Genome-wide Specificity of Highly Efficient TALENs and CRISPR/Cas9 for T Cell Receptor Modification

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INTRODUCTION

In T cells with transgenic high-avidity T cell receptors (TCRs), endogenous and transferred TCR chains compete for surface expression and may pair inappropriately, potentially causing autoimmunity. To knock out endogenous TCR expression, we assembled 12 transcription activator-like effector nucleases (TALENs) and five guide RNAs (gRNAs) from the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system. Using TALEN mRNA, TCR knockout was successful in up to 81% of T cells. Additionally, we were able to verify targeted gene addition of a GFP gene by homology-directed repair at the TALEN target site, using a donor suitable for replacement of the reporter transgene with therapeutic TCR chains. Remarkably, analysis of TALEN and CRISPR/Cas9 specificity using integrase-defective lentiviral vector capture revealed only one off-target site for one of the gRNAs and three off-target sites for both of the TALENs, indicating a high level of specificity. Collectively, our work shows highly efficient and specific nucleases for T cell engineering.

TALENs are functional dimers consisting of DNA-binding domains fused to a FokI endonuclease catalytic domain. Upon co-localization of the FokI subunits tethered to the two TALEN-monomers, a DSB is introduced at the target site (Figure 1A).9 The chimeric guide RNA (gRNA) of the CRISPR/Cas9 system promotes Cas9-mediated DSB introduction by base-pairing between its 5’ sequence of 20 bases and a DNA target site. The full target site for SpCas9 must contain a target profile of GN_{14}NGG, with the terminal three bases referred to as a protoscaler adjacent motif (PAM).10,11 Designer nucleases have been reported to induce DSB not only at their target sites, but also at other genomic loci that contain sequence similarity, termed off-target sites. To examine the quantity and characteristics of these events, several studies have established in silico prediction methods,12–14 in vitro cleavage site analysis,13 systematic mismatching of various target site positions,14 and genome-wide off-target detection.15–20

Studies to date designed to prevent the assembly of TCRs with unknown specificity have employed RNAi-mediated TCR knockdown and ZFN- or TALEN-mediated TCR knockout.6,20–22 Here, we report the generation and employment of highly efficient and specific TALENs and CRISPR/Cas9 to safely edit the human TCR locus. In order to disrupt the endogenous TCR, we generated 12 TALENs and five CRISPR/Cas9 gRNAs specific for the constant regions of the human TCRα and TCRβ genes. In this proof-of-concept study, we demonstrated the ability to efficiently target the TCR locus by TALEN or CRISPR/Cas9.

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the TCR α and β chain genes (TRAC and TRBC1/2) (Figure 1B). To assess the specificity of the reagents, we performed an unbiased genome-wide off-target analysis by integrase-defective lentiviral vector (IDLV) capture into TALEN- and CRISPR/Cas9-mediated DSBs in cultured cells. This analysis showed near exclusivity of DSB generation at the TALEN and CRISPR/Cas9 target sites, demonstrating a high level of specificity. We furthermore show HDR-mediated, targeted integration of a GFP-expression cassette into the TRAC locus. The donor IDLV is designed for subsequent exchange of the GFP cassette for user-defined TCR genes, thereby representing a tool for the generation of therapeutic T cells with high-avidity TCR.

RESULTS

Design and Construction of Designer Nucleases

We designed a set of TALENs and CRISPR/Cas9 gRNAs to disrupt endogenous TCR expression. Aiming at a direct comparison of both platforms while excluding locus inherent effects, we chose partly overlapping target sites. We constructed eight TALENs to induce specific DSBs in the constant region of the TCR α chain (TRAC, αT2-9) and four TALENs targeting both constant regions of the TCR β chain (TRBC1 and TRBC2; βT1-4) (Figures 2A and 2D; Table S1). For constructing the TALEN candidates, we used the pTAL3 scaffold (TP) published by Cermak et al.,23 the GoldyTALEN (TG),23 and the comparable CAG-T7-TALEN (Sangamo)-Destination (TS) scaffold that possess shortened linkers between DNA-binding domain and FokI domain, as well as alternate mutations for promoting heterodimeric pairing [TSOH; CAG-T7-TALEN(Sangamo)-FokI-ELD-Destination and CAG-T7-TALEN(Sangamo)-FokI-KKR-Destination] (Table S1).24 This TALEN architecture has been reported to have a strong impact on TALEN activity as well as specificity.25-27 Six of the TALENs with the pTAL3 scaffold shared one or both monomers with other TALENs to create TALENs separated by 12 and 15 bp spacers, respectively. Obligate heterodimeric (OH) FokI domains were used to prevent homodimeric pairing of left or right TALEN arrays, thereby minimizing the risk for off-target activity.28 In addition, we subcloned TALEN

Figure 1. DSB Repair and Targeted Genome Editing of the TCR Loci Using Designer Nucleases

(A) During NHEJ-repair of TALEN- and CRISPR/Cas9-induced DSBs, frameshift mutations can result in gene knockout, or episomal IDLV can be integrated into DSBs, allowing for the permanent marking of off-target DSBs. If donor DNA is provided, HDR can lead to targeted integration of an expression cassette, i.e., therapeutic TCR chains. (B) The TCR α and β locus are composed of a number of variable (V), joining (J), constant (C), and, in the case of TRBC, diversity (D) gene segments (numbers of functional genes from IMGT/GENE-DB53 version 3.1.16, December 14, 2016). The positions of TALEN and gRNA target sequences for TCR knockout in the TCR α constant region (TRAC) and a homologous sequence shared by both TCR β constant regions (TRBC1/2) are marked by scissor symbols. DSB, DNA double-strand break; gRNA, guide RNA; HDR, homology-directed repair; IDLV, integrase-defective lentiviral vector; LTR, long terminal repeat; NHEJ, non-homologous end joining; PAM, protospacer adjacent motif; RVD, repeat variable di-residue.
T4 and T4 into the RCIsrcript Goldy backbone that is suitable for in vitro transcription.27

In parallel, we evaluated RNA-guided nucleases of the CRISPR/Cas9 system for TCR gene editing. We designed two and three different gRNAs for the constant regions of the TCR α chain (αC1 and αC2) and the TCR β chain (βC1-3), respectively, that overlapped with the corresponding TALEN target sites (Figures 2A and 2D; Table S1). Using in silico predictive software, we chose sites containing high sequence fidelity for the Cas9 nuclease. In addition, to ascertain

Figure 2. Evaluation of TALEN and CRISPR/Cas9 Activity
(A) Positions of TALEN and CRISPR/Cas9 gRNA binding sites at the TRAC target site. (B) TALEN and CRISPR/Cas9 activity in the TRAC locus in K562 cells. (C) Activity of obligate heterodimeric TALENs in the TRAC locus in 293T cells. (D) TALEN and gRNA binding sites at TRBC1 and TRBC2 target locus. (E) TALEN activity in the TRBC1 and TRBC2 locus in 293T cells. (F) CRISPR/Cas9 activity in the TRBC1 and TRBC2 locus in K562 cells. (B, C, E, and F) PCR amplification of the target regions in the TCR loci produces upper bands. T7EI-mediated cleavage of NHEJ-originated heteroduplex DNA results in additional cleavage bands, marked by arrowheads. A SNP in the TRBC2 locus results in additional bands, marked by arrows (>). Ctrl, negative control; M, marker; Sp., length of spacer between TALEN binding sites in base pairs; TALENα, GoldyTALEN; TALENβ(α+), pTAL3 (obligate heterodimeric FokI domain); TALENβ, CAG-T7-TALEN(Sangamo)-Destination.

αT4 and βT4 into the RCIsrcript Goldy backbone that is suitable for in vitro transcription.27

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After delivery of TALEN or Cas9/gRNA-expressing plasmids to K562 cells by nucleofection or to 293T cells using polyethyleneimine (PEI) transfection, TALEN and CRISPR/Cas9 activities were examined using the T7 endonuclease I (T7EI) assay. All TALENs with monomers separated by a 14 bp or 15 bp spacer induced specific DSBs at their target sites, whereas TALENs separated by 12 bp spacers failed to do so (Figure 2; Figure S1). In contrast to previous reports, obligate heterodimeric TALENs were less efficient than wild-type FokI domains (Table 1; Figure S1).29,30 When expressed from the TSOH vectors, the heterodimeric TALENs did not show locus-specific activity at the resolution of the T7EI assay (data not shown).

To quantify the nuclease cleavage activities, we further analyzed PCR-amplified nuclease target sites by deep sequencing. TALEN and CRISPR/Cas9 target locus activity in K562 cells resulted in up to 42.3% and 76.5% of sequences with insertions or deletions (indels), respectively (Table 1). Although the α chain TALENs delivered as plasmid showed superior efficiency compared with the β chain TALENs, nucleofection with TALEN mRNA increased the cleavage efficiency of βT4, but not of αT4. The deep sequencing results of all analyzed TRBC samples showed higher editing rates in the C2 region than in the C1 region (Figures 2 and 3; Table 1). Using an alternative C1 forward primer that binds with high target specificity upstream of the C1/C2 homologous region, we showed that in a proportion of the cells, simultaneous cleavage at the respective target sites in C1 and C2 resulted in the deletion of the complete sequence between both target sites (Figure S2).

Knockout of Endogenous TCR Expression in T Lymphocytes
After confirming TALEN and CRISPR/Cas9 activity in K562 cells, we evaluated their efficacy for TCR knockout in primary T cells. Using electroporation-based gene transfer of TALEN-expressing plasmid DNA (αT4p), we achieved up to 12.2% TCR knockout in primary T cells, determined by loss of CD3 surface expression (Figure S3). Flow cytometric analysis of T cells electroporated with CRISPR/Cas9-expressing plasmids revealed a TCR knockout efficiency rate of 19.9% (16.6% ± 1.7%, mean ± SD) for TRAC-specific αT2 and 16.6% (14.2% ± 2.1%) for TRBC1/C2-specific βC2 (Figure 3A).

The nuclease activity was additionally confirmed by T7EI assay and deep sequencing analysis of PCR products. Nineteen percent of sequences of αC2-treated cells, 6.3% of TRBC1, and 12.5% of TRBC2 sequences of βC2-treated cells contained indels (Figure 3B).

Because previous reports have demonstrated enhanced disruption rates following mRNA delivery of nucleases, we delivered TALEN αT4 and βT4 mRNA (αT4m and βT4m) to primary T cells by electroporation. We observed an increase in cell viability of transfected T cells, as well as a marked increase in TCR knockout efficiency rate of 78.8% (75.1% ± 2.5%) for αT4m and 81.2% (76.6% ± 3.8%) for βT4m on day 6 after electroporation (Figure 3C; Figure S4). This result was confirmed by T7EI assay and deep sequencing of the PCR products showing that 79.5% of the TRAC sequences and 52.1% and 71.4% of the TRBC1 and TRBC2 sequences, respectively, had nuclease-generated indels (Figure 3D). Modified T cells were able to be kept in culture for 20 days with high viability and stable TCR disruption rates as determined by flow cytometric analysis (Figure S5). The higher knockout efficiencies in T cells also resulted in higher deletion rates between the TRBC1 and TRBC2 regions than in K562 cells (Figure S2).

| Table 1. Indel Frequency at TALEN and CRISPR/Cas9 Target Sites |
|-----------------|------------------|
| **Nuclease**    | **% Indel**      |
| TRAC Locus      |                  |
| αT2a            | 42.3             |
| αT2OH           | 3.5†             |
| αT4p            | 19.4†/26.2       |
| αT4c            | 27.0             |
| αT6m            | 18.4             |
| αT8c            | 16.7             |
| αT9c            | 21.4             |
| αC1             | 44.7             |
| αC2             | 76.5             |
| TRBC1 Locus     |                  |
| βT2a            | 42.2†            |
| βT3p            | 0.0†             |
| βT4p            | 3.0†/8.6         |
| βT4OH           | 0.1†             |
| βT4c            | 4.1              |
| βT6m            | 9.5              |
| βC2             | 52.3             |
| TRBC2 Locus     |                  |
| βT2a            | 4.5†             |
| βT3p            | 0.0†             |
| βT4p            | 1.9†/16.7        |
| βT4OH           | 1.3†             |
| βT4c            | 10.3             |
| βT6m            | 20.5             |
| βC1             | 59.5             |
| βC3             | 24.7             |

% Indel denotes the frequency of sequences with insertions or deletions at the respective nuclease target sites analyzed by deep sequencing. Values with superscript T were obtained in 293T cells; all other results were generated in K562 cells. C, CRISPR/Cas9; TG, GoldyTALEN; Tm, TALEN delivered as mRNA; TPOOH, pTAL3 (obligate heterodimeric FokI domain); TS, CAG-T7-ATTRIBUTE(Sangamo)-Destination.

the relative accuracy of in silico modeling, we also included one gRNA (BC3) with a low “quality score” intended as a control for off-target analysis. For CRISPR/Cas9 generation we used the pX330 expression plasmid.10

**TALEN and CRISPR/Cas9 Activity at Their Target Sites**

After delivery of TALEN or Cas9/gRNA-expressing plasmids to K562 cells by nucleofection or to 293T cells using polyethyleneimine (PEI) transfection, TALEN and CRISPR/Cas9 activities were examined using the T7 endonuclease I (T7EI) assay. All TALENs with monomers separated by a 14 bp or 15 bp spacer induced specific DSBs at their target sites, whereas TALENs separated by 12 bp spacers failed to
TRAC and TRBC TALENs and CRISPR/Cas9 Are Specific for Their Target Sites

Off-target activity has implications for the translational application of programmed nucleases, and each class has previously shown off-target activity.14,16–20 To analyze the nuclease specificity for these clinically relevant loci, we transduced K562 cells with an IDLV cargo that can be captured at genomic sites where DSBs occur.15,17,20 The transduced cells were subsequently transfected with CRISPR/Cas9 and bC1-3 plasmids and the three scaffold variants of TALEN aT4 and bT4, including mRNA. IDLV-marked DSBs were subsequently identified using linear amplification-mediated (LAM)-PCR, non-restrictive (nr) LAM-PCR, and deep sequencing.32–35 Maximally, 3,268 IDLV integration sites were found in cells treated with or without nucleases. When two or more of these events are mapped within less than 100 nt or in more than one LAM-PCR sample replicate, they are defined as clustered integrations sites (CLISs) and are evidence of on- or off-target activity. Low-frequency off-target sites can manifest as single integration sites rather than CLISs. To validate true off-target sites, we used the sequence pattern match tool “scan-for-matches.”36 For this, regions upstream and downstream of all integration sites were scanned for potential binding sites with a maximum distance of 10 bp between integration site and predicted cleavage position and up to six or eight mismatches for gRNA or TALENs, respectively.36 Identified on- and off-target sites were examined for NHEJ-derived indel formation by targeted deep sequencing and the CRISPResso tool37 in order to validate the LAM-PCR data. CLISs were observed proximal to the respective target site in all nuclease-treated samples, but not in the IDLV-only control (Table S2). In total, we detected six off-target sites for the analyzed TALEN candidates (Figure 4; Table S3). Four of these (aT4 OT3, bT4 OT1-3) were detected only in samples treated with TALEN mRNA (Tables S2 and S3). One off-target site (bT4 OT2) was indicated only by a single IDLV integration site that was located in the spacer region of a potential TALEN binding site with seven mismatches to the target site. Five off-target sites were detected in gene-coding regions and one in an...
intergenic region. All off-target sites except αT4 OT3 were cleaved by a homodimer of the respective left TALEN binding domain (Table S3). Deep sequencing confirmed cleavage at all target sites with mutation frequencies of up to 16.9% (TRAC), 11.3% (TRBC1), and 17.9% (TRBC2). Four of the off-target sites were confirmed by deep sequencing in K562 cells with mutation frequencies of 0.1% to 0.6% (Table S4). Of the CRISPR/Cas9 candidates, only one (βC1) showed an off-target CLIS, localized in an intergenic region (Figure 4). In contrast, gRNA βC3 that was included as control with a low “quality score” showed 24 CLISs at off-target sites (Tables S2 and S5). In addition, we chose two web-based tools in order to compare their predicted with our experimentally determined off-target sites. Both the CRISPR Design tool38 that was used for designing the TCR β chain CRISPR/Cas9 gRNA and the COSMID tool39 predicted 7 of the 24 βC3 off-target sites identified in this study when allowing the highest possible number of mismatches (CRISPR Design: four mismatches, COSMID: three mismatches). The COSMID tool predicted all identified off-target sites when one insertion and one deletion were allowed in addition to two mismatches. However, the total number of predicted off-target sites, in this case, was 1,197 at 823 genomic sites. Only the COSMID tool predicted the single off-target site for βC1 (Table S5).

The off-target analyses were performed in K562 because of the low proliferation rates of T cells that lack CD3 surface expression leading to insufficient dilution of episomal IDLV and amplification of predominantly non-integrated IDLV by LAM-PCR. Therefore, the off-target sites that were detected in K562 cells were validated in primary T cells by deep sequencing. The target site disruption frequency in T cells was 83.5% for TRAC, 52.7% for TRBC1, and 73.8% for TRBC2. Similar to the on-target editing efficiencies, the frequency of off-target sites was more prevalent in T cells than K562 (Figure 4; Table S4).

**Targeted Gene Addition through HDR**

Having demonstrated successful TCR knockout, the capability of αT4 to stimulate efficient targeted gene addition by HDR was evaluated. For gene addition to the TRAC locus, we designed and

![Figure 4. Quantification of NHEJ at TALEN and CRISPR/Cas9 On- and Off-Target Sites, Analyzed by Deep Sequencing and the CRISPResso Tool](image-url)
constructed two different donor templates containing a GFP expression cassette flanked by 200 bp (TA200G) or 800 bp (TA800G) sequences homologous to the αT4 target site. The design strategy allows for the use of the GFP reporter gene for detection and quantification, and is constructed such that it can be replaced by functional TCR sequences. After cultivation for 2 weeks, the background expression of GFP in cells not treated with TALENs was 3.8% ± 1.6% (TA200G) and 3.9% ± 1.9% (TA800G) (mean ± SD). In contrast, 11.2% ± 0.1% of the K562 cells, nucleofected with the TA800G donor and TALEN αT4P plasmids, expressed GFP. Notably, compared with the 800 bp homology donor, the 200 bp homology regions resulted in a lower efficiency of targeted integration of the GFP gene (5.8% ± 2.8%) (Figure 5A). HDR-mediated targeted transgene integration at the nuclease target sites was additionally validated by bidirectional targeted integration PCR using primers binding in the GFP cassette and outside of the donor homology regions at the endogenous TRAC locus (Figure 5B).

After validating HDR from donor plasmids for transgene addition in K562 cells, we packaged the TA800G donor into IDLV particles and transduced CD3/CD28-activated primary T cells followed 24 hr later by αT4m nuclease electroporation. GFP expression and TCR knockout were subsequently analyzed by flow cytometry. Seven days after transduction, 64.2% ± 5% of the cells were CD3⁺/GFP⁺, 6.9% ± 3.6% of the cells were CD3⁺/GFP⁻, and 1.1% ± 0.5% were CD3⁻/GFP⁺ (Figure 5C). After 2 and 3 weeks of cultivation, the frequency of GFP-expressing CD3⁺ cells decreased to 4.5% ± 2% and 4.5% ± 2%, respectively (Figure 5C). In comparison, 3.5% ± 1.6% of IDLV control cells showed a very low GFP expression on day 7, which was reduced to 0.4% ± 0.3% and 0.4% ± 0.2% on days...
et al., this represents the highest TALEN-mediated TCR knockout edge, along with the TRAC gene. In contrast, delivery of TALEN toxicity in primary T cells, and this can limit the effective dose CRISPR/Cas9 and TALENs, respectively. We observed DNA-dependent knockdown of the endogenous TCR, codon optimization, or modification of the amino acid sequence for the preferential pairing of transferred TCR chains. Homodimerization of two identical TALEN monomers is of great importance regarding off-target activity because two left or two right homodimers may co-localize promiscuously and cleave unintended sequences. Indeed, many reported off-target sites are cleaved by homodimers of TALENs and ZFNs. However, specificity may come at a tradeoff for activity, as evidenced by our observation that TALENs containing the wild-type FokI domains consistently outperformed the homodimers of the respective TALEN left TALEN monomer of TALEN off-target sites were cleaved by homodimers of the respective TALEN delivered as plasmid DNA. However, the lower indel frequency at the respective target sites indicates lower general cleavage efficiencies for TALENs delivered as plasmid DNA. This may result in dose-dependent prevention of off-target cleavage or in cleavage activity below the detection limit of the T7EI capture assay. In accordance with this, deep sequencing of the on- and off-target sites in T cell samples resulted not only in markedly elevated target site indel frequencies, but also considerably higher off-target indel frequencies (Figure 4; Table S4). The two off-target sites that were not confirmed by deep sequencing in K562 cells (zT4 OT3 and zT4 OT3) were observed in T cells, however, with very low indel frequencies (Figure 4; Table S4). Importantly, five of the six detected TALEN off-target sites were cleaved by homodimers of the respective left TALEN monomer of zT4 and zT4. Thus, usage of optimized heterodimeric versions of TALENs would prevent these off-target sites completely.

Only one off-target CLIS was found for gRNA bC1, indicating a high degree of specificity for the CRISPR/Cas9 gRNA examined in this study. In contrast, 24 off-target CLISs were detected for the control gRNA bC3. This demonstrates that currently available bioinformatics predictive tools for target sites design are able to a priori exclude target sites with the possibility of exhibiting significant off-target effects. However, although number and position of mismatches between target site and off-target site play an important role, in silico homology-based predictions can only serve as a guide for nuclease design, but do not uniformly predict bona fide off-target sites. This is demonstrated in our observations that only 7 of the 24 experimentally determined off-target sites for bC3 were predicted by two different tools that identify off-target sites with up to four or three mismatches. When allowing insertions and deletions in addition to that cleavage of both TRBC target sites can result in a deletion of the complete sequence separating them (C1-C2 deletion) (Figure S2). We hypothesize that these chromosomal deletions resulted in a biased quantification of knockout efficiencies by deep sequencing that suggested lower knockout rates in the TRBC1 locus as compared with the TRBC2 locus. Due to the homology between the C1 and the C2 region, the forward primers we used for PCR amplification for T7EI assay and deep sequencing have up to two mismatches. Thus, mispriming of the C2 forward primer in the C1 region can result in the amplification of sequences with a C1-C2 deletion. In contrast, the target specificity of the C1 reverse primer prevents a successful amplification of these deletion-containing sequences (Figure S2A).

Multiple studies analyzing on- and off-target activity of different classes of designer nucleases have been published and are of great importance for translational cellular engineering. The genome-wide IDLV capture methodology we employed is predicted to detect off-target sites with a sensitivity threshold of 1% of mutated sequences. Using this approach, we detected a high frequency of on-target IDLV CLISs in all nuclease-treated cells, whereas none of the integration sites in IDLV-only-treated cells was located at these target sites (Table S2). Four of the six detected TALEN off-target sites were found only in samples treated with TALEN mRNA. This may be because of the differing scaffolds of TALENs delivered as plasmid DNA. However, the lower indel frequency at the respective target sites indicates lower general cleavage efficiencies for TALENs delivered as plasmid DNA. This may result in dose-dependent prevention of off-target cleavage or in cleavage activity below the detection limit of the T7EI capture assay. In accordance with this, deep sequencing of the on- and off-target sites in T cell samples resulted not only in markedly elevated target site indel frequencies, but also considerably higher off-target indel frequencies (Figure 4; Table S4). The two off-target sites that were not confirmed by deep sequencing in K562 cells (zT4 OT3 and zT4 OT3) were observed in T cells, however, with very low indel frequencies (Figure 4; Table S4). Importantly, five of the six detected TALEN off-target sites were cleaved by homodimers of the respective left TALEN monomer of zT4 and zT4. Thus, usage of optimized heterodimeric versions of TALENs would prevent these off-target sites completely.

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two mismatches, the COSMID tool predicted all of the off-target sites; however, experimental validation of the 1,197 off-target sites at 823 genomic sites predicted using these parameters would be highly laborious.

We and others have previously shown that the number of off-target sites for the same type of nuclease varies greatly depending on the respective target sequence composition. In our current study we observed that up to 11 mismatches were tolerated by both TALEN candidates (Figure 4; Table S3). These findings highlight the necessity of an individual and comprehensive analysis of off-target cleavage for each nuclease, particularly in the context of future translational use.

Transgenic TCR chains or chimeric antigen receptors (CARs) can be delivered efficiently to primary T cells by lentiviral or retroviral vectors that integrate at unknown positions in the genome. As a more specific alternative, with our TCR-specific GFP-expressing vector, we showed HDR-mediated targeted integration of a reporter gene into the TRAC locus (Figure 5C). At all analyzed time points the TALEN-treated cells comprised a population showing high GFP expression that was absent in the IDLV-only control cells (Figure 5C). The majority of these cells were CD3+, indicating simultaneous TCR knockout and transgene integration into the TRAC target locus. The small population of CD3- and GFP+ cells most likely represents cells with specific transgene integration into the silenced/non-productive TRAC allele. The donor construct is designed for subsequent transgene exchange that can be used to introduce user-defined TCR or CAR genes into the TRAC locus. This powerful approach supports designer cellular engineering and would allow for CD3-dependent or antigenic restimulation of the cells, if required, for expansion to therapeutic numbers.

Taken together, we report a set of TALENs and CRISPR/Cas9 reagents for specific and efficient genome editing in the constant regions of the TCR α and β chains. The demonstrated efficiency and specificity are additive to the field and further support translational application of designer nucleases for engineering T cells for adoptive cell therapy.

MATERIALS AND METHODS

TALEN and CRISPR/Cas9 gRNA Design and Generation

TALEN target sites were chosen using two versions of the online tool TAL Effector-Nucleotide Targeter (TALE-NT; https://tale-nt.cac.cornell.edu/TALENT/). The old version was used for TALEN εT2-5 and βT1-5; the new version was used for εT6-9 design. TALEN assembly was accomplished by Golden Gate cloning using the Golden Gate TALEN and TAL Effector Kit 2.0. Complete TALEN arrays of TALEN εT2-5 and BT1-5 were cloned into the mammalian expression vector pCMV-MCS (Agilent) after the introduction of supplementary recognition sites for AfiI and XhoI. In addition, repeating the second Golden Gate reaction, εT2, εT4, βT2, and βT4 were cloned into pCAG-T7-TALEN(Sangamo)-Destination vector for homodimeric pairing and pCAG-T7-TALEN(Sangamo)-FokI-KKR-Destination or pCAG-T7-TALEN(Sangamo)-FokI-ELD-Destination vectors for heterodimeric pairing. For TALEN εT6-9 the Tε backbone was used during the Golden Gate cloning procedure. TALEN εT4 and βT4 were further subcloned into RCIScript-TG backbone. For in vitro transcription, the TALEN plasmids were linearized with SacI-HF and mRNA was generated using the mMESSAGE mMACHINE T3 kit (Thermo Fisher Scientific) with polyA addition using Poly(A) Tailing Kit (Thermo Fisher Scientific). The amino acid substitutions for obligate heterodimeric TALENs were introduced with the QuikChange II Site-Directed Mutagenesis kit (Agilent) and primers containing point mutations (see list of primers in the Supplemental Information). TRAC CRISPR/Cas9 gRNA were designed manually following the guidelines published by Cong et al. For TRBC CRISPR/Cas9 gRNA design and in silico off-target prediction, the gRNA design tool (http://crispr.mit.edu/) was applied. Chosen gRNA sequences were ordered as 5’ phosphorylated oligonucleotides and cloned into the pX330-U6-Chimeric_BB-BBh-hSpCas9 vector.

Cell Culture

Cells were cultured with 5% CO2 at 37°C. K562 and HEK293T cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco), supplemented with 10% fetal bovine serum (FBS) and 0.1 mg/mL penicillin and streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (blood) using Ficoll density centrifugation. T cells were isolated from these using the Dynabeads Untouched Human T Cells Kit (Thermo Fisher Scientific) or the EasySep Human T Cell Isolation Kit (STEMCELL Technologies). Prior to transfection or transduction, T cells were activated for 40 hr with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) and cultured in T cell medium (RPMI 1640 medium; Gibco), supplemented with 5% AB human serum (Thermo Fisher Scientific), 0.1 mg/mL penicillin and streptomycin, and recombinant human IL-7 and IL-15 (Miltenyi Biotec) at 5 ng/mL.

Transfection of Cells

For transfection of 293T cells, 2.5 µg of each TALEN monomer in IMDM was mixed with 18 µg PEI (Sigma-Aldrich) in IMDM, incubated at room temperature (RT) for 30 min and given to 1 × 10⁶ cells dropwise. A total of 2 × 10⁵ K562 cells was nucleofected in 16-well Nucleocuvette Strips with the Amaxa 4D-Nucleofector (Lonza) using the SF Cell Line 4D-Nucleofector X Kit and 1–2 µg nuclease plasmid, 4 µg TALEN mRNA, and/or 3 µg donor DNA. Delivery of TALEN plasmids (2 µg) or mRNA (2–4 µg) and CRISPR/Cas9 plasmids (1 µg) to 2–3 × 10⁵ T cells was done by Neon Transfection System (Thermo Fisher Scientific) in resuspension buffer T using 10 µL Neon tips and the following electroporation parameters: 1,400 V, 10 ms, 3 pulses. Transfected cells were initially plated in 96-well plates in 200 µL of antibiotic-free medium and transferred to medium containing antibiotics after 24 hr. For viability analysis, cells were counted 24 hr after electroporation using trypan blue exclusion. In the context of HDR-mediated gene transfer experiments, T cells were electroporated 24 hr after transduction or 48–72 hr after activation.

IDLV Production and Transduction of Cells

IDLV particles were produced in 293T cells using calcium phosphate transfection of a GFP transfer vector or the TA800G donor vector and...
the components of a third generation lentiviral packaging system as described previously. For DSB capture, K562 cells were transduced with GFP-IDLV (MOI 10) followed by nucleofection of $1 \times 10^6$ transduced cells per sample with 4–8 μg of TALEN- or CRISPR/Cas9-expressing plasmids 24 hr later. For T cell transduction 48 hr after activation or 24 hr after electroporation, 6 $\times 10^5$ cells were transduced with TA800G-IDLV (MOI 50) in 200 μL of T cell medium. After 24 hr, fresh T cell medium was added and cells were cultivated at a density of $5 \times 10^5$ to $1.5 \times 10^6$ cells/mL or counted for electroporation.

**TTEI Assay and On- and Off-Target Deep Sequencing**

Forty-eight hours after nuclease transfection, genomic DNA was isolated and the target sites were amplified by PCR using primers for TRAC, TRBC1, or TRBC2 included in the list of primers (see Supplemental Information). The PCR was performed with the following conditions: initial denaturation 5 min at 95°C, 30–35 cycles, denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s (for variations, see list of primers in the Supplemental Information), and elongation at 72°C for 40 s; final elongation of 5 min at 72°C. 1 μL of buffer NEBuffer 2 was added to 8.5 μL of the PCR product, and the DNA was denatured and re-annealed (95°C for 5 min, 95°C to 85°C at $-2$°C/s, and then 85°C to 25°C at $-0.1$°C/s). Five units of T7E1 was added for digestion of the PCR product for 15 min at 37°C. For validating deletions between the nuclease target sites in C1 and C2, PCRs were conducted with the T7E1 primers and the TRBC1S primer included in the list of primers (see Supplemental Information). The PCRs were performed with the following parameters: initial denaturation 5 min at 94°C, 28–35 cycles, denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s, and elongation at 72°C for 90 s; final elongation at 72°C for 5 min. For deep sequencing, the on- and off-target site primers were directly fused to sequencing adapters and barcodes for multiplexed Illumina MiSeq sequencing, or a two-step PCR was performed using primers containing the MiSeq adapters and barcodes (list of primers is provided in the Supplemental Information). The PCR was performed with the following conditions: initial denaturation 5 min at 95°C, 30–35 cycles, denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s (for variations, see list of primers in the Supplemental Information), and elongation at 72°C for 40 s; final elongation of 5 min at 72°C. The frequency of insertions and deletions was analyzed using version 0.9.9 of the CRISPResso tool. The chosen minimum average read quality was 30; only mutations occurring within a 20 bp window centered on each predicted on- or off-target site were included, and substitutions were ignored in NHEJ quantification. Reference sequences were taken from University of California, Santa Cruz (UCSC) Assembly hg19 (February 2009) (TRBC1/ TRBC2 sequences from hg38 [December 2013]). For NHEJ quantification, indel frequency of controls was subtracted from the respective values of treated samples.

**Flow Cytometry**

For flow cytometric analysis of CD3 expression, 5–6 days after transfection with nucleases, T cells were washed with staining buffer (FBS with 2% FBS) and stained for 45 min at 4°C. The allophycocyanin (APC)-eFluor 780-conjugated mouse anti-human CD3 antibody (1:100; eBioscience) or the fluorescein isothiocyanate (FITC)- or APC-conjugated mouse anti-human CD3 antibody (FITC 1:200, APC 1:50; BD Biosciences) was diluted in fluorescence-activated cell sorting (FACS) buffer. Stained cells were washed twice and resuspended in staining buffer containing 0.5 μg/mL propidium iodide (PI) for dead cell staining. For analysis of GFP expression of K562 or T cells, the cells were washed and resuspended in FACS buffer containing 0.5 μg/mL PI, and washing was repeated. Flow cytometric analyses were performed using LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

**IDLV Integration Site Analysis**

GFP expression of transduced cells was determined by flow cytometry after transfection with nucleases. Genomic DNA was isolated using the High Pure PCR Template Preparation Kit (Roche) after 3–4 weeks of cultivation when the frequency of GFP-expressing cells was stable. Each sample was analyzed by at least two repetitions of 3° LAM-PCR with enzymes MseI and MluCI and 3° nLAM-PCR using 500 ng of genomic DNA each. For sequencing with the Illumina MiSeq platform, barcode-containing adapters were added to LAM-PCR products by an additional PCR step, and the integration site data were analyzed using the high-throughput insertion site analysis pipeline (HISAP). Additional in silico off-target prediction for βC1 and βC3 was accomplished with the web-based COSMID tool. For all analyses, the UCSC Assembly hg19 (February 2009) was used as reference genome.

**Donor Construction and Targeted Integration PCR**

For the 200 bp α chain donor construct (TA200G), we ordered a GeneArt Strings DNA Fragment (Thermo Fisher Scientific) containing two 200 bp regions flanking the αT4 cutting site in the TRAC locus and restriction sites AsISI and SbfI for cloning into a lentiviral transfer vector in antisense direction. A GFP-expression cassette comprising a phosphoglycerate kinase (PGK) promoter and a polyA signal was cloned between the two homology sites in antisense direction using MfeI and NheI. For TA800G construction, the 200 bp homology regions were exchanged by 800 bp homology regions, amplified from Jurkat genomic DNA with primers containing AsISI or NheI restriction sites for the 5' homology arm and SbfI or SphI sites for the 3' homology arm. Targeted integration PCR was performed with the following conditions: initial denaturation 5 min at 94°C; followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s, and a final elongation of 5 min at 72°C. For 5' targeted integration amplification, forward primers Alpha_donor200for or Alpha_donor800for and reverse primer DonorPGK were used. 3' Targeted integration PCR was done with forward primer DonorGFPpolyA and reverse primers Alpha_donor200rev or Alpha_donor800rev (see list of primers in the Supplemental Information).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, five tables, and a list of primers and can be found with this article online at http://dx.doi.org/10.1016/j.omtm.2017.01.005.
AUTHOR CONTRIBUTIONS
Conceptualization, R.G., M.S., and C.v.K.; Methodology, F.K., R.G., M.S., and K.P.; Investigation, F.K.; Resources, M.J.O., J.T., and H.G.; Writing – Original Draft, F.K., R.G., M.J.O., and M.S.; Supervision, R.G., C.v.K., and M.S.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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