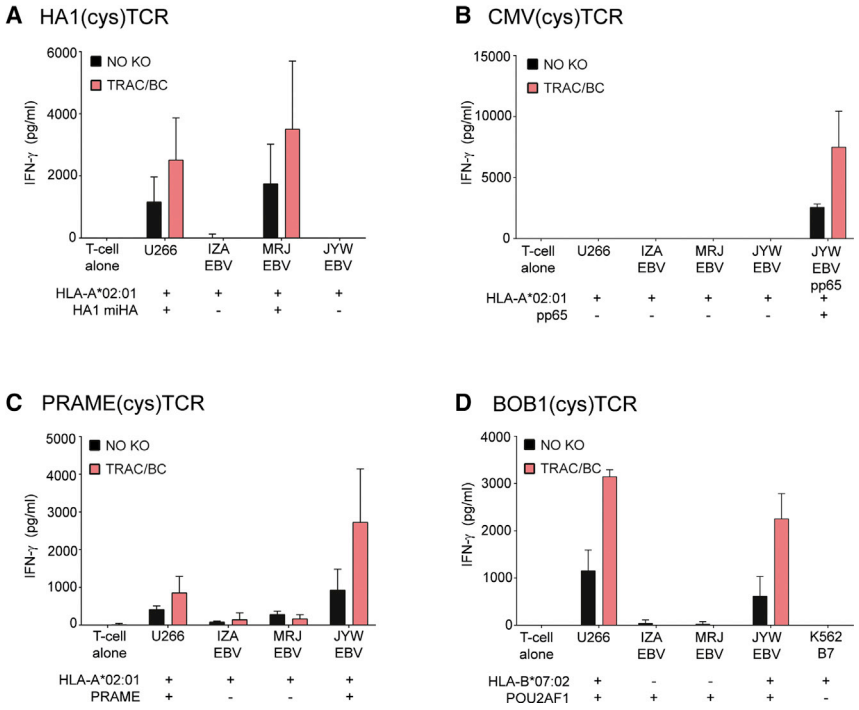


**Figure 4. Enhanced *In Vitro* Antigen-Specific Recognition by *TRAC/BC* KO CD8 T Cells Transduced to Express Clinically Designed TCRs Incorporating cys-Modification of TCR Constant Domains**

Primary CD8 T cells were transfected on day 2 post-stimulation with crRNA-trRNA RNPs targeting both *TRAC* and *TRBC* loci or without RNP (no KO). On day 3 (A) HA1(cys) TCR, (B) CMV(cys) TCR, (C) PRAME(cys) TCR, or (D) BOB1(cys) TCR was retrovirally transduced into CD8 T cells. On day 10, transduced T cells were co-cultured overnight with cell lines known to endogenously express each TCR-specific antigen in the context of HLA class I. Supernatant was subsequently harvested, and recognition of each target cell was measured by IFN-γ ELISA. Each figure represents combined analysis of two separate experiments and two different donors (n = 2). Error bars represent mean and SD.

populations.[21,22] Our chosen method for CRISPR/Cas9 editing was RNPs, which are shown to be highly efficient in inducing genomic editing in primary T cells without interfering with cell viability and expansion.[24,28] Following transfection, RNPs permit instantaneous genomic editing, are transiently expressed, and are therefore beneficial for cellular therapeutics by reducing the risk of Cas9 immunogenicity.[29] We showed that, using RNPs, simultaneous KO of *TRAC* and *TRBC* loci is achieved at exceptionally high efficiencies of approximately 90% within bulk T cell populations following a single transfection step and thereby easily adapted into current T cell production protocols. To further increase the safety in an allogeneic transplant setting, the remaining unedited T cells can be depleted from T cell products prior to TCR gene transfer. However, in an autologous setting, the low frequency of remaining unedited T cells would not be harmful to the patient and the risk of neo-reactivities is dramatically reduced due the high *TRAC/BC* KO efficiencies.

The generation of T cell populations depleted of endogenous TCRαβ improved the expression frequencies of four clinically relevant TCRs in transduced T cells. Notably, with the exception of HA1 TCR, the increased frequency of tetramer binding cells rarely exceeded 50% of total transduced T cells. Historically, low tetramer frequencies in unedited CD8 T cells were often rationalized as a result of competition for cell-surface expression. This is no longer a sufficient explanation when TCR is introduced into a TCRαβ-depleted T cell population. Although we do not have a clear explanation for this, the low tetramer binding frequencies may be a result of differences in TCR turnover at the cell surface, different tetramer binding thresholds, or even due to the inclusion of structural modifications of introduced TCR. Consequently, increased tetramer binding may not reflect absolute frequency of introduced TCR expressed. Nevertheless, it reflected the increased functional efficacy that we describe and resulted in an enhanced cellular therapeutic in a preclinical xenograft model for human multiple myeloma.

Our data also supported findings from previous studies demonstrating that removal of endogenous TCRαβ is an essential approach to improve TCR gene therapies.[21,22] Prior to CRISPR/Cas9, the challenges of a double KO strategy have perhaps prevented its application into general protocols for T cell production. Furthermore, single KO approaches have been shown to be easier and were sufficiently safe by preventing graft-versus-host disease (GvHD) in xenograft models.[30] Our data also corroborated this, as allogeneic responses were also reduced in single KO T cells. However, most studies are focused toward a single TCR construct, and our data demonstrate that the essentiality of simultaneous *TRAC/BC* KO largely depends on the pairing capabilities of the TCR being introduced. The requirement for *TRAC/BC* KO was especially relevant for weak competitor HA1 TCR, a codon-optimized construct without any structural modifications. In such circumstances, single KO may encourage mixed dimerization with the remaining TCRα or β chains, as without competition, the introduced TCRαβ chains would preferentially mispair with almost any alternative TCRαβ available, leading to unpredictable neo-reactivities.[16] Removing competition in T cells also allowed for unmodified HA1 TCR to reach expression levels comparable to the cys-modified alternative. As a result of double TCRαβ depletion, introduced TCR can now retain its native configuration without the need for structural modifications to aid preferential binding.

Improvements on TCR expression is just one step toward creating a successful clinical therapeutic. Persistence of an infused T cell product

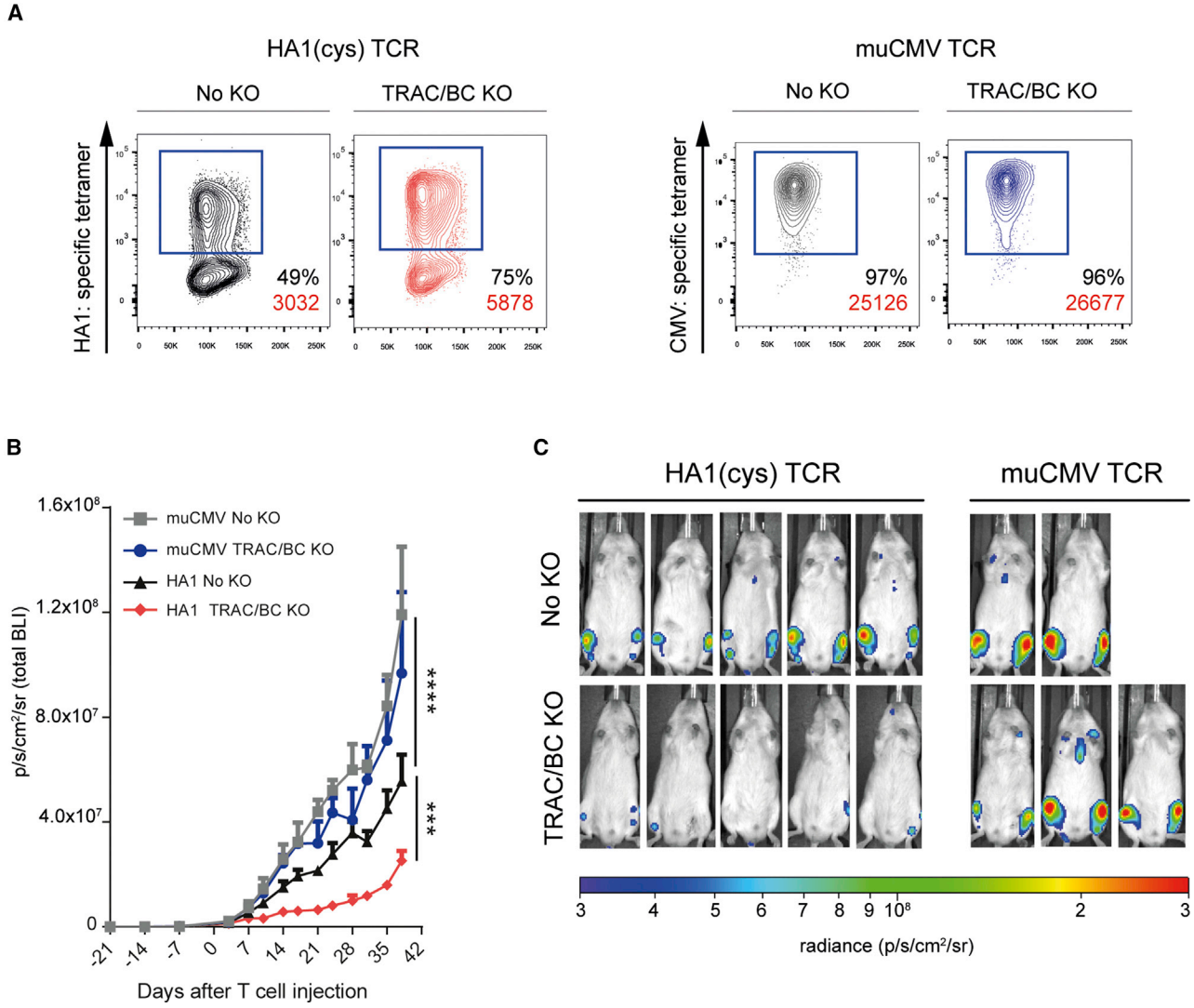


**Figure 5. Enhanced Expression of TCR in *TRAC/BC* KO CD8 T Cells Improves the *In Vivo* Efficacy of TCR Gene Transfer Cellular Therapeutics in a Xenograft Model of Multiple Myeloma**

NSG mice were infused with a luciferase-expressing U266 multiple myeloma cell line day −21 prior to T cell infusion. Primary CD8 T cells were transfected with *TRAC/BC*-RNP or without RNP (no KO) and subsequently retrovirally transduced with HA1(cys) TCR or muCMV TCR as a negative control. Transduced cells were enriched on marker-gene expression, and (A) antigen-specific tetramer was used to detect introduced TCR expression prior to T cell infusion. Data represents the total frequency of tetramer-positive T cells (black) and the geometric mean of the tetramer-positive population (red). Mice were then infused intravenously with $0.75 \times 10^6$ transduced T cells. (B) Tumor burden was measured by luminescence of U266 over time for each mouse infused with T cells expressing HA1(cys) TCR (no KO, n = 5; *TRAC/BC* KO, n = 5) or muCMV TCR (no KO, n = 2; *TRAC/BC* KO, n = 3). (C) Representative images at day 21 of U266 tumor burden. Error bars represent mean and SE.

as well as increased efficacy is an important consideration. Recent studies have demonstrated that the phenotype of T cells present in the infused product has a predictive effect of response rates in CART patients, and the self-renewal potential of a cell is considered advantageous for adoptive T cell therapies.[14,31–33] Although currently the standard for TCR and CAR introduction into T cells, retroviral and lentiviral systems may potentially hinder persistence of T cells by inducing early exhaustion due to rapid re-expression and therefore continual stimulation following antigen encounter.[34] Recently, the endogenous regulation of TCR and CAR was described using CRISPR/Cas9 genome-editing strategies and was demonstrated as a promising approach for future therapeutics;[23,24] however, expression frequencies have yet to compare to lentiviral and retroviral methods. Whichever strategy is used, the risk of genome editing creating oncogenic mutations in a cellular therapeutic is ever present. An increased potential for chromosomal translocations in a double KO approach is to be considered; furthermore, off-target editing by CRISPR-Cas9 can also pose a risk. As with early retroviral work, safety of CRISPR-Cas9

genome editing is still under scrutiny, and understanding the risks associated is currently being explored.

In conclusion, all tgTCRs used in this study demonstrated improved expression frequencies in T cells depleted of endogenous TCRαβ, resulting in improved efficacy of T cell therapeutics. The simplicity of the CRISPR/Cas9 gene-editing approach, alongside retroviral insertion of TCR, means it can be applied to any primary T cell population and give rise to large numbers of tumor-reactive TCR-expressing cells without increasing production time. In conclusion, simultaneous KO of endogenous TCRαβ is now a feasible approach that creates an enhanced cellular therapeutic for adoptive T cell therapy of malignancies.

## MATERIALS AND METHODS

### Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Isopaque separation from blood donated from healthy individuals after informed consent, according to the declaration of Helsinki, and cryo-preserved. Cell lines, U266 (multiple myeloma), Epstein-Barr virus-transformed lymphoblastic cell lines (EBV-LCL)s (IZA-EBV, MRJ-EBV, JYW-EBV), T2, and K562 were cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1.5% 200 mM L-glutamine (Lonza, Switzerland), and 1% 10,000 U/mL penicillin/streptomycin (Lonza, Switzerland). Prior to experiments, K562 was retrovirally transduced to express HLA-A*02:01 (K562-A2) or HLA-B*07:02 (K562-B7), and magnetic-activated cell sorting (MACS) enriched for nerve growth factor receptor (NGFR) marker gene expression using anti-allophycocyanin (APC) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were cultured in T cell medium (TCM) consisting of IMDM supplemented with 5% heat-inactivated FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 5% human serum, 1.5% 200 mM L-glutamine (Lonza, Switzerland), 1% 10,000 U/mL penicillin/streptomycin (Lonza, Switzerland), and 100 IU/mL interleukin-2 (IL-2) (Novartis, Switzerland). This study was approved by the institutional review board of the Leiden University Medical Centre (approval number B16.039).

### RNP Production

RNPs were generated by complexing CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) with *Streptococcus pyogenes* (sp)Cas9 as previously described.[28] In brief, guide RNA (gRNA) sequences (Table S1) were ordered as modified crRNA (Integrated DNA Technologies [IDT] Coralville, IA, USA) and annealed with trRNA (IDT) at a 1:1 ratio for 5 min 95°C. *TRAC* gRNA was previously published,[23] while *TRBC* gRNA was designed using the online genetic perturbation platform offered by the Broad Institute.[35] (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). gRNA sequences were checked for on-target and off-target predicted binding using an online tool provided by IDT (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE). Off-target prediction sites are listed (Tables S2 and S3). Per transfection, 90 pmol of crRNA:trRNA duplex was then combined with 30 pmol (5 μg) Cas9 nuclease V3 (IDT) (3:1 gRNA to Cas9 molar ratio) and incubated for 15 min at room temperature to form an individual RNP complex. RNPs were then either immediately electroporated into T cells or aliquoted and cryopreserved at −80°C. *TRAC*-RNP and *TRBC*-RNP were formed separately and were combined only during transfection.

### Generation of Gene-Edited CD8 T Cells

CD8 T cells were isolated from frozen PBMCs following magnetic separation using anti-CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched CD8 T cells were directly stimulated with irradiated autologous PBMCs (3,500 rads) at an effector:stimulator (E:S) ratio of 1:5 in the presence of 0.8 μg/mL phytohemagglutinin (PHA) (Oxoid Microbiology Products, Thermo Fisher Scientific, Waltham, MA, USA). Every 2–3 days, T cells were refreshed with TCM. On day 2 post-stimulation, 1–5 × $10^6$ T cells per transfection were electroporated with *TRAC*-RNPs and/or *TRBC*-RNPs using the NEON transfection system (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. In short, T cells were resuspended in 90 μL T buffer before addition to 10 μL $dH_2O$ containing *TRAC*-RNP and/or *TRBC*-RNP and 11 μM electroporation enhancer (IDT). Using 100 μL Neon Tips (Thermo Fisher Scientific, Waltham, MA, USA), T cells and RNPs were electroporated (1600 V, 10 ms, 3 pulses) as previously described.[36] Electroporated cells were immediately returned to fresh prewarmed TCM and incubated overnight at a concentration of 1 × $10^6$ cells/mL. On day 3 post-stimulation, T cells were retrovirally transduced with retrovirus encoding TCRαβ chains. Day 6/7 post-stimulation, transduced T cells were MACS enriched, following manufacturer's instructions, for NGFR marker gene expression or murine TCRβ expression using anti-APC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), unless otherwise stated.

### Virus Production and Retroviral Transduction of TCR

Codon-optimized TCRαβ chains were linked via 2A sequences and cloned into pLZRS-I-NGFR. For determining endogenous TCR KO efficiencies, single TCRα chains were cloned into pLZRS-I-GFP, and single TCRβ chains were cloned into pLZRS-I-NGFR. TCRαβ with murinized constant domains were cloned into MP71-TCR-FLEX retroviral vectors as previously described.[3] TCR germline sequences can be found in the international immunogenetics information system (IMGT). HA1 TCR expressed IMGT: TRAV25*01 and TRBV7-9*03 and is specific for miHA HA1, expressed in hematopoietic cells.[20] CMV TCR expressed IMGT: TRAV18 and TRBV13 and is specific for HLA-A*02:01-restricted peptide derived from CMV protein pp65,[19] a reactivity involved in the control CMV reactivation following hematopoietic stem cell transplant.[37] PRAME TCR expressed IMGT: TRAV8*04 and TRBV9 and recognizes a HLA-A*02:01-restricted peptide derived from PRAME, a known tumor-associated antigen (TAA).[12] BOB1 TCR expressed IMGT: TRAV13-1*01 and TRBV4 and is specific for HLA-B*07:02-restricted peptide derived from transcription factor BOB1, expressed in healthy and malignant B cells.[4] cys-modification of TCR constant domains

was as described previously.[27] Virus supernatant was harvested from transfected Phoenix-A cells and frozen at −80°C. For retroviral transduction, 24-well flat-bottom suspension culture plates (Greiner Bio-One) were preincubated with 30 μg/mL Retronectin (Takara, Clontech, Kusatsu, Japan) and blocked with 2% human serum albumin (Sanquin, Amsterdam, the Netherlands). Thawed virus supernatant was added to the plates and centrifuged at 3,000 × *g* for 20 min at 4°C. Virus supernatant was then removed, and $0.3 \times 10^6$ T cells were added to each well before subsequent overnight incubation at 37°C. Afterward, T cells were transferred to 24-well culture plates and given fresh TCM to expand.

### Flow Cytometry

On days 7–10 post-stimulation, fluorescence-activated cell sorting (FACS) analysis was performed on either the LSRII (BD Biosciences, NJ, USA) or Fortessa X20 (BD Biosciences, NJ, USA), and data were analyzed using FlowJo V10 software (TreeStar, OR, USA). $0.05 \times 10^6$ T cells were aliquoted into 96-well v-bottom plates, and all cells were preincubated with 1/1,000 zombie aqua (BioLegend, San Diego, CA, USA) for 25 min at 4°C. For determining endogenous TCR KO, T cells were subsequently incubated with anti-NGFR-APC (ME20.4, Sanbio/Southern Biotech Association, Uden, the Netherlands) and anti-huTCRαβ-PercpCy5.5 (IP26, BioLegend San Diego, U.S.) for 20 min at 4°C. For tetramer binding of retrovirally-transduced cells, T cells were initially incubated with phycoerythrin (PE)-labeled peptide-major histocompatibility complex (pMHC) tetramers for 20 min at 4°C and subsequently incubated with anti-NGF-R-APC (ME20.4, Sanbio/Southern Biotech Association Uden, the Netherlands) or with anti-muTCRβ- APC (H57-597, BD/Pharmingen, NJ, USA) for 20 min at 4°C.

### T Cell Reactivity Assays

T cell recognition was measured by IFN-γ ELISA (Sanquin, Amsterdam, the Netherlands). On days 10–14, T cells (1:5 or 1:30 E: S ratios) were co-cultured with 30,000 target cells in 60 μL TCM per 384-well flat-bottom plates (Greiner Bio-One, Austria). Supernatants were harvested after overnight incubation to measure IFN-γ release. In peptide pulsed conditions, T2, K562-A2, or K562-B7 were preincubated with the relevant 1 μM peptide (Table S4) for 30 min RT before addition to T cells.

T cell cytotoxicity was measured by $^{51}$chromium release assay. Target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 h at 37°C and thoroughly washed. Labeled target cells were then co-cultured with T cells at indicated effector:target (E:T) ratios in 100 μL 10% IMDM for 6 h before harvesting 25 μL of supernatant and transferring to a 96-well LumaPlates (PerkinElmer, Waltham, MA, USA). $^{51}$Cr release was measured using a 2450 Microbeta$^2$ plate counter (PerkinElmer, Waltham, MA, USA). % killing was calculated as follows; (test release − spontaneous release)/(maximum release − spontaneous release) × 100. Maximum release was determined upon incubation of target cells with 1% Triton-X.

### *In Vivo* Anti-myeloma Efficacy

Gender- and age-matched NOD.Cg-*Prkdc*(scid)*Il2rg*(tm1Wjl)/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained in individually ventilated cages in groups, and food and water was provided *ad libitum*. Mice of 8–12 weeks of age were injected intravenously (i.v.) with $5 \times 10^6$ U266 cells that were transduced to express luciferase (pCDH-EF1-Luc2-P2A-tdTomatoRed, a gift from Kazuhiro Oka to Addgene, plasmid #72486, Cambridge, MA, USA) and sorted to > 98% purity by flow cytometry. To monitor tumor growth, mice were anesthetized with 3%–4% isoflurane after they received an intraperitoneal (i.p.) injection of 200 μL 7.5 mM D-luciferine (Cayman Chemical Company, Ann Arbor, MI, USA). Whole-body bioluminescence images were obtained using a CCD camera (IVIS spectrum, PerkinElmer, Waltham, MA, USA). Three weeks after tumor transfer, mice were injected i.v. with $0.75 \times 10^6$ transduced CD8 T cells, and efficacy was assessed at indicated time points until sacrifice. All animal studies were conducted in accordance with institutional guidelines after obtaining permission from the national Ethical Committee for Animal Research (AVD116002017891).

### Statistics

Statistical analysis was performed using GraphPad Prism7 software. Statistical significance of TCR expression was calculated using Friedman multiple-comparison test with adjusted p values. Each dataset from each donor was paired. Statistical significance of *in vivo* efficacy was calculated using two-way ANOVA Tukey's multiple-comparison test with multiplicity-adjusted p values.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.10.001.

## AUTHOR CONTRIBUTIONS

L.T.M. designed, performed, analyzed, and interpreted the *in vitro*/*in vivo* experiments and wrote the manuscript. R.M.R. designed, performed, analyzed, and interpreted the *in vivo* experiments and critically revised the manuscript. A.K.W. provided technical assistance and performed *in vitro* experiments. C.K. provided technical assistance and performed *in vivo* experiments. D.F.G.R. provided technical assistance and designed TCR constructs. C.R.P. performed *in vitro* experiments. J.H.F.F. critically revised the manuscript and supervised. M.H.M.H. conceptualized, supervised, interpreted data, and critically revised the manuscript.

## CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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