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Original Article

Protective T cell receptor identification for orthotopic reprogramming of immunity in refractory virus infections

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Viral infections cause life-threatening disease in immunocompromised patients and especially following transplantation. T cell receptor (TCR) engineering redirects specificity and can bring significant progress to emerging adoptive T cell transfer (ACT) approaches. T cell epitopes are well described, although knowledge is limited on which TCRs mediate protective immunity. In this study, refractory adenovirus (AdV) infection after hematopoietic stem cell transplantation (HSCT) was treated with ACT of highly purified Hexon5-specific T cells using peptide major histocompatibility complex (pMHC)-Streptamers against the immunodominant human leukocyte antigen (HLA)-A*0101-restricted peptide LTDLGQNLLY. AdV was successfully controlled through this oligoclonal ACT. Novel protective TCRs were isolated *ex vivo* and preclinically engineered into the TCR locus of allogeneic third-party primary T cells by CRISPR-Cas9-mediated orthotopic TCR replacement. Both TCR knockout and targeted integration of the new TCR in one single engineering step led to physiological expression of the transgenic TCR. Reprogrammed TCR-edited T cells showed strong virus-specific functionality such as cytokine release, effector marker upregulation, and proliferation capacity, as well as cytotoxicity against LTDLGQNLLY-presenting and AdV-infected targets. In conclusion, *ex vivo* isolated TCRs with clinical proven protection through ACT could be redirected into T cells from naive third-party donors. This approach ensures that transgenic TCRs are protective with potential off-the-shelf use and widened applicability of ACT to various refractory emerging viral infections.

INTRODUCTION

Persistent viruses cause self-limiting infections in most healthy individuals but lead to life-threatening disease in the absence of a protective T cell immunity in the immunocompromised host. Approved antiviral treatment options are often lacking. Restoring the patient's T cell immunity with adoptive T cell transfer (ACT) is an attractive approach for the treatment of viral complications due to T cell deficiency.[1–4] In immunocompetent individuals, protective T cell immunity is mainly directed against viral peptide epitopes. Adenovirus (AdV) causes persistent infections and is an unsolved medical problem in the immunocompromised host but is easily controlled in the healthy population by T cell responses against the adenoviral hexon capsid protein.[5] The naturally presented human leukocyte antigen (HLA)-A*0101-restricted peptide LTDLGQNLLY (LTDL) is an immunodominant T cell epitope within the hexon protein.[6,7] Hexon-specific T cells isolated from healthy donors have already been used to successfully treat patients with refractory adenoviral infections.[8,9] However, potential graft-versus-host disease (GvHD) mediated by allogeneic T cell receptors (TCRs) and availability of HLA-identical donors with relevant frequencies of protective hexon-specific T cells limit ACT strategies. Advanced T cell engineering can overcome these hurdles by knockout (KO) of the donor's endogenous TCRs as well as redirecting any T cell specificity via transgenic TCR knockin. In this study, we performed a proof of concept of this approach using TCRs that specifically recognize the immunodominant hexon protein LTDL presented in the context of HLA-A*0101.

Recombinant TCR-expressing T cells are most commonly engineered using retroviral or lentiviral vectors bearing various risks.[10] First, transgenic TCR surface expression competes for CD3 molecules with the endogenous TCR.[11] Second, TCR mispairing can occur between transgenic and endogenous TCR chains, potentially generating

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harmful specificities and off-target effects.[12–14] Third, random integration of viral vectors into the human genome is a potential safety hazard and prevents physiological TCR regulation upon antigen-specific stimulation.[12,15,16] Therefore, novel virus-free methods with targeted integration strategies, such as clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) appear to be highly attractive alternatives.[17,18]

In the present study, we treated life-threatening refractory AdV infection after hematopoietic stem cell transplantation (HSCT) with Streptamer-isolated LTDL-specific T cells. *In vivo* expansion of the LTDL-specific T cells was correlated with viral clearance, enabling *ex vivo* isolation of these novel, clinically protective LTDL-specific TCR sequences from peripheral blood. TCRs isolated from such a clinical context are promising for further use in ACT, since they are expected to be highly functional and safe. In a non-viral "two in one" procedure, this protective LTDL-specific TCR was successfully knocked into the endogenous TCR locus together with a highly efficient KO of the endogenous TCR using CRISPR-Cas9 gene editing in primary human T cells. This approach ensures that transgenic TCRs are protective and allows the generation of highly specific and safe T cell products for allogeneic ACT against life-threatening viral infections. Herein, presented data with LTDL-specific TCR-edited T cells serve as a proof-of-concept step in developing off-the-shelf ACTs.

RESULTS

ACT of LTDL-specific CD8$^+$ T cells controls refractory systemic AdV infection after HSCT

ACT using the major histocompatibility complex (MHC)-multimer technique was performed to isolate a specific T cell population directed against the immunodominant AdV hexon peptide epitope LTDL presented in the context of HLA-A*0101 (Figure 1A). MHC class I-Streptamer selection[19–21] allowed highly efficient enrichment (533-fold) of LTDL-specific cytotoxic T cells from the same haploidentical parent, who donated stem cells for HSCT previously (0.11% peripheral blood mononuclear cells [PBMCs] to 58.7% in the T cell product, Figure 1B). The LTDL-specific T cell product showed high viability (88%) and contained 58% cytotoxic T cells (Figure 1B). 1.75×10^3 LTDL-specific CD8 T cells/kg were infused on day 0 (252 days after HSCT, Figure 1C). An *in vivo* increase of LTDL-specific T cells (<0.05%, 3.2%, and 12.6% at days 0, 8, and 28) after LTDL-ACT was detected by flow cytometry in the peripheral blood (Figure 1D). The increasing frequencies of LTDL-specific T cells correlated with a reduction of viral load (from 600,000 copies/mL on day −2), which was completely cleared by day 36 (below detection limit, Figure 1C). High LTDL-specific T cell frequencies were detectable up to 86 days after transfer (10.2%), and viral clearance was confirmed by PCR until day 111 after T cell transfer. Two and a half years after LTDL-ACT the patient was still free of AdV infections, and a sustained LTDL-specific T cell population was detectable in the patient's peripheral blood.

Identification of novel protective LTDL-specific TCRs

In order to identify novel, protective LTDL-specific TCRs from the patient's sustained T cell response (0.28%), a double peptide MHC (pMHC)-Streptamer staining was performed in order to sort single CD8$^+$ LTDL-specific T cells (Figures 1A and 2A). TCR α and β chains from sorted single cells were amplified using a rapid amplification of cDNA ends (RACE)-PCR protocol[22] and sequenced. Two full α/β TCRs were identified. While their β chains differ in D (TRBD) and J segments (TRBJ), and thus in their CDR3 regions, the α chains are highly similar and differ in their CDR3 regions only (Figure 2D). In order to predict *in vivo* functional avidity of the identified TCRs, retrovirally transduced T cells were used for a dissociation (K_{off}) rate assay to determine their structural avidity. Mean K_{off} rates of 97.2 and 117 s were observed for AdV-TCR_1 and AdV-TCR_2, respectively (Figure 2B). Dashed lines indicate the half time ($t_{1/2}$) of previously described TCRs with confirmed low (40.6 s) and high (178.3 s) functionality,[23] suggesting that the identified AdV TCRs had typical structural avidities of protective antiviral T cell populations. In co-culture experiments with LTDL-pulsed target cells, LTDL-TCR-transduced T cells showed significantly increased specific proliferation compared to co-culture with non-immunogenic GSEE-pulsed targets for TCR_1 and TCR_2 T cells (Figure 2C). After stimulation with LTDL peptide, TCR_1 and TCR_2 T cells specifically expressed interferon γ (IFNγ) (12.08% of TCR_1 and 14.23% of TCR_2 T cells), tumor necrosis factor (TNF)-α (12.3% of TCR_1 and 16.9% of TCR_2), and CD107a (17.7% and 19.0%; Figure 2E). After 6 days of co-culture with irradiated LTDL-pulsed phytohemagglutinin (PHA) blasts, LTDL-TCR-transduced T cells specifically secreted soluble Fas ligand (sFasL) (Figure 2E). Additionally, effector markers granulysin, perforin, granzyme A, and granzyme B were secreted by LTDL-TCR-transduced T cells upon LTCDL stimulation (Figure 3). Transgenic cells showed specific killing against LTDL-pulsed target cells (42.8% TCR_1, 55.3% TCR_2, Figure 2F) in an effector-to-target ratio-dependent manner. A setting using AdV-infected target cells showed that transgenic LTDL-TCR_1 cells specifically lysed 97% of infected monocytes (Figure 2F).

Replacement of endogenous TCRs by recombinant LTDL-TCR via CRISPR-Cas9-mediated homology-directed repair (HDR)

For the CRISPR-Cas9-mediated engineering and replacement of the endogenous TCR, a double-stranded DNA template based on TCR_1 was designed for HDR (Figure 1A). This template provides a full TCRβ chain sequence including specific V, D, and J segments and the complete β chain constant region. The T2A-linked α chain includes V and J segments, as well as the first 273 nt of *TRAC* exon 1, thereby utilizing the endogenous α chain constant region downstream of the Cas9-induced double-strand break. Two homology arms allow targeted and in-frame integration into the *TRAC* locus (Figure 4A), thereby disrupting the endogenous TCRα chain. Additionally, *TRBC* was knocked out simultaneously to prevent mispairing with the remaining β chain. As a proof of concept for this approach, we performed this editing approach with PBMCs of HLA-A1-positive donors. A1/hexon 5 (LTDL) MHC class I-Streptamer staining confirmed successful knockin and recombinant TCR complex assembly in up to 7.3% of CD8$^+$ T cells (Figures 4B and 4C). CD3 expression was completely lost in cells with TRAC and TRBC double KO, whereas simultaneous knockin of the recombinant LTDL-TCR rescued CD3 expression (Figure 4B). In-frame and site-directed

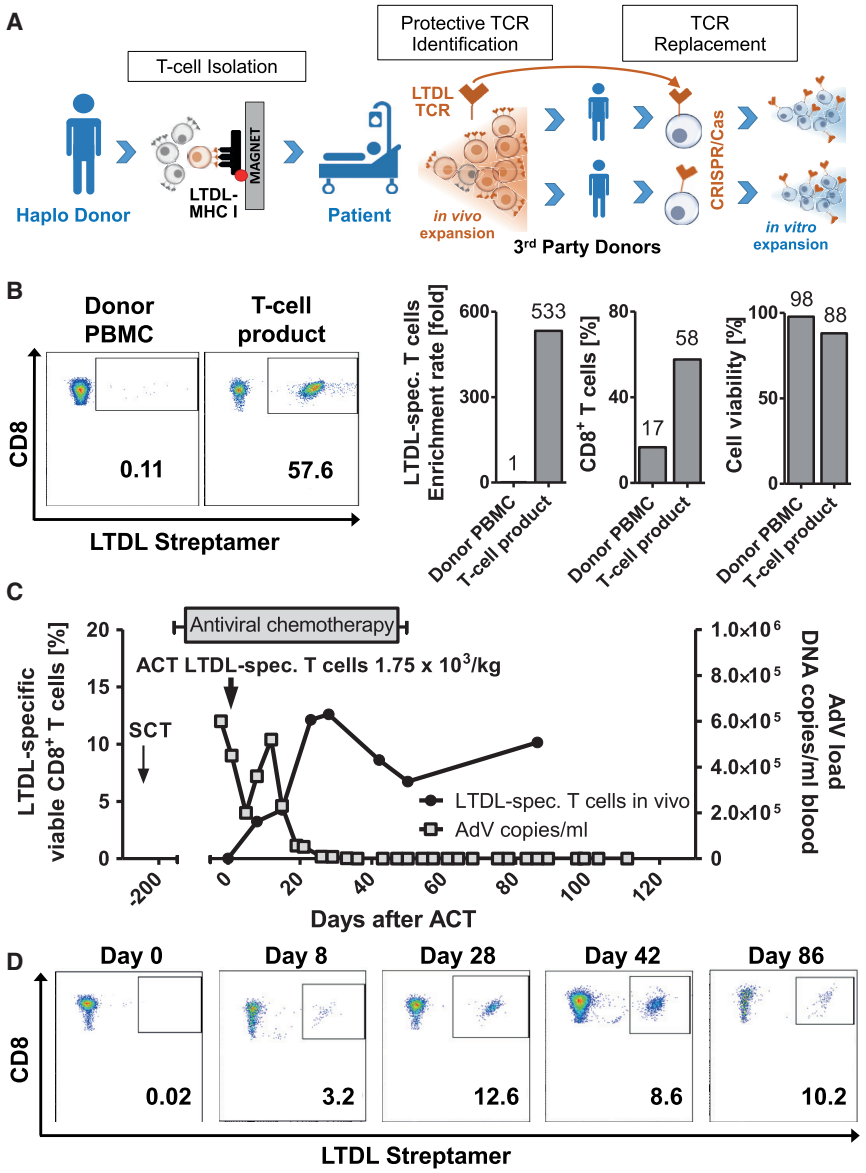
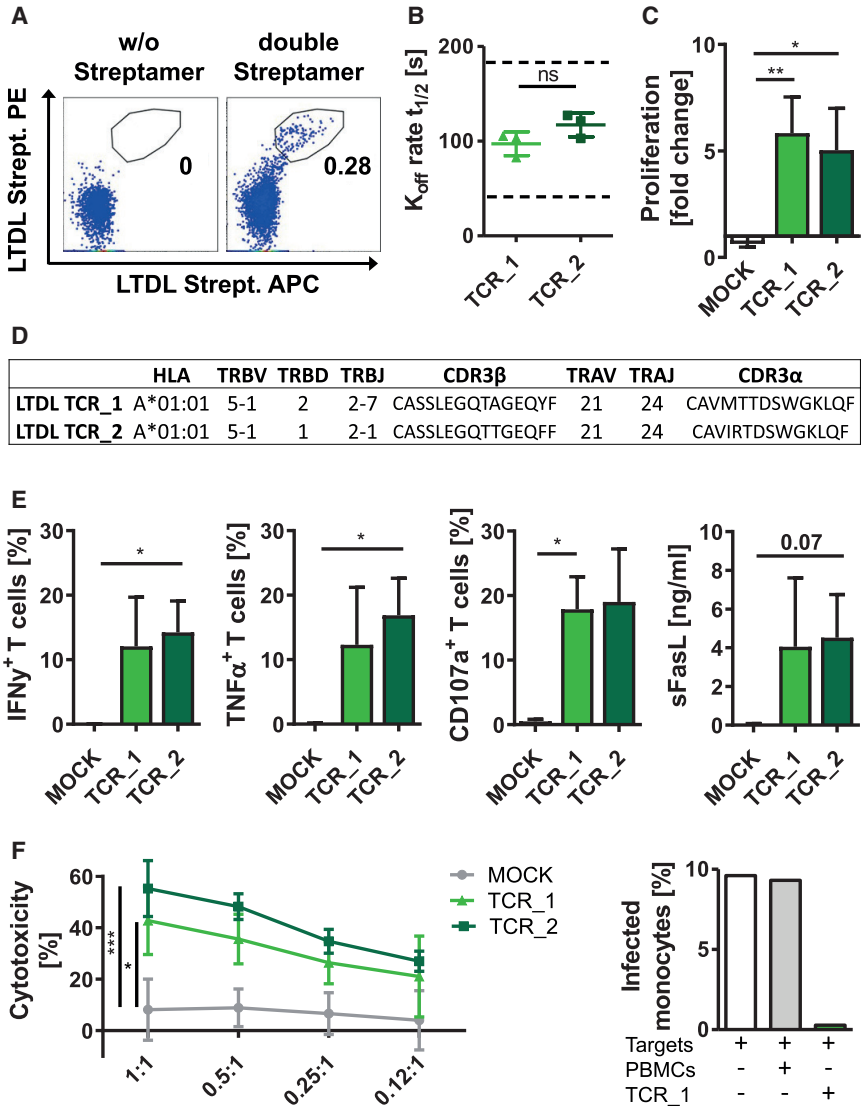


Figure 1. Adoptive transfer of LTDL-specific CD8⁺ T cells successfully controls refractory systemic AdV infection after haploidentical stem cell transplantation

(A) Approach of protective TCR isolation from a patient successfully treated with LTDL-specific T cells after allogeneic stem cell transplantation and CRISPR-Cas9-mediated TCR replacement in a third-party AdV sero-negative donor in order to generate protective LTDL-specific transgenic T cells for future adoptive T cell transfer. (B) Flow cytometric plots of LTDL-Streptamer-stained donor PBMCs and T cell product. Enrichment rate, CD8 T cell frequency, and cell viability in donor PBMCs and in the T cell product determined by flow cytometry. (C) Frequency of LTDL-specific T cells and viral load of the patient successfully treated with adoptive T cell transfer. Time points of stem cell transplantation (SCT) and ACT are indicated with arrows. (D) Detection of LTDL-specific T cells in peripheral blood of the patient via Streptamer staining.

integration of the LTDL-TCR was confirmed on the genetic level via PCR. For this, a PCR primer pair was designed binding upstream of the 5′ homology arm in the endogenous *TRAC* region and within the variable region of the inserted TCRβ chain (Figure 4D).

Proliferation, effector markers, cytotoxicity, and secreted functional analytes were investigated in LTDL-TCR knockin T cells. 71% of LTDL-TCR T cells showed highly specific functionality in terms of IFNγ secretion upon co-culture with LTDL-presenting target cells (Figure 5A). LTDL-TCR T cells proliferated specifically 2.3-fold within 3 days of co-culture with LTDL-pulsed target cells, whereas cell counts of mock control cells decreased about 20% compared to unspecific stimulation with GSEE peptide (Figure 5B). TNF-α expression was upregulated specifically upon antigen-specific stimulation with



	HLA	TRBV	TRBD	TRBJ	CDR3β	TRAV	TRAJ	CDR3α
LTDL TCR_1	A*01:01	5-1	2	2-7	CASSLEGQTAGEQYF	21	24	CAVMTTDSWGKLQF
LTDL TCR_2	A*01:01	5-1	1	2-1	CASSLEGQTTGEQFF	21	24	CAVIRTDSWGKLQF

Figure 2. Identification of novel protective LTDL-specific TCRs

(A) Double pMHC-Streptamer staining of patient-derived PBMCs with LTDL-Streptamer labeled with two different fluorochromes 2 years after ACT. Double-positive cells were single-cell sorted and subsequently used for TCR sequence identification. (B) Structural avidity of identified LTDL-TCRs. The plot shows the quantification of half-life times measured in a K_{off} rate assay. Dashed lines represent $t_{1/2}$ of previously described TCRs with confirmed low (40.6 s) and high (178.3 s) functionality. Mean ± SD of technical triplicates. (C) Proliferation potential of LTDL-TCR-transduced T cells after co-culture with LTDL-pulsed, irradiated PHA blasts. Data show fold changes of proliferated LTDL-specific T cells after co-culture with LTDL-pulsed PHA blasts compared to co-culture with GSEE control-pulsed cells. Mean ± SD of three independent experiments. (D) Composition of the TCRs identified by single-cell sequencing. The table shows the composition of the TCRs identified by single-cell sequencing. (E) Intracellular cytokine staining for IFNγ and TNF-α as well as CD107a staining of LTDL-TCR-transduced and mock T cells after stimulation with LTDL peptide for 6 h, respectively. Data show frequencies among $CD8^+$ T cells. Mean ± SD of three donors. Engineered T cells were co-cultured with irradiated LTDL-pulsed PHA blasts for 6 days. Supernatants were harvested for detection of secreted sFasL in a bead-based immunoassay. Mean ± SD of three independent experiments. (F) Cytotoxic capacity of T cells during co-culture with LTDL-pulsed PHA blasts for 48 h. PHA blasts were used as target cells at different effector-to-target ratios, as indicated. Mean ± SD of three donors (left graph). Frequency of AdV-GFP-infected monocytes after co-culture (right graph) without and with PBMCs or LTDL-TCR-transduced T cells (AdV-TCR_1). *p < 0.05, **p < 0.01 (Student's t test).

LTDL peptide from 0.4% of mock T cells to 6.5% of cytotoxic T cells in the LTDL-TCR knockin sample (Figure 5C). Up to 46% specific cytotoxic killing of LTDL-pulsed PHA blasts could be observed in an effector-to-target ratio-dependent manner (Figure 5D). Supernatants of co-cultures containing LTDL-TCR transgenic cells and peptide-pulsed cells revealed a significant increase of granulysin, granzyme B, perforin, and sFasL as well as an upregulation of granzyme A upon LTDL-specific stimulation (Figure 5F). T cell phenotypes revealed that the knockin procedure influences T cell phenotypes during *in vitro* expansion. At day 8 after CRISPR-Cas9-mediated knockin, most T cells demonstrated a central memory phenotype in the LTDL-TCR knockin T cells as well as in the untreated control population. During *in vitro* cultivation, LTDL-TCR knockin cells developed more mature effector memory phenotype (effector memory T [Tem] cells, 43%) compared to untreated control cells (25%, Figure 5E).

DISCUSSION

Absent T cell immunity in immunocompromised hosts following HSCT gives rise to life-threatening viral infections. ACT can restore protective T cell immunity, but available HLA-identical donors with sufficient frequency of specific T cells are rather limited.[24] Potential allogeneic donors often lack either the required HLA match or the protective T cell specificity required to cure the infected patient.[24] Viruses such as AdV are a major threat, especially for pediatric patients following HSCT.[25] Despite the high prevalence of AdV infections in general, HSCT donors often lack AdV-specific or at least hexon-specific T cells, thus increasing the recipient's risk for uncontrolled viral spread.[7,24] Thus, we aimed to establish a safe and highly specific procedure for the generation of TCR-engineered epitope-specific protective T cells from virus-naive third-party donors or partially HLA-matched HSCT donors for readily available future ACT treatment of refractory

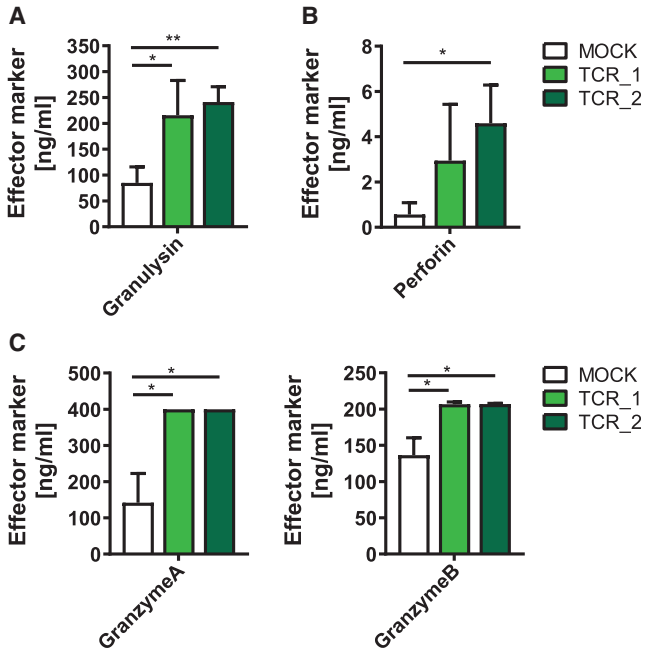


Figure 3. Characterization of novel protective LTDL-specific TCRs

Cells were co-cultured with irradiated LTDL-pulsed PHA blasts for 6 days. Supernatants were harvested for a bead-based immunoassay. (A) Detection of granulysin. Mean ± SD of three independent experiments. (B) Detection of perforin. Mean ± SD of three donors. (C) Detection of granzyme A and granzyme B. Mean ± SD of three independent experiments. Granzyme A secretion exceeded detection maximum of 400 ng/mL. *$p < 0.05$, **$p < 0.01$ (Student's t test).

virus infections in the immunocompromised host. However, it is still unclear what represents a protective TCR. pMHC class I-Streptamer-selected T cells represent a variety of TCRs that are all directed against one single epitope. We aimed at *in vivo* selection of those TCRs that are protective through re-isolation of TCRs *ex vivo* after ACT. With this strategy, we identified two novel protective TCRs *ex vivo* using a clinical successful ACT and engineered allogeneic T cells by orthotopic TCR replacement via CRISPR-Cas9-mediated HDR.

The heterodimeric nature of the TCR, competition and mispairing with the endogenous TCR, and unphysiological transgene expression due to viral promotors provided by retroviral vectors, as well as random integration after conventional gene transfer, represent major challenges in the field of TCR engineering. CRISPR-Cas9 HDR provides solutions for these problems; that is, replacement of the endogenous TCR using CRISPR-Cas9 HDR and simultaneous KO of the endogenous TCR α and β chains prevent harmful TCR mispairing and competition of CD3 molecules.[12] Targeted integration into the endogenous TRAC locus circumvents random integration into potential oncogenes, but it allows regulation of the recombinant TCR by its natural promotor, thereby enabling physiological regulation upon antigen stimulation.[12]

LTDL has been described as an AdV-derived immunodominant T cell epitope.[26] We observed in a single case close correlation between increasing LTDL-specific T cell frequencies and decreasing viral loads below detection limits following adoptive transfer with LTDL-Streptamer-sorted T cells.

The presented proof of concept is a step toward off-the-shelf protective TCR banks enabling T cell products from HSCT donors with redirected T cells.[7,24,27–29] LTDL is restricted to presentation on HLA A*01:01, which has an allele frequency of about 0.14 in Europe.[30] The identification of further immunogenic epitopes and their corresponding TCRs restricted to other HLA alleles is crucial for the establishment of off-the-shelf protective TCR banks, providing appropriate TCRs for all recipients. However, partially matched T cells may not persist life-long due to rejection by the host's immune system, but they are expected to exert a transient immune protection. For long-term persistence of T cells in future off-the-shelf ACT, rejection will have to be solved by KO of HLA and an additional solution for NK cell-mediated missing-self responses. Nevertheless, since the transgene derives from sequences of a human TCR, the risk of rejection of the modified T cells due to the transgene itself is low. The transgene TCR is isolated *ex vivo* and therefore approved to be protective and to have a low risk for an uncontrolled cytokine release if the TCR was overly stimulated *in vivo*. Additionally, the simultaneous KO of the endogenous TCR will prevent harmful alloreactivity.[31]

Compared to current standards of ACT, this novel approach does not require a protective response in the donor. TCR replacement enables ACT from any T cell source including autologous cells. In addition, this could be performed together with options of additional engineering to overcome immunosuppression.[32] Since low doses of virus-specific T cells have already been shown to be efficient,[33] *in vitro* expansion of the generated T cell product is not needed for clinical application. This short-term procedure maintains early T cell phenotypes that can exploit their proliferative potential following ACT.[34] However, final enrichment using pMHC-Streptamers will be required, since CRISPR-Cas9-mediated knockin rates are lower compared to retroviral transduction. Isolation using pMHC-Streptamers is a commonly used technique for T cell enrichment to high purities while maintaining T cell phenotypes and functionality.[20,35] The lower editing efficacies of the HDR CRISPR-Cas9 technique compared to conventional retroviral or lentiviral transduction methods is in accordance with published reports on replacement of the endogenous TCR with a NY-ESO-1 TCR with up to 12% editing efficacy in primary human T cells. The knockin efficacies using large HDR templates, such as those coding for TCRs, have currently a technical limit. Future research will have to clarify the mechanisms of this technical hurdle. In the present study, functional assays were performed 2–3 weeks after genetic engineering to reduce unspecific background due to initial strong but unspecific T cell activation via CD3/CD28. This promoted a more maturated T cell phenotype. Regarding clinical applications, ACT with redirected T cells would be preferred within 1 week after genetic engineering when T cells mainly harbor a central memory phenotype.

In conclusion, we provide a strategy to use protective T cell immunity in a safe and highly efficient procedure for precision ACT treatment.

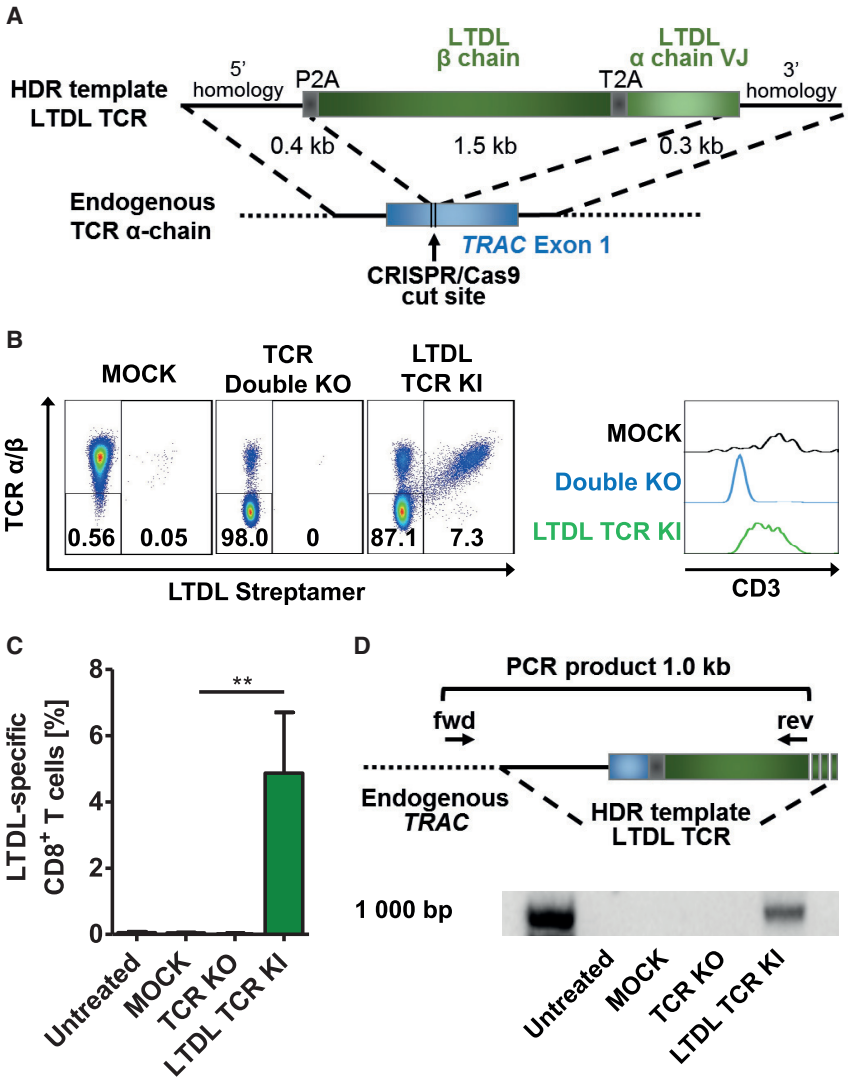


Figure 4. Successful replacement of the endogenous TCR with a recombinant LTDL-TCR via CRISPR-Cas9-mediated HDR

(A) HDR template for LTDL-TCR including homology arms for integration within the first exon of the endogenous TCR α chain constant region. (B) Representative flow cytometry plots showing TCR α and β chain double knockout (KO) and TCR knockin (KI) efficiency 8 days after electroporation. Numbers in gates indicate percentage of parental $CD8^+$ population. Correlation of TCR and CD3 expression is shown in the histogram. (C) Frequency of LTDL-Streptamer$^+$$CD8^+$ T cells 8 days after electroporation. Mean ± SD of five independent experiments of three different donors. $^{**}p < 0.01$ (Student's t test). (D) Confirmation of in-frame integration of the AdV-TCR HDR template into the *TRAC* locus by PCR. PCR was performed with a primer pair binding upstream of the left homology arm (forward) and in the variable part of the HDR template β chain (reverse).

Orthotopic knockin of new transgenic TCRs, editing both endogenous TCR chains, prevents mispairing and leads to physiological regulation of transgenic TCRs. This approach enables generation of specific T cells from virus-naive third-party donors, will widen future availability of ACT, and could be applied also to various emerging infectious pathogens and other indications of T cell immunotherapy.

MATERIALS AND METHODS

Subject details

A 17-year-old male patient suffered from refractory AdV infection 5 months after haploidentical HSCT. The patient received stem cell donor-derived AdV-specific T cells as ACT at the age of 18. Written informed consent was obtained from the patient and his family for scientific analysis, and publication was approved by the local Institutional Ethics Review Board. The work was done in accordance with the Declaration of Helsinki.

Cell culture and cell lines

K32 cell lines expressing HLA-A*01 linked to LTDL or FSECNALGSY, respectively, were grown in very low endotoxin (VLE) Dulbecco's modified Eagle's medium (DMEM) (Biochrom) supplemented with 10% fetal bovine serum (Sigma) and penicillin-streptomycin (10,000 U/mL) (Gibco) at 37°C and 5% CO_2. 293Vec-Galv (BioVec Pharma) cells were cultured in VLE DMEM (Biochrom) with 10% fetal bovine serum (Sigma), penicillin-streptomycin (10,000 U/mL) (Gibco), and 2% L-glutamine (Biochrom) at 37°C and 5% CO_2. 293Vec-RD114 cells (BioVec Pharma) were cultured in VLE DMEM (Biochrom) with 10% fetal bovine serum (Sigma), penicillin-streptomycin (10,000 U/mL) (Gibco), and 2% L-glutamine (Biochrom) at 37°C and 5% CO_2. PBMCs were obtained from HLA A*01-positive male and female healthy donors after informed consent was obtained, and the use of the blood samples was approved according to national law by the local Institutional Review Board (Ethics Committee of Ludwig Maximilians University Hospital in Munich). T cells were cultured in TexMACS medium (Miltenyi Biotec) with 2.5% human AB serum, 10 ng/mL IL-7, and 10 ng/mL IL-15 (Miltenyi Biotec) unless indicated otherwise. Allogeneic feeder PBMCs (PHA blasts) were cultured in VLE RPMI 1640 (Biochrom) with 10% fetal bovine serum (Sigma) and 1% L-glutamine (Biochrom). PBMCs were stimulated with PHA-L (Invitrogen/Sigma) according to the manufacturer's recommendations for 3 days. For expansion, cells were cultured with 200 IU/mL IL-2 (Novartis) for an additional 3 days. Subsequently, cells were pulsed with peptides as indicated or further expanded with 100 IU/mL IL-2.

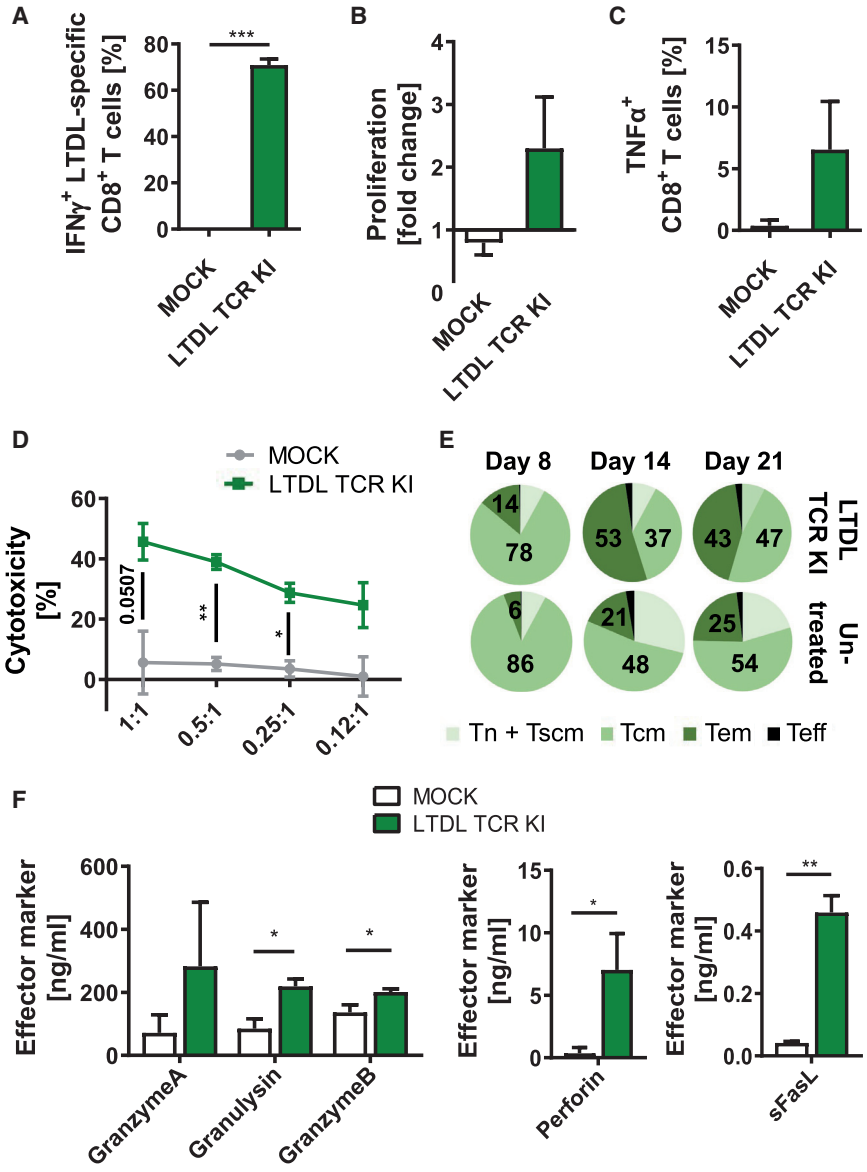


Figure 5. Characterization of CRISPR-Cas9-engineered LTDL-specific T cells

(A) Intracellular IFNγ staining of LTDL-TCR KI cells after co-culture with K32 target cells expressing LTDL peptide linked to HLA-A*01 on the surface. Mean ± SD of three independent experiments. (B) Proliferation of mock and LTDL-TCR KI cells after 3 days of co-culture with irradiated LTDL-pulsed PHA blasts. Values show fold changes of proliferated viable LTDL-Streptamer$^+$ T cells after co-culture with LTDL compared to GSEE-pulsed cells. Mean ± SD of three donors. (C) Intracellular cytokine staining of TNF-α after stimulation with LTDL peptide. Frequencies among CD8$^+$ T cells. Mean ± SD of three donors. (D) Cytotoxic capacity of T cells upon co-culture with target cells for 48 h. T cells were co-cultured with LTDL-pulsed PHA blasts in different effector-to-target ratios, as indicated. Mean ± SD of three donors. (E) T cell phenotypes of untreated cells and cells of the LTDL-TCR KI sample after 8, 14, and 21 days of untreated culture or after genetic engineering, respectively. Cells were cultivated in TexMACS medium with 10 ng/mL IL-7 and 10 ng/mL IL-15. Phenotypes were defined via CD62L and CD45RO: CD62L$^+$CD45RO$^-$ naive (Tn) and stem cell-like (Tscm) T cells, CD62L$^+$CD45RO$^+$ central memory (Tcm) T cells, CD62L$^-$CD45RO$^+$ effector memory (Tem) T cells, or CD62L$^-$CD45RO$^-$ effector T (Teff) cells. Mean of at least three independent experiments is shown. (F) Cells were co-cultured with irradiated LTDL-pulsed PHA blasts for 6 days. Supernatants were harvested for detection of secreted analytes in a bead-based immunoassay (mean ± SD of three independent experiments). Granzyme A exceeded the detection maximum of 400 ng/ml. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test).

sent was obtained, using Ficoll-density centrifugation (Biocoll, Biochrom).

For *in vitro* assays T cells were enriched from PBMCs using untouched magnetic separation (EasySep human T cell enrichment kit, STEMCELL). Subsequently, T cells were cultured in TexMACS medium (Miltenyi Biotec) with 2.5% human AB serum, 10 ng/mL IL-7, and 10 ng/mL IL-15 (Miltenyi Biotec). T cells were activated with magnetic CD3/CD28 Dynabeads (Gibco) and 30 IU/mL IL-2 (Novartis) for 2–3 days.

Isolation of T cells

For the immunomagnetic separation of LTDL-specific T cells, the Clinical Streptamer isolation kit was used as previously described.[36] Briefly, the purified positive fraction was incubated with 1 mM D-biotin and washed at 4°C to allow for dissociation and removal of the reversible AdV epitope-specific Streptamer reagents, yielding "minimally manipulated" target cells. The target cell population was washed and resuspended in clinical-grade human serum albumin (HSA) (20%) for immediate use. The following MHC-Streptamer was used for multimerization with Strep-Tactin phycoerythrin (PE) (cell staining) or Strep-Tactin magnetic beads (cell isolation): HLA-A*0101/hexon (LTDL). All reagents were obtained from Stage Cell Therapeutics (Göttingen, Germany; now part of Juno, Seattle, WA, USA). PBMCs were isolated from healthy donors after informed con-

Streptamer and antibody staining

0.3 μg of pMHC class I molecules with a Strep-tag were multimerized using 1.5 μL of Strep-Tactin-PE (IBA Lifesciences) to form a fluorescent Streptamer in a total volume of 15 μl of fluorescence-activated cell sorting (FACS) buffer (PBS+1% fetal calf serum [FCS]) per 1×10^6 cells. T cells were stained for 45 min at 4°C in the dark with LTDL-Streptamers followed by antibody staining with 7-aminoactinomycin D (7AAD) (BioLegend), CD56-allophycocyanin (APC) (BD Biosciences), CD20-APC (BioLegend), CD14-APC (Miltenyi Biotec), CD8-APC/Cy7 (BioLegend), CD4-PE/Cy7 (BioLegend),

and human TCRα/β-fluorescein isothiocyanate (FITC) (BioLegend). For analysis of CD3 re-expression CD3-FITC (Miltenyi Biotec) instead of TCRα/β-FITC was used. Flow cytometric analysis was done on a BD FACSCanto II (BD Biosciences). Lymphocytes were identified by forward scatter (FSC)/side scatter (SSC), and 7AAD-positive dead cells were excluded. Since TCR KO induces downregulation of CD3, T cells were identified by the absence of CD56 (natural killer [NK] cell marker), CD20 (B cell marker), and CD14 (monocyte marker). Next, cells were analyzed for CD4 and CD8 surface expression, and the frequency of LTDL-Streptamer$^+$ cells was measured (Figure S1).

Phenotypic characterization

For categorization in different T cell phenotypes, cells were stained with CD56-APC (BD Biosciences), CD20-APC (BioLegend), CD14-APC (BioLegend), CD4-PE/Cy7 (BioLegend), CD8-APC/Cy7 (BioLegend), CD45RO-PE (BioLegend), and CD62L-FITC (BioLegend). 7AAD staining (BioLegend) was performed for live/dead discrimination. Flow cytometric analysis was performed on a BD FACSCanto II (BD Biosciences). Lymphocytes were identified by FSC/SSC, and 7AAD-positive dead cells were excluded. T cells were identified by the absence of CD56 (NK cell marker), CD20 (B cell marker), and CD14 (monocyte marker). The frequencies of CD45RO$^+$ and CD62L$^+$ cells among CD4$^+$ and CD8$^+$ cells were evaluated.

One week after genetic engineering, 5×10^5 cells were cultured without interleukins overnight, followed by stimulation with 0.5 μg of peptide and addition of brefeldin A and CD107a-APC antibody. After 6 h, cells were stained with CD4-VioGreen, CD3-FITC, and CD8-APC/Vio770. Subsequently, cells were fixed, permeabilized, and intracellularly stained with IFNγ-PE, TNF-α-PE/Vio770, and CD154-VioBlue antibodies (all reagents were obtained from Miltenyi Biotec). Flow cytometric analysis was performed on a MACSQuant (Miltenyi Biotec). Lymphocytes were identified by FSC/SSC, and TCR-expressing T cells were identified by CD3 staining.

Two weeks after genetic engineering cells were co-cultured with K32 cells, expressing HLA-A*01:01 linked to LTDL in a 1:1 ratio or stimulated with *Staphylococcus* enterotoxin B (SEB) for 6 h at 37°C. Live/dead discrimination was done with 7AAD (BioLegend). For surface marker staining, CD56-FITC (BD Biosciences), CD20-FITC (BioLegend), CD14-FITC (BioLegend), CD8-APC/Cy7 (BioLegend), and LTDL-Streptamer-PE were used (panel A). Subsequently, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and intracellularly stained with IFNγ-APC (BD Biosciences). Lymphocytes were identified by FSC/SSC, and 7AAD-positive dead cells were excluded. T cells were identified by the absence of CD56 (NK cell marker), CD20 (B cell marker), and CD14 (monocyte marker). The frequency of IFNγ$^+$ cells among LTLD-Streptamer$^+$ CD8$^+$ cells was evaluated. Alternatively, cells were stained with CD8-VioBlue, CD4-VioBright-FITC, CD3-APC-Vio770, and IFNγ-PE. For live/dead discrimination, viability dye was used (all reagents from Miltenyi Biotec, panel B). Lymphocytes were identified by FSC/SSC, and viability dye-positive dead cells were excluded. T cells with intact TRC-CD3 surface complexes were identified by CD3 staining. The frequency of IFNγ$^+$CD8$^+$ cells was evaluated. Flow cytometric analysis was performed on a BD FACSCanto II (BD Biosciences, for panel A) or MACSQuant (Miltenyi Biotec, panel B).

Retroviral transduction

TCRs were retrovirally transduced in primary human TCR KO T cells using stable RD114 producer cell lines. For the production of stable producer cell lines, 293Vec-Galv cells (kindly provided by BioVec Pharma) were transfected with 3 μg of pMP71 expression vector (containing the TCR construct) using Lipofectamine 2000 (Invitrogen). 293Vec-RD114 cells (kindly provided by BioVec Pharma) packaging cells were transduced by adding virus-containing supernatant from transfected 293Vec-Galv cells. Virus supernatant from stable transduced 293Vec-RD114 cells was coated on RetroNectin (TaKaRa)-treated well plates. 2 days after electroporation, 1×10^6 T cells were transduced via spinoculation on virus-coated plates.

Sanger sequencing for TCR editing validation

Genomic DNA was isolated (QIAamp DNA mini kit, QIAGEN) 7 days after electroporation. PCR was performed with the following primers and AccuPrime polymerase system (Invitrogen): TRAC forward 5′-ATCACGAGCAGCTGGTTTCT-3′, reverse 5′-CCCGTGTCATTCTCTGGACT-3′; TRBC forward 5′-TACCAGG ACCAGACAGCTCTTAGA-3′, reverse 5′-TCTCACCTAATCTC CTCCAGGCAT-3′; TRAC HDRT KI forward 5′- CCCAACTTAA TGCCAACATACCA-3′, reverse 5′- GAAGTACTGCTCCCCCG C-3'. After initial denaturation at 95°C for 2 min, 45 cycles with denaturation at 95°C for 40 s, followed by annealing at 55°C for TRAC/TRBC and 59°C for TRAC HDRT KI, respectively, for 40 s and elongation at 68°C for 60 s were performed. Final elongation was done at 68°C for 10 min. For TCR KO validation, PCR products were purified (Clean & Concentrator-5, Zymo Research), sent for Sanger sequencing (Eurofins) with forward and/or reverse primers, and analyzed with TIDE software.[37]

Cytotoxic killing assay

Frozen autologous PBMCs were thawed and cultured in VLE RPMI 1640 (Biochrom) with 10% fetal bovine serum (Sigma) and 1% L-glutamine (Biochrom). Cells were cultured with PHA (Invitrogen/Sigma) for 3 days according to the manufacturer's recommendations. Subsequently, medium was replaced and supplemented with 200 IU/mL IL-2 (Novartis). After 3 days, medium was again replaced with 100 IU/mL IL-2 for 1 day. 1×10^6 PHA blasts were pulsed with 1 μg of respective peptide (kindly provided by Stefan Stefanovic, University of Tuebingen, Tuebingen, Germany) overnight at 37°C. On the next day, cells were labeled with CellTrace Violet (CTV, Invitrogen). Two weeks after genetic engineering, T cells were magnetically depleted for CD56$^+$ cells (CD56 microbeads, Miltenyi Biotec) and enriched for LTDL-Streptamer$^+$ cells (Strep-Tactin microbeads, IBA Lifesciences). Mock control cells were enriched using CD8 microbeads (Miltenyi Biotec). After 1 week, T cells were co-cultured with pulsed PHA blasts in the indicated effector-to-target ratios in TexMACS medium without phenol red (Miltenyi Biotec) for 48 h. Cells were stained

with propidium iodide (Miltenyi Biotec) for live/dead discrimination. Cytotoxic killing capacity was assessed by determination of CTV-negative target cells compared to target-only controls.

T cell proliferation and cytokine release

Frozen autologous PBMCs were thawed and cultured in VLE RPMI 1640 (Biochrom) with 10% fetal bovine serum (Sigma) and 1% L-glutamine (Biochrom). Cells were cultured with PHA (Invitrogen/Sigma) according to the manufacturer's recommendations for 3 days. Subsequently, medium was replaced and supplemented with 200 IU/mL IL-2 (Novartis). After 3 days, 1×10^6 PHA blasts were pulsed with 10 μg of peptide (kindly provided by Stefan Stefanovic, University of Tuebingen, Tuebingen, Germany) overnight at 37°C, followed by irradiation at 30 Gy. T cells were magnetically depleted for $CD56^+$ cells (CD56 microbeads, Miltenyi Biotec) 2 weeks after genetic engineering. T cells were labeled with CTV (Invitrogen) and co-cultured with pulsed PHA blasts in a 1:1 ratio in TexMACS medium without phenol red (Miltenyi Biotec). After 3 days cells were stained with LTDL-Streptamer-PE followed by surface staining with CD56-FITC (BD Biosciences), CD20-FITC (BioLegend), CD14-FITC (BioLegend), CD8-APC (BD Biosciences), CD4-VioGreen (Miltenyi Biotec), and 7AAD (BioLegend) for live/dead discrimination. Lymphocytes were identified by FSC/SSC, and T cells were identified by the absence of CD56, CD20, and CD14. Specifically, proliferated cells were determined as $7AAD^-$, CTV^-, and LTDL-$Streptamer^+$ T cells compared to respective controls (Figure S2). Supernatants were harvested after 6 days by centrifugation and secreted cytokines were analyzed in a bead-based immunoassay (LEGENDplex, BioLegend) in technical duplicates. Granzyme A exceeded detection limits of 8,000 pg/mL even at high dilutions (1:50), and therefore values were set to a maximum of 399 μg/mL.

Viral dissemination assay

PBMCs were thawed and $CD14^+$ monocytes were isolated by magnetic bead-based separation (CD14 microbeads, Miltenyi Biotec) according to the manufacturer's instructions. Isolated monocytes were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) (800 U/mL) for 3 days followed by subsequent infection with AdV11pGFP or AdV5F35GFP at a multiplicity of infection of 0.05–0.1 for 3 h. Virus was removed and AdV-TCR_1-transduced PBMCs were added in a 1:1 ratio. After 3 days of co-culture, the frequency of infected monocytes was analyzed using a FACSCalibur flow cytometer as described previously.[38,39]

Determination of K_{off} rates

K_{off} rates were determined as dissociation of reversible pMHC-Streptamers upon addition of D-biotin (IBA Lifesciences) in a flow cytometry-based assay (CyAn ADP Lx 9 color flow cytometer, Beckman Coulter) as described previously.[40] Briefly, pMHC molecules were multimerized with Strep-Tactin-APC (IBA Lifesciences) according to the manufacturer's instructions and incubated with 5×10^6 transduced T cells for 45 min. After 25 min, cells were additionally stained with CD8a-PE (eBioscience). For live/dead discrimination, cells were incubated with propidium iodide (Life Technologies). 1×10^5 to 1×10^6 pre-cooled cells were analyzed by flow cytometry. After 30 s, D-biotin was added to a final concentration of 1 mM while analysis continued. Dissociation kinetics were analyzed using FlowJo software (FlowJo) and GraphPad Prism software (GraphPad).

TCR identification and template design

TCRs were identified by single-cell TCR-single-cell analysis (SCAN) RACE PCR[22] from FACS-sorted LTDL-$Streptamer^+CD8^+$ T cells. DNA templates for CRISPR-Cas9-mediated HDR are based on TCR_1 and were designed *in silico* and synthesized as double-stranded gBlocks (Integrated DNA Technologies) or plasmid (Puc57-BsaI-free, BioCat). The HDR template has the following structure: 5′ homology arm (370 bp), P2A, complete TCR-β with human *TRBC* (NC_000007.14), T2A, variable region of TCR-α and first *TRAC* (NC_000014.9) exon until Cas9-induced double strand break, 3′ homology arm (280 bp). The HDR template was amplified by PCR using Q5 high-fidelity DNA polymerase (NEB) and PCR products were purified using Clean & Concentrator-5-Kit (Zymo Research) according to the manufacturers' instructions. DNA constructs for retroviral transduction had the following structure: human Kozac sequence, followed by TCR-β with minimally murinized *TRBC* with additional cysteine bridge. A subsequent P2A sequence is followed by TCR-α, including minimally murinized *TRAC* with an additional cysteine bridge,[41] cloned into pMP71 vectors.

CRISPR-Cas9-mediated TCR editing

80 μM target-specific CRISPR RNA (crRNA) (IDT) was mixed with 80 μM universal trans-activating CRISPR RNA (tracrRNA) (IDT) and incubated at 95°C for 5 min to form guide RNA (gRNA). Cas9 (IDT) was diluted to 40 μM with PBS and slowly added to the cooled-down gRNA. The Cas9-to-gRNA ratio was 2:1:1. Electroporation enhancer (IDT) was added to a final concentration of 20 μM and the ribonucleoprotein (RNP) complex was incubated for 15 min at room temperature. CD3/CD28 Dynabeads (Invitrogen) were magnetically removed from activated primary human T cells prior to electroporation. 1×10^6 T cells were re-suspended in 100 μL of chilled buffer M[42] and 26 μL of RNP was added, respectively. For CRISPR-Cas9-mediated knockin, 3μg of PCR amplified, purified HDR template was added to the RNP complex with TRAC and TRBC gRNA. Cells were electroporated with pulse code T-023 in an Amaxa Nucleofector IIb (Lonza). After electroporation, cells were cultured in 500 μL of TexMACS medium (Miltenyi Biotec) without interleukins for 30 min. Subsequently, 500 μL of TexMACS medium with 20 ng/mL IL-7 and 20 ng/mL IL-15 (Miltenyi Biotec) and 10 μL of HDR enhancer (IDT) were added. HDR enhancer was removed after 24 h. For mock controls, cells were electroporated without CRISPR-Cas reagents.

Statistical analysis

FlowJo software (FlowJo) was used for the analysis of flow cytometric data. GraphPad Prism was used for all statistical analyses. All experiments were repeated at least three times except the determination of K_{off} rates, which was done once in technical triplicates and the viral dissemination assay. Statistical analysis was carried out by unpaired

and paired t tests, respectively (GraphPad Prism). $p < 0.05$ was considered statistically significant. Statistical information for each experiment can be found in the figure legends. N represents number of independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.05.021.

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AUTHOR CONTRIBUTIONS

T.F. and D.H.B. set up the concept of the approach. The patient was treated by T.F., M.D., F.B., J.F., P.L., and R.H. Experiments were conceived and designed by T.K., T.A.S., F.B., S.W., T.F., and D.H.B. TCR isolation, identification, and template design were done by T.R.M. and K.S. Experiments were performed by T.A.S., L.J., T.R.M., and K.M.D. Data analysis and manuscript preparation were done by T.A.S., T.K., and T.F. The manuscript was critically reviewed by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Leen, A.M., Christin, A., Myers, G.D., Liu, H., Cruz, C.R., Hanley, P.J., Kennedy-Nasser, A.A., Leung, K.S., Gee, A.P., Krance, R.A., et al. (2009). Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. Blood *114*, 4283–4292.

2. Feuchtinger, T., Matthes-Martin, S., Richard, C., Lion, T., Fuhrer, M., Hamprecht, K., Handgretinger, R., Peters, C., Schuster, F.R., Beck, R., et al. (2006). Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br. J. Haematol. *134*, 64–76.

3. Walter, E.A., Greenberg, P.D., Gilbert, M.J., Finch, R.J., Watanabe, K.S., Thomas, E.D., and Riddell, S.R. (1995). Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N. Engl. J. Med. *333*, 1038–1044.

4. Kaeuferle, T., Krauss, R., Blaeschke, F., Willier, S., and Feuchtinger, T. (2019). Strategies of adoptive T -cell transfer to treat refractory viral infections post allogeneic stem cell transplantation. J. Hematol. Oncol. *12*, 13.

5. Leen, A.M., Sili, U., Vanin, E.F., Jewell, A.M., Xie, W., Vignali, D., Piedra, P.A., Brenner, M.K., and Rooney, C.M. (2004). Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-reactive and subgroup-specific $CD8^+$ T cells. Blood *104*, 2432–2440.

6. Günther, P.S., Peper, J.K., Faist, B., Kayser, S., Hartl, L., Feuchtinger, T., Jahn, G., Neuenhahn, M., Busch, D.H., Stevanović, S., and Dennehy, K.M. (2015). Identification of a novel immunodominant HLA-B*07: 02-restricted adenoviral peptide epitope and its potential in adoptive transfer immunotherapy. J. Immunother. *38*, 267–275.

7. Dörrie, J., Krug, C., Hofmann, C., Müller, I., Wellner, V., Knippertz, I., Schierer, S., Thomas, S., Zipperer, E., Printz, D., et al. (2014). Human adenovirus-specific γ/δ and $CD8^+$ T cells generated by T-cell receptor transfection to treat adenovirus infection after allogeneic stem cell transplantation. PLoS ONE *9*, e109944.

8. Feucht, J., Opherk, K., Lang, P., Kayser, S., Hartl, L., Bethge, W., Matthes-Martin, S., Bader, P., Albert, M.H., Maecker-Kolhoff, B., et al. (2015). Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. Blood *125*, 1986–1994.

9. Roex, M.C.J., van Balen, P., Germeroth, L., Hageman, L., van Egmond, E., Veld, S.A.J., Hoogstraten, C., van Liempt, E., Zwaginga, J.J., de Wreede, L.C., et al. (2020). Generation and infusion of multi-antigen-specific T cells to prevent complications early after T-cell depleted allogeneic stem cell transplantation-a phase I/II study. Leukemia *34*, 831–844.

10. Berdien, B., Reinhard, H., Meyer, S., Spöck, S., Kröger, N., Atanackovic, D., and Fehse, B. (2013). Influenza virus-specific TCR-transduced T cells as a model for adoptive immunotherapy. Hum. Vaccin. Immunother. *9*, 1205–1216.

11. Ahmadi, M., King, J.W., Xue, S.A., Voisine, C., Holler, A., Wright, G.P., Waxman, J., Morris, E., and Stauss, H.J. (2011). CD3 limits the efficacy of TCR gene therapy in vivo. Blood *118*, 3528–3537.

12. Schober, K., Müller, T.R., Gökmen, F., Grassmann, S., Effenberger, M., Poltorak, M., Stemberger, C., Schumann, K., Roth, T.L., Marson, A., and Busch, D.H. (2019). Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nat. Biomed. Eng. *3*, 974–984.

13. Bendle, G.M., Linnemann, C., Hooijkaas, A.I., Bies, L., de Witte, M.A., Jorritsma, A., Kaiser, A.D., Pouw, N., Debets, R., Kieback, E., et al. (2010). Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat. Med. *16*, 565–570, 1 p. following 570.

14. van Loenen, M.M., de Boer, R., Amir, A.L., Hagedoorn, R.S., Volbeda, G.L., Willemze, R., van Rood, J.J., Falkenburg, J.H., and Heemskerk, M.H. (2010). Mixed T cell receptor dimers harbor potentially harmful neoreactivity. Proc. Natl. Acad. Sci. USA *107*, 10972–10977.

15. Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S.J., Hamieh, M., Cunanan, K.M., Odak, A., Gönen, M., and Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature *543*, 113–117.

16. van Loenen, M.M., Hagedoorn, R.S., de Boer, R., van Egmond, E.H., Falkenburg, J.H., and Heemskerk, M.H. (2011). Rapid re-expression of retrovirally introduced versus endogenous TCRs in engineered T cells after antigen-specific stimulation. J. Immunother. *34*, 165–174.

17. Roth, T.L., Puig-Saus, C., Yu, R., Shifrut, E., Carnevale, J., Li, P.J., Hiatt, J., Saco, J., Krystofinski, P., Li, H., et al. (2018). Reprogramming human T cell function and specificity with non-viral genome targeting. Nature *559*, 405–409.

18. Stadtmauer, E.A., Fraietta, J.A., Davis, M.M., Cohen, A.D., Weber, K.L., Lancaster, E., Mangan, P.A., Kulikovskaya, I., Gupta, M., Chen, F., et al. (2020). CRISPR-engineered T cells in patients with refractory cancer. Science *367*, eaba7365.

19. Schmitt, A., Tonn, T., Busch, D.H., Grigoleit, G.U., Einsele, H., Odendahl, M., Germeroth, L., Ringhoffer, M., Ringhoffer, S., Wiesneth, M., et al. (2011). Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific $CD8^+$ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. Transfusion *51*, 591–599.

20. Knabel, M., Franz, T.J., Schiemann, M., Wulf, A., Villmow, B., Schmidt, B., Bernhard, H., Wagner, H., and Busch, D.H. (2002). Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nat. Med. *8*, 631–637.

21. Neuenhahn, M., Albrecht, J., Odendahl, M., Schlott, F., Dössinger, G., Schiemann, M., Lakshmipathi, S., Martin, K., Bunjes, D., Harsdorf, S., et al. (2017). Transfer of minimally manipulated CMV-specific T cells from stem cell or third-party donors to treat CMV infection after allo-HSCT. Leukemia *31*, 2161–2171.

22. Dössinger, G., Bunse, M., Bet, J., Albrecht, J., Paszkiewicz, P.J., Weißbrich, B., Schiedewitz, I., Henkel, L., Schiemann, M., Neuenhahn, M., et al. (2013). MHC multimer-guided and cell culture-independent isolation of functional T cell receptors from single cells facilitates TCR identification for immunotherapy. PLoS ONE *8*, e61384.

23. Nauerth, M., Weißbrich, B., Knall, R., Franz, T., Dössinger, G., Bet, J., Paszkiewicz, P.J., Pfeifer, L., Bunse, M., Uckert, W., et al. (2013). TCR-ligand k_{off} rate correlates with the protective capacity of antigen-specific $CD8^+$ T cells for adoptive transfer. Sci. Transl. Med. *5*, 192ra187.

24. Feuchtinger, T., Richard, C., Joachim, S., Scheible, M.H., Schumm, M., Hamprecht, K., Martin, D., Jahn, G., Handgretinger, R., and Lang, P. (2008). Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. J. Immunother. *31*, 199–206.

25. Baldwin, A., Kingman, H., Darville, M., Foot, A.B., Grier, D., Cornish, J.M., Goulden, N., Oakhill, A., Pamphilon, D.H., Steward, C.G., and Marks, D.I. (2000). Outcome and clinical course of 100 patients with adenovirus infection following bone marrow transplantation. Bone Marrow Transplant. *26*, 1333–1338.

26. Günther, P.S., Peper, J.K., Faist, B., Kayser, S., Hartl, L., Feuchtinger, T., Jahn, G., Neuenhahn, M., Busch, D.H., Stevanović, S., and Dennehy, K.M. (2015). Identification of a novel immunodominant HLA-B*07:02-restricted adenoviral peptide epitope and its potential in adoptive transfer immunotherapy. J. Immunother. *38*, 267–275.

27. Haque, T., Wilkie, G.M., Jones, M.M., Higgins, C.D., Urquhart, G., Wingate, P., Burns, D., McAulay, K., Turner, M., Bellamy, C., et al. (2007). Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: Results of a phase 2 multicenter clinical trial. Blood *110*, 1123–1131.

28. Leen, A.M., Bollard, C.M., Mendizabal, A.M., Shpall, E.J., Szabolcs, P., Antin, J.H., Kapoor, N., Pai, S.Y., Rowley, S.D., Kebriaei, P., et al. (2013). Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. Blood *121*, 5113–5123.

29. O'Reilly, R.J., Prockop, S., Hasan, A.N., Koehne, G., and Doubrovina, E. (2016). Virus-specific T-cell banks for "off the shelf" adoptive therapy of refractory infections. Bone Marrow Transplant. *51*, 1163–1172.

30. González-Galarza, F.F., Takeshita, L.Y.C., Santos, E.J.M., Kempson, F., Maia, M.H.T., da Silva, A.L.S., Teles e Silva, A.L., Ghattaoraya, G.S., Alfirevic, A., Jones, A.R., and Middleton, D. (2015). Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. Nucleic Acids Res. *43*, D784–D788.

31. Stenger, D., Stief, T.A., Kaeuferle, T., Willier, S., Rataj, F., Schober, K., Vick, B., Lotfi, R., Wagner, B., Grünewald, T.G.P., et al. (2020). Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. Blood *136*, 1407–1418.

32. Kaeuferle, T., Deisenberger, L., Jablonowski, L., Stief, T.A., Blaeschke, F., Willier, S., and Feuchtinger, T. (2020). CRISPR-Cas9-mediated glucocorticoid resistance in virus-specific T cells for adoptive T cell therapy posttransplantation. Mol. Ther. *28*, 1965–1973.

33. Stemberger, C., Graef, P., Odendahl, M., Albrecht, J., Dössinger, G., Anderl, F., Buchholz, V.R., Gasteiger, G., Schiemann, M., Grigoleit, G.U., et al. (2014). Lowest numbers of primary $CD8^+$ T cells can reconstitute protective immunity upon adoptive immunotherapy. Blood *124*, 628–637.

34. Berger, C., Jensen, M.C., Lansdorp, P.M., Gough, M., Elliott, C., and Riddell, S.R. (2008). Adoptive transfer of effector $CD8^+$ T cells derived from central memory cells establishes persistent T cell memory in primates. J. Clin. Invest. *118*, 294–305.

35. Wang, X.-C., Pang, H., Xu, X., Schmitt, A., Freund, M., Schmitt, M., and Chen, B.-A. (2013). Streptamer versus tetramer-based selection of functional cytomegalovirus-specific T cells. J. Formos. Med. Assoc. *112*, 338–345.

36. Freimüller, C., Stemberger, J., Artwohl, M., Germeroth, L., Witt, V., Fischer, G., Tischer, S., Eiz-Vesper, B., Knippertz, I., Dörrie, J., et al. (2015). Selection of adenovirus-specific and Epstein-Barr virus-specific T cells with major histocompatibility class I streptamers under good manufacturing practice (GMP)-compliant conditions. Cytotherapy *17*, 989–1007.

37. Brinkman, E.K., Chen, T., Amendola, M., and van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. *42*, e168.

38. Galperin, M., Farenc, C., Mukhopadhyay, M., Jayasinghe, D., Decroos, A., Benati, D., Tan, L.L., Ciacchi, L., Reid, H.H., Rossjohn, J., et al. (2018). $CD4^+$ T cell-mediated HLA class II cross-restriction in HIV controllers. Sci. Immunol. *3*, eaat0687.

39. Keib, A., Mei, Y.F., Cicin-Sain, L., Busch, D.H., and Dennehy, K.M. (2019). Measuring antiviral capacity of T cell responses to adenovirus. J. Immunol. *202*, 618–624.

40. Nauerth, M., Stemberger, C., Mohr, F., Weißbrich, B., Schiemann, M., Germeroth, L., and Busch, D.H. (2016). Flow cytometry-based TCR-ligand K_{off}-rate assay for fast avidity screening of even very small antigen-specific T cell populations ex vivo. Cytometry A *89*, 816–825.

41. Cohen, C.J., Li, Y.F., El-Gamil, M., Robbins, P.F., Rosenberg, S.A., and Morgan, R.A. (2007). Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. Cancer Res. *67*, 3898–3903.

42. Chicaybam, L., Sodre, A.L., Curzio, B.A., and Bonamino, M.H. (2013). An efficient low cost method for gene transfer to T lymphocytes. PLoS ONE *8*, e60298.