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ORIGINAL ARTICLE

Genome-wide off-target analyses of CRISPR/Cas9-mediated T-cell receptor engineering in primary human T cells

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Abstract

Objectives. Exploiting the forces of human T cells for treatment has led to the current paradigm of emerging immunotherapy strategies. Genetic engineering of the T-cell receptor (TCR) redirects specificity, ablates alloreactivity and brings significant progress and off-the-shelf options to emerging adoptive T-cell transfer (ACT) approaches. Targeted CRISPR/Cas9-mediated double-strand breaks in the DNA enable knockout or knock-in engineering. **Methods.** Here, we perform CRISPR/Cas9-mediated TCR knockout using a therapeutically relevant ribonucleoprotein (RNP) delivery method to assess the safety of genetically engineered T-cell products. Whole-genome sequencing was performed to analyse whether CRISPR/Cas9-mediated DNA double-strand break at the TCR locus is associated with off-target events in human primary T cells. **Results.** TCRα chain and TCRβ chain knockout leads to high on-target InDel frequency and functional knockout. None of the predicted off-target sites could be confirmed experimentally, whereas whole-genome sequencing and manual Integrative Genomics Viewer (IGV) review revealed 9 potential low-frequency off-target events genome-wide. Subsequent amplification and targeted deep sequencing in 7 of 7 evaluable loci did not confirm these low-frequency InDels. Therefore, off-target events are unlikely to be caused by the CRISPR/Cas9 engineering. **Conclusion.** The combinatorial approach of whole-genome sequencing and targeted deep sequencing confirmed highly specific genetic engineering using CRISPR/Cas9-mediated TCR knockout without potentially harmful exonic off-target effects.

Keywords: CRISPR/Cas9, genetic engineering, off-target, T-cell receptor, T-cell therapy, whole-genome sequencing

INTRODUCTION

T cells are a powerful tool of adaptive immunity being able to mediate tumor rejection and pathogen clearance. Adoptive T-cell transfer (ACT) of antigen-specific T cells paved the way to transfer therapeutic immunity.[1] Genetic engineering of T cells opens up a new era of adoptive T-cell therapies to treat a variety of infectious diseases and cancer. Engineering has become an enabler for almost unlimited options of synthetic immunity, introducing antigen-specific receptors and other attributes intended to improve therapeutic efficacy and safety.[2–4] Historically, the genetic manipulation of primary T cells began with recombinant retroviral vectors, still remaining the most prevalent vectors in clinical studies of TCR- or CAR-modified T cells. However, genetic engineering approaches using transposons, and retro- or lentiviral vectors bear various risks, such as random/semi-random integration into the human genome resulting in unphysiological promoter control and incidental manipulation of proto-oncogenes or tumor suppressor genes.[5–8] 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.[9–12]

Technically, CRISPR/Cas9 approaches advance the field of genetic engineering by enabling gene knockout, and simultaneous knockout and site-directed knock-in in a two-in-one process: CRISPR/Cas9-induced targeted double-strand breaks result in gene knockout because of the error-prone endogenous DNA repair mechanism (non-homologous end joining) typically leading to small insertions and deletions (InDels), often causing frameshift mutations.[13] In case homology-containing templates are present at the same time, the double-strand break can be corrected *via* transgene integration (homology-directed repair). Homology-directed repair leads to a knockout of the endogenous function with simultaneous knock-in of a transgene in a two-in-one process.[14]

Genetic engineering of the T-cell receptor (TCR) does not only redirect specificity by concurrent preservation of physiological TCR regulation,[6–8] but also ablates alloreactivity, thereby bringing significant progress to future off-the-shelf options of emerging adoptive T-cell transfer approaches.[15] However, the transfer of genetically engineered T cells has to meet particularly high safety standards, which has so far limited the approach in its applicability. A complication of CRISPR/Cas9-mediated knockout of endogenous TCRs could be the binding of the Cas9 nuclease to unintended genomic sites that share a sequence homology with the on-target site.[16] In addition to typical double-strand breaks and InDel mutations, Cas9 nucleases can also lead to chromosome rearrangements.[16] Therefore, the identification of putative off-target sites is of utmost importance for the safety of the generated T-cell products considering future clinical application.

In the present study, the safety of CRISPR/Cas9-mediated TCR-engineered T-cell products was assessed in terms of unspecific nuclease-induced off-target events using not only a biased target prediction-based approach but also an unbiased whole-genome sequencing and targeted deep sequencing combinatorial approach. This approach allows the genome-wide identification of off-target events and a quantitative analysis of their frequencies in the T-cell product.

RESULTS

CRISPR/Cas9-mediated knockout of the endogenous TCR

Genetic engineering of the TCR in donor-derived T cells opens up a new era of emerging adoptive T-cell transfer (ACT) approaches (Figure 1a). Aiming to study TCR-engineered primary human T cells, we knocked out the genes encoding for TCRα chain (*TRAC*) and TCRβ chain (*TRBC*) using CRISPR/Cas9 technology. The knockout was phenotypically and genetically highly efficient with 87% (*TRAC* KO) and 79% (*TRBC* KO) TCR-negative T cells and insertion/deletion (InDel) frequencies of 87% (*TRAC* KO) and 78% (*TRBC* KO; Figure 1b and c). Locus-dependent InDel patterns after CRISPR/Cas9-mediated gene knockout have been determined after PCR amplification of the *TRAC* and *TRBC* loci and are shown in Figure 1d. Although there seems to be a trend towards slightly higher *TRAC* knockout rates, frequencies of TCR-negative T cells and InDel frequencies were not significantly different between *TRAC* and *TRBC* loci ($P = 0.3$ and $P = 0.2$, respectively; Figure 1b). Functionally, TCR knockout was confirmed by significantly impaired IFN-γ production upon super-antigen

staphylococcal enterotoxin B (SEB) stimulation (2.4% IFN-γ^+ TCR knockout cytotoxic T cells vs. 22.7% IFN-γ^+ MOCK cytotoxic T cells; Figure 1e). Read coverage of next-generation sequencing revealed reduced read frequencies at the double-strand break sites, thereby confirming successful knockout of the *TRAC* and *TRBC* genes (Figure 1f).

Analysis of predicted gRNA-dependent Cas9-induced off-target sites

The safety of the T-cell product was assessed in terms of unspecific nuclease-induced off-target events in T cells genetically engineered at these TCR loci. CRISPR/Cas9-induced off-target events

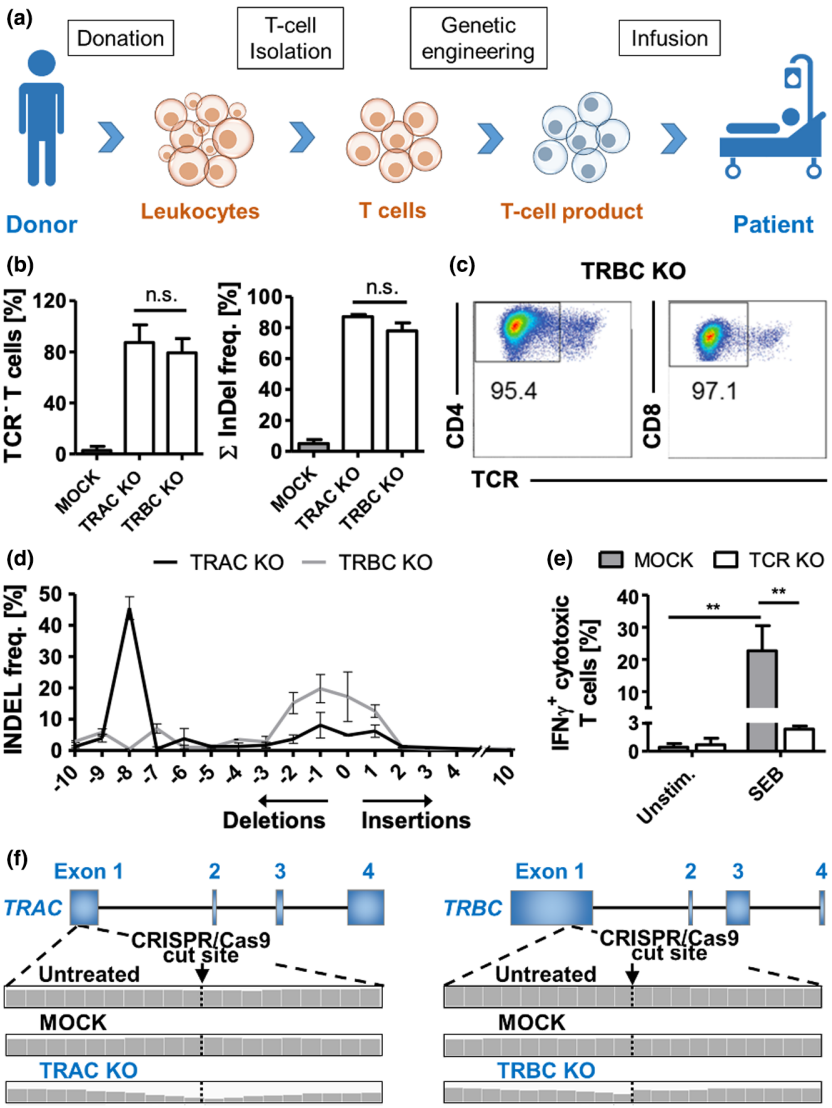

Figure 1. CRISPR/Cas9-mediated knockout of *TRAC* and *TRBC* in primary human T cells. **(a)** Adoptive transfer of TCR-engineered T-cell product approach. **(b)** Frequencies of TCR-negative (TCR$^-$) T cells after *TRAC* or *TRBC* knockout detected by flow cytometric analysis. Data show mean ± SD of ≥ 3 independent experiments. Total insertion and deletion frequency (InDel freq.) at on-target site determined by TIDE after knockout of either *TRAC* or *TRBC*. Mean ± SD of 2 WGS donors. Student's *t*-test; n.s. = not significant. **(c)** Flow cytometric density plots of anti-TCRα/β-stained CD4 and CD8 T cells exemplary shown for *TRBC* knockout. **(d)** TIDE-generated insertion and deletion patterns at on-target sites of *TRAC* and *TRBC* KO T cells. Mean ± SD of 2 WGS donors. **(e)** Intracellular cytokine staining for IFN-γ without stimulation (Unstim.) or Staphylococcal enterotoxin B (SEB) stimulus in MOCK-electroporated and TCR KO T cells. Mean ± SD of 5 independent experiments. Student's *t*-test; ** $P < 0.01$. **(f)** Schematic *TRAC* and *TRBC* gene at CRISPR/Cas9 on-target site region. Read coverage in untreated, MOCK, and *TRAC*/*TRBC* KO cells based on IGV exemplary shown for one donor. CRISPR/Cas9 cut sites are indicated with dotted lines.

were investigated using whole-genome sequencing (WGS), and InDel mutations were evaluated on predicted sites and genome-wide.

Cas-OFFinder *in silico* predicted 138 off-target sites for *TRAC* gRNA and 208 off-target sites for *TRBC* gRNA allowing four mismatches between gRNA and target sequence (Supplementary tables 1 and 2). Therefore, no off-target sites allowing 0 or 1 mismatch between gRNA and target sequence were predicted for *TRAC* or *TRBC* gRNA, 1 off-target site allowing 2 mismatches was predicted for *TRBC* gRNA, and 5 or 26 off-target sites allowing 3 mismatches were predicted for *TRAC* or *TRBC* gRNA. Allowing up to 4 mismatches led to 133 additional predicted off-targets for *TRAC* gRNA and 181 for *TRBC* gRNA (Figure 2a).

In silico predicted off-target sites were experimentally analysed for verification on whole-genome sequencing data sets of 2 independent CRISPR/Cas9-engineered T-cell products. T-cell samples from healthy male and female donors were engineered with either *TRAC* or *TRBC* gRNA, whereas untreated and MOCK (electroporated only) T cells served as controls (Figure 2b). Whole-genome sequencing (WGS) yielded a stable overall depth of genomic coverage over all samples and donors (32.9x mean of untreated, 32.6x of MOCK, 33.5x of *TRAC* and 33.1x of *TRBC* sample;

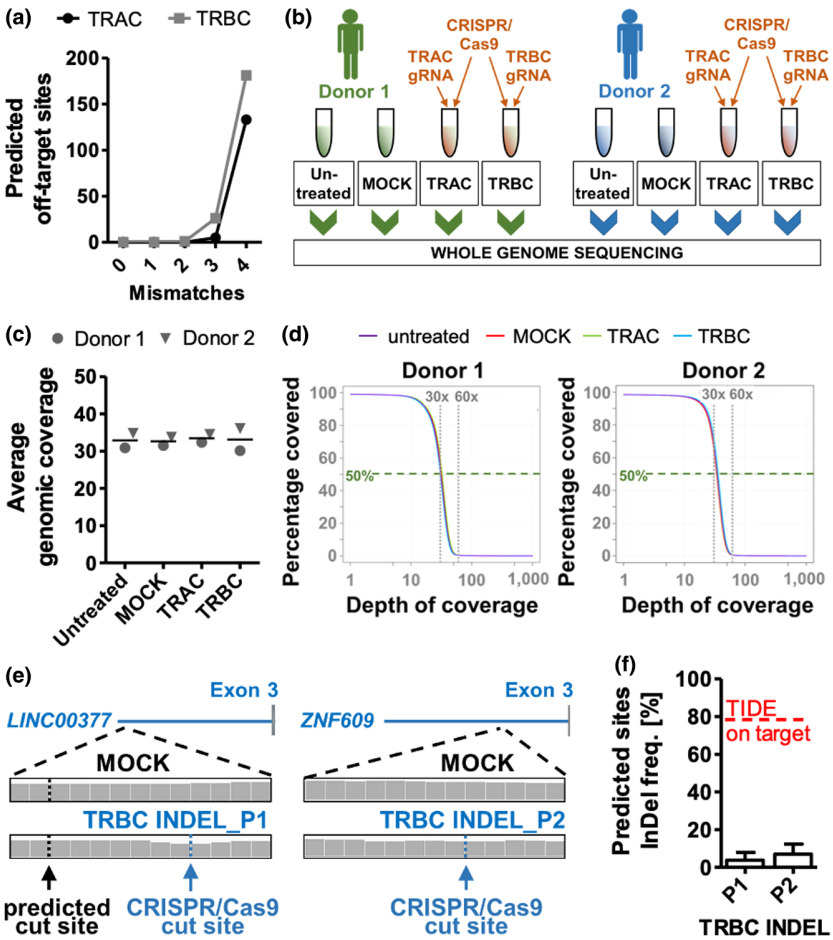

Figure 2. Predicted gRNA-dependent Cas9-induced off-target sites. **(a)** Numbers of Cas-OFFinder predicted gRNA-dependent off-target sites with up to 4 mismatches to the target sequence. **(b)** Samples subjected to whole-genome sequencing. **(c)** Depth of average genomic coverage (x-fold) including duplicated fragments. **(d)** Coverage plot showing base coverage distribution (including duplicated fragments) from high-quality aligned data of Donor 1 and Donor 2 samples. **(e)** Schematic *LINC00377* and *ZNF609* genes at CRISPR/Cas9 off-target site region. Read coverage in untreated, MOCK and *TRAC/TRBC* KO cells based on IGV exemplary shown for one donor. Identified CRISPR/Cas9 cut sites are indicated with blue dotted lines. Predicted cut site (if different from identified) is indicated with black dotted line. **(f)** Frequency of mutated reads among total reads at candidate off-target InDel sites. Mean ± SD of 2 donors.

Figure 2c). Figure 2d demonstrates the uniformity of coverage distribution of aligned data in all analysed samples. The samples of Donor 1 reached 30x coverage in 51–61% of the respective reference genome, and Donor 2 samples reached 30x coverage in 66–74%.

One of the *in silico* predicted off-target sites showed nuclease-induced candidate InDel mutation (TRBC INDEL_P2) upon Integrative Genomics Viewer (IGV) review. TRBC INDEL_P2 is located in the intronic region of *ZNF609* encoding a zinc finger protein (Figure 2e) and showed a mutated read frequency of 7% ± 3.8 (Figure 2f). Extending IGV-based mutation analysis to off-target regions up to 40 nucleotides up- and downstream of the predicted off-target site revealed a second candidate InDel in the *TRBC* sample (TRBC INDEL_P1). TRBC INDEL_P1 was located 7 bases downstream of the predicted locus, in the intronic region of non-coding RNA *LINC00377* (Figure 2e), and showed mutated read frequencies of 3.8% ± 2.9 (Figure 2f). However, neither candidate InDel TRBC INDEL_P1 nor candidate InDel TRBC INDEL P2 passed the quality filters for genome-wide variant calling (GATK).

Whole-genome analysis of gRNA-dependent Cas9-induced off-target effects

In order to extend CRISPR/Cas9-induced off-target identification from predicted sites to an unbiased analysis approach, sequencing data were analysed genome-wide. Starting from high numbers of InDel variants, various filtering steps were performed (Figure 3a). After filtering high-quality calls, InDels of untreated and MOCK control samples were subtracted from InDels in the CRISPR/Cas9-treated samples to identify mutations attributed to TCR genetic engineering. From 33,284 InDels in the *TRAC*-engineered sample and 31,692 InDels in the *TRBC* sample, 316 and 272 InDels were present in the respective samples of both donors (Figure 3c, Supplementary tables 3 and 4).

In order to exclude false positives because of agnostic filtering, candidate InDels were reviewed *via* Integrative Genomics Viewer (IGV) leading to

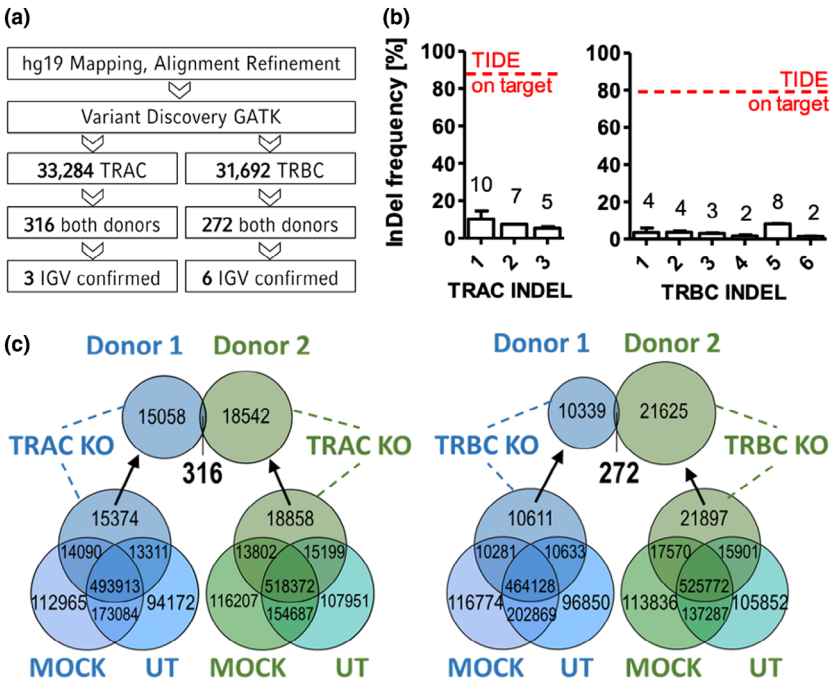

Figure 3. Whole-genome analysis of gRNA-dependent Cas9-induced off-target effects. **(a)** Workflow and InDel numbers after variant calling and filtering. After GATK variant discovery, InDels in control samples were subtracted from *TRAC* and *TRBC* KO samples. The respective TRAC and TRBC candidate InDels present in both donors were reviewed manually using IGV. **(b)** Frequency of mutated reads among total reads at the IGV-confirmed off-target sites. Mean ± SD of 2 donors. **(c)** Venn diagram of candidate CRISPR/Cas9-induced off-target InDels identified by subtraction of control sample InDels from CRISPR/Cas9-treated samples and overlapping InDels of both donors. UT = untreated control.

confirmation of 3 InDels in the *TRAC* sample (TRAC INDEL_1-3) and 6 InDels in the *TRBC* sample (TRBC INDEL_1-6). Frequencies of mutated reads for the confirmed InDels were ranging from 5.4% to 10.1% in the *TRAC* samples and 1.7% to 8.2% in the *TRBC* sample (Figure 3b). Of those 9 IGV-confirmed InDels, 3 were located in non-coding genomic regions and 2 in intronic regions of pseudogenes, whereas 4 InDels were located in introns of the coding genes *MAP4*, *DLG2*, *DMGDH* or *WNK1* (Table 1). No common InDels have been observed in both *TRAC* and *TRBC* samples. The size of confirmed insertions varied from 1 to 3 bp and of confirmed deletions from 1 to 12 bp (Table 1); thus, no structural variants were detected. None of these potential off-target InDels have been predicted *in silico*.

Targeted deep sequencing of potential off-target events identified in whole-genome sequencing

In order to further evaluate potential low-frequency off-target events identified by whole-genome sequencing, the respective loci were PCR-amplified and subjected to targeted deep sequencing (Figure 4a). The quality control parameter high-quality bases (Q30) showed stable values ranging from 82% to 92% in all samples (Figure 4b). GC content and mean read length showed minor amplicon-dependent variations from 34% to 47% and from 250 to 287 bp, respectively (Figure 4b).

For technical reasons because of 2 highly repetitive microsatellite and poly(T) regions (TRAC INDEL_3 and TRBC INDEL_2), targeted deep sequencing results from 7 of 9 loci were eligible for further off-target analysis. Targeted deep sequencing revealed that *TRAC*/*TRBC* knockout samples did not show any mutations in all 3 donors at the pre-identified genomic positions of 4 loci (TRAC INDEL_1, TRBC INDEL_4, TRBC INDEL_5 and TRBC INDEL_6; Supplementary table 5). Knockout independent InDel mutations have been detected at TRBC INDEL_1 locus in 2 of 3 donors and at TRBC INDEL_3 locus in 3 of 3 donors, InDel mutation frequencies being not significantly different in *TRAC*/*TRBC* knockout samples and MOCK controls (Figure 4c). Whole-genome sequencing showed a 6AT deletion at TRBC INDEL_1 locus, while targeted deep sequencing additionally revealed 5AT and 7AT deletions at lower frequencies (Supplementary table 5). At TRBC INDEL_3 locus, whole-genome sequencing showed a GC insertion and targeted deep sequencing additionally revealed GCAC and GCACAC insertions at lower frequencies (Supplementary table 5). TRAC INDEL_2 locus showed a low-frequency SNP (8.64%) in one donor (Supplementary table 5).

Influence of electroporation procedure on the genome's mutation frequency

In order to assess whether the electroporation procedure itself induces genetic mutations in

Table 1. Summary of WGS-identified confirmed off-target InDels

Name	Chromosome	Position	Ref	Alt	Mutation	Size	Region	Gene	Function
TRAC									
TRACINDEL_1	3	48121439	C	CA	Insertion	1	Intron	*MAP4*	Microtubule-associated protein
TRACINDEL_2	5	161864874	G	GT	Insertion	1	–	–	–
TRACINDEL_3	11	84145161	CA	C	Deletion	1	Intron	*DLG2*	Membrane-associated guanylate kinase
TRBC									
TRBCINDEL_1	1	148187613	CATATATATATAT	C	Deletion	12	–	–	–
TRBCINDEL_2	5	78433265	T	TAC	Insertion	2	Intron	*DMGDH*	Catabolism of choline
TRBCINDEL_3	6	67152308	A	AGC	Insertion	2	–	–	–
TRBCINDEL_4	7	57660861	ACG	A	Deletion	2	Intron	pseudogene	–
TRBCINDEL_5	12	913582	G	GT	Insertion	1	Intron	*WNK1*	Controlling the transport of sodium and chloride ions
TRBCINDEL_6	16	33848983	CTCT	C	Deletion	3	Intron	pseudogene	–

Name = identified mutation, chromosome and position of affected chromosome, Ref = reference (hg19) sequence, Alt = altered (identified) sequence, mutation and mutation size (nucleotides), region, gene name and function at the altered locus. WGS = whole-genome sequencing.

general, SNP, insertion and deletion frequencies were compared between untreated samples and MOCK-electroporated samples. The untreated samples showed a mean of 4.1×10^6 SNPs, 3.8×10^5 insertions and 3.9×10^5 deletions compared with human reference genome hg19, which was not significantly influenced by electroporation ($P = 0.94$, $P = 0.28$ and $P = 0.34$, respectively; Figure 4d). Thus, the total number of genetic mutations did not differ between untreated and MOCK-electroporated sample ($P = 0.78$). Further, comparison of the insertion and deletion size leads to a non-significant reduction ($P = 0.08$) in maximum deletion size in the MOCK-electroporated sample (313 vs. 218 bp; Figure 4e).

DISCUSSION

Genetic engineering of the T-cell receptor (TCR) redirects specificity and ablates alloreactivity, thereby bringing significant progress and off-the-shelf options to emerging adoptive T-cell transfer (ACT) approaches. Targeted CRISPR/Cas9-mediated manipulation revolutionised genetic engineering approaches, but raises legitimate and serious concerns regarding their safety profile to exclude additional unwanted Cas9 off-target activity when used in humans.[17]

Studies analysing Cas9 off-target activity in a broad range of organisms and with various gRNAs have already been published.[17] However, off-target activity is dependent not only on the sequence similarity of on- and off-target sites but also on the target cell type, the method used to deliver the gRNA/nuclease and the variant of the nuclease.[17] Further, identification of off-target activity is dependent on the method used for its detection.[13] Various methods have been developed during the last years: Off-target detection by biased methods, such as targeted deep sequencing, is restricted to pre-selected sites, whereas unbiased approaches, such as GUIDE-Seq and whole-genome sequencing, allow detection of off-target sites anywhere in the genome.[13] Biased targeted deep sequencing of predicted sites is on the one hand fast and widely available, but on the other hand depends on the reliability of *in silico* off-target prediction tools.[18] GUIDE-Seq is an unbiased method to detect off-target effects with InDel frequencies as low as 0.1%.[19] However, through the dependency on the incorporation of its tags into break sites, it is dependent on various factors, such as efficiency of tag transfection.[20] Furthermore, this method can only detect double-strand breaks present at the time of labelling, and thus may miss earlier breaks that have already been repaired.[18] Whole-genome sequencing, as an unbiased approach, does not only detect off-target sites at expected and unexpected sites but is also capable of identifying other than InDel mutations, such as structural variants of up to 800 bp in size.[13,20,21] Our analysis showed that CRISPR/Cas9 engineering did not lead to any off-target structural variants, the mutation biggest in size passing variant calling was a deletion of 12 bp. Because of its disadvantage of high volume, data on unbiased whole-genome analyses of Cas9 off-target events in primary human T cells are rare and such data of T-cell products as a whole or analysed by combinatorial approaches of whole-genome sequencing/targeted deep sequencing are lacking. Thus, the present study aimed to assess the safety of *TRAC*- and *TRBC*-engineered T-cell products in terms of CRISPR/Cas9-induced off-targets and their frequencies among bulk T cells by whole-genome sequencing and subsequent targeted deep sequencing of whole-genome sequencing-identified potential off-target events.

Whole-genome sequencing, variant analyses and quality filtering revealed low off-target activity with 3 low-frequency InDels for *TRAC* gRNA and 6 low-frequency InDels for *TRBC* gRNA in the present study. Knipping *et al*. performed a similar approach delivering Cas9/gRNA-expressing plasmids to a cultured human erythroleukaemic cell line (K562) to knock out the T-cell receptor.[14] Subsequently, tagging double-strand breaks genome-wide by linear double-stranded integrase-defective lentiviral vectors (IDLV) was used to identify nuclease off-target sites in an unbiased approach.[14] Because of the differences in gRNAs, target cell type, delivery of gRNA/nuclease and off-target detection method, it is not surprising that the off-target sites detected by Knipping *et al*. were different compared with those identified in the present study. However, with 1 identified off-target site for a *TRBC* gRNA with high 'quality score' and 24 for a *TRBC* gRNA with low 'quality score', numbers of identified off-targets were in the same range as in the present study.[14]

Because of the putative technical limitation of whole-genome sequencing in terms of evaluation of rare events,[22] amplicons of the whole-genome

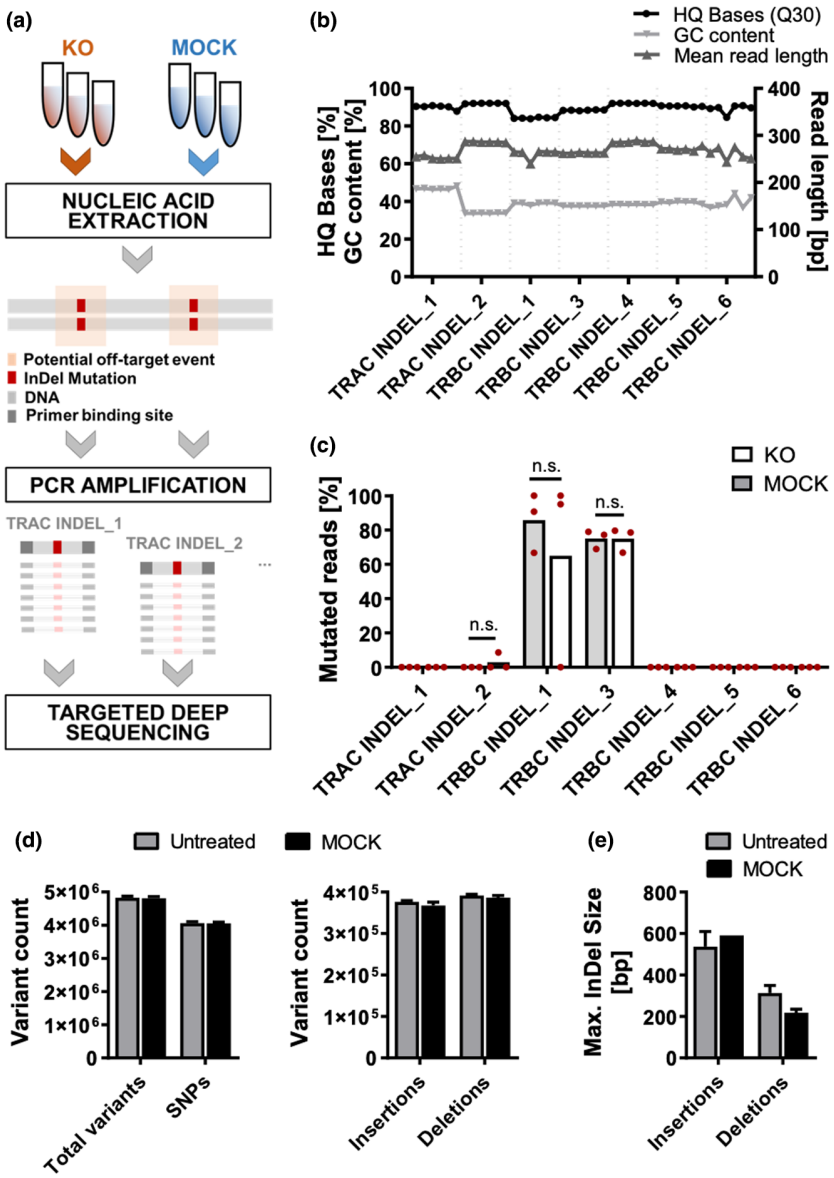

Figure 4. Targeted deep sequencing of WGS-pre-identified off-target events. **(a)** Whole-genome sequencing (WGS)-pre-identified off-target sites of three healthy donors were amplified with PCR and subjected to next-generation sequencing. **(b)** Sequence Quality overview at 7 pre-identified InDels. HQ bases (Q30) shows the percentage of high-quality bases having at least Phred quality 30. GC Content: GC content in percentile of high-quality sequencing. Mean read length (bp): average read length in bp of high-quality sequencing reads. **(c)** Mutated read frequency in untreated (MOCK) and *TRAC*/*TRBC* knockout samples (KO) in per cent (%). Mean of $n = 3$. Dots represent individual donors. n.s. = not significant. **(d)** Numbers of total variants, single nucleotide polymorphisms (SNPs), insertions and deletions in untreated and MOCK-electroporated samples. Mean ± SD of 2 donors. **(e)** Maximum size of insertions and deletions in untreated and MOCK-electroporated samples. Mean ± SD of 2 donors.

sequencing-identified low-frequency off-target sites were additionally subjected to targeted deep sequencing. Off-target events that have been pre-identified in whole-genome sequencing and variant calling may be because of technical artefacts. Actually, higher depth in targeted deep sequencing analysis revealed that 7 of 7 pre-identified off-target mutations were either not detectable or also detectable in the non-engineered controls. Two loci consisted of highly repetitive microsatellite and poly (T) regions. Therefore, it was technically not possible to PCR-amplify or deep sequence these genomic regions. Hence, our combinatorial approach using whole-genome sequencing, variant calling and

targeted deep sequencing confirmed that no CRISPR/Cas9-mediated off-target events were detectable. For evaluation of *in silico* predictions versus experimental confirmation of gRNA-specific off-target cleavage, using a positive control gRNA with known off-target sites will be an added value and provide additional proof of concept for the whole-genome sequencing approach.

In conclusion, the present study assesses the safety of the TCR knockout T cell in terms of nuclease-induced off-target events. In a combinatorial approach using biased target prediction and unbiased whole-genome sequencing followed by targeted deep sequencing, the study confirmed high CRISPR/Cas9-mediated TCR knockout efficiency without any confirmed off-target InDel frequencies. Still, further *in vivo* investigations are needed to draw conclusions about the clinical safety of the genetically engineered T-cell product.

METHODS

CRISPR/Cas9-mediated TCR knockout

Target-specific crRNAs 5′-GAGAATCAAAATCGGTGAAT-3′ *TRAC* crRNA[23] and 5′-GGAGAATGACGAGTGGACCC-3′ *TRBC* crRNA[24] were mixed with universal tracrRNA according to the manufacturer's instructions (Integrated DNA Technologies). Cas9 and Electroporation Enhancer (both Integrated DNA Technologies) were added according to the manufacturer's instructions. CD3/CD28 Dynabeads were magnetically removed from activated T cells prior to electroporation. Cells were electroporated with RNP complex in Buffer M[25] using pulse code T-023 in an Amaxa Nucleofector IIb (Lonza). MOCK control cells were electroporated without CRISPR/Cas9 reagents. After electroporation, all cells were cultured in TexMACS medium supplemented with human AB serum, interleukin-7 and interleukin-15.

Determination of TCR knockout efficiency

For phenotypic determination of TCR knockout efficiency, CRISPR/Cas9-engineered T cells were stained with eFluor 780 for life/dead discrimination, CD56, CD20, CD14, CD8, CD4 and human TCR α/β (Biolegend, San Diego, CA, USA). Subsequently, the cells were analysed by flow cytometry.

For genotypic investigation of TCR knockout, PCR from genomic DNA was performed using the following primers:

TRAC fwd 5′-ATCACGAGCAGCTGGTTTCT-3′,

TRAC rev 5′-CCCGTGTCATTCTCTGGACT-3′; and

TRBC fwd 5′-TACCAGGACCAGACAGCTCTTAGA-3′,

TRBC rev 5′-TCTCACCTAATCTCCTCCAGGCAT-3′.

TRBC primers were adapted from Ren *et al*. Sanger-sequenced PCR products (Eurofins Scientific, Luxemburg) were further analysed using TIDE software as described elsewhere.[26]

Functional knockout confirmation

Genetically engineered T cells were stimulated with Staphylococcal enterotoxin B (SEB) for 6h. Brefeldin A (Sigma-Aldrich®, Merck KG) was added for 4 h. CD8, CD4 and CD3 surface markers were stained, and Viobility Dye was used for live/dead discrimination (all reagents from Miltenyi Biotec, Bergisch Gladbach). Subsequently, cells were fixated and permeabilised using Cytofix/Cytoperm (BD Biosciences, Franklin Lakes, NJ, USA) and intracellularly stained with IFN-γ–APC (BD) for flow cytometric analysis.

Whole-genome sequencing

PBMCs were isolated from peripheral blood of male and female healthy donors ($n = 2$) after informed consent. Ethics approval was obtained from the local Research Ethics Committee, and the study was performed in accordance with the Declaration of Helsinki. Four days after electroporation with *TRAC* or *TRBC* gRNA, respectively, genomic DNA was isolated (QIAamp DNA Mini Kit, Qiagen) and sequencing was performed on the Genome Sequencer Illumina HiSeq platform by GATC Biotech.

Whole-genome analysis of Cas9 off-target sites

Reads were mapped to the reference genome hg19 using BWA v0.7.15. PCR duplicates from amplification within library preparation were removed using Picard v1.131 (http://picard.sourceforge.net). InDel calling was performed using Genome Analysis Toolkit (GATK)'s HaplotypeCaller v3.7. Parameters for variant calling were set as follows: QD < 2.0, FS > 200.0, MQ < -12.5; MQRankSum -12.5, LowCov ≤ 20; HaplotypeFilter > 13.0 and ReadPosFilter < -20.0; ReadPosRankSum < -20.0; and $15 <$ DP < 100. Variants detected were annotated based on their gene context using snpEff V4.3. GATK's VariantAnnotator module was used to evaluate the quality of variants based on several metrics. GATK-identified variants that were identically reported in both donors, but neither in untreated control nor in MOCK control samples, were defined as candidate off-target events. In order to remove false positives, candidate off-target sites were manually reviewed using Integrative Genomics Viewer.[27] If variants were confirmed by IGV to appear in CRISPR/Cas9-treated sample of both donors, but neither in the untreated control nor in MOCK control samples, these were defined as confirmed variants.

Analysis of predicted Cas9 off-target sites

Off-target events were predicted using Cas-OFFinder.[28] Parameters used for subsequent GATK analysis were the same as for whole-genome analysis of Cas9 off-target sites (see above). Because of the limited numbers of Cas-OFFinder predicted off-target sites, all of those were

analysed by Integrative Genomics Viewer (IGV[27]). During IGV review, sequences of knockout and control cell samples were compared. IGV review defined candidate InDels as those that appeared only in sequences of the respective CRISPR/Cas9-treated cells in both donors, but not in MOCK or untreated cells.

Analysis of electroporation-dependent increase in mutation rate

Reads were mapped to the reference genome hg19 using BWA V0.7.15, and PCR duplicates from amplification within library preparation were removed using Picard V1.131 (http://picard.sourceforge.net) as for whole-genome analysis of Cas9 off-target sites. The SNP and InDel calling was done using GATK's Haplotype Caller according to McKenna *et al*. and DePriso *et al*.[29,30]

Targeted deep sequencing

After electroporation with *TRAC* and *TRBC* gRNA, samples from 3 additional healthy donors were prepared as described above. Potential off-target loci (identified in WGS) were amplified by PCR using the following primer pairs:

TRAC INDEL_1 fwd: 5′-TGACACAGCAAGACTCCATC-3′,
rev: 5′-CCACCTTTTTCTTTTCCACAC-3′;
TRAC INDEL_2 fwd: 5′-TTTAGACATGAAGTCCTTGCC-3′,
rev: 5′-ACTGAGCACAGATTATCACAAC-3′;
TRAC INDEL_3 fwd: 5′-AGCAATGAGTACAAATGCCAACG-3′,
rev: 5′-CCTTGTGTCTCTAGCGCCTT-3′;
TRBC INDEL_1 fwd: 5′-TCTCCCGCTTGCTCTATAC-3′,
rev: 5′-TGATGCCTTGACCTCACAC-3′;
TRBC INDEL_3 fwd: 5′-GGCCTGTACCACTGCTTCAA-3′,
rev: 5′-TGGAGAGCCAGAAGAACTCC-3′;
TRBC INDEL_4 fwd: 5′-GCAACAAGAGTGAAACCCC-3′,
rev: 5′-CATAGATGAAAGAGTTCCCATGAAG-3′;
TRBC INDEL_5 fwd: 5′-AGTAACATCTCCCTGCCTCC-3′,
rev: 5′-AGCCTGTAATCTCAGCTACTC-3′; and
TRBC INDEL_6 fwd: 5′-GCACACACAGAAGTCATGAG-3′,
rev: 5′-GCGATGAAGAATTACAGCACC-3′.

Amplicons were generated by using Q5 High-Fidelity DNA Polymerase (TRAC INDELS_1-3 and TRBC INDELS_2-6) and Phusion DNA Polymerase (TRBC INDEL_1) according to the manufacturer's instructions (both from New England BioLabs, Ipswich, MA, USA).

Targeted deep sequencing was carried out by Eurofins Genomics Europe Sequencing GmbH. Therefore, integrity and quantity of the starting material were determined followed by an indexing PCR with flow cell adapters. After a bead-based purification and an additional Quality Control check, deep sequencing was performed on the Illumina MiSeq platform using 2x300 bp Sequence mode. High-quality sequence reads were aligned to the reference genome hg19 using BWA with default parameters 22. Variant calling was done using GATK, and variants were annotated using GATK's Variant Annotator module. Following filters were applied for single nucleotide variant sites: QD < 2.0, LowCovFilter ≤ 20, MQ < 40, FSFilter > 60.0, HaplotypeFilter > 13.0, ReadPosFilter < −8.0 and MQRankSum Low < −12.5. Filter applied for Insertion/Deletion variant sites was as follows: QD < 2.0, ReadPosFilter < −20.0 and FSFilter > 200.0.

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CONFLICT OF INTERESTS

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTION

Theresa Kaeuferle: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing. **Tanja A Stief:** Formal analysis; Investigation; Methodology; Validation; Visualization. **Stefan Canzar:** Formal analysis; Investigation; Methodology; Software; Validation; Visualization. **Nayad Natali Kutlu:** Data curation; Investigation; Visualization; Writing – review & editing. **Semjon Willier:** Formal analysis; Methodology; Validation. **Dana Stenger:** Investigation; Validation. **Paulina Ferrada-Ernst:** Investigation. **Nicola Habjan:** Investigation; Methodology. **Annika Peters:** Formal analysis; Investigation. **Dirk H Busch:** Conceptualization; Formal analysis; Methodology; Supervision. **Tobias Feuchtinger:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Visualization; Writing – original draft; Writing – review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.