Optimized two-step electroporation process to achieve efficient nonviral-mediated gene insertion into primary T cells

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The development of gene editing technologies over the past years has allowed the precise and efficient insertion of transgenes into the genome of various cell types. Knock-in approaches using homology-directed repair and designer nucleases often rely on viral vectors, which can considerably impact the manufacturing cost and timeline of gene-edited therapeutic products. An attractive alternative would be to use naked DNA as a repair template. However, such a strategy faces challenges such as cytotoxicity from double-stranded DNA (dsDNA) to primary cells. Here, we sought to study the kinetics of transcription activator-like effector nuclease (TALEN)-mediated gene editing in primary T cells to improve nonviral gene knock-in. Harnessing this knowledge, we developed a rapid and efficient gene insertion strategy based on either short single-stranded oligonucleotides or large (2 Kb) linear naked dsDNA sequences. We demonstrated that a time-controlled two-step transfection protocol can substantially improve the efficiency of nonviral transgene integration in primary T cells. Using this approach, we achieved modification of up to ~30% of T cells when inserting a chimeric antigen receptor (CAR) at the T-cell receptor alpha constant region (TRAC) locus to generate ‘off-the shelf’ CAR-T cells.

Cell-based therapies have emerged in the past few years as a very successful approach to treat cancer and rare diseases. CAR-T therapy, a targeted cellular immunotherapy that uses engineered T cells to specifically eliminate antigen-bearing tumor cells, has been in the spotlight achieving remarkable results in both preclinical and clinical applications for a variety of tumors [1]. Many methodologies have since been developed to optimize the performance of chimeric antigen receptor-expressing T cell (CAR-T cells), such as removing the T-cell receptor (TCR) from healthy donor cells to generate universal CAR-T cells [2]. Because universal CAR-T cells are engineered out of third-party healthy donor T cells, donors can be carefully chosen for potency and cells can be manufactured, formulated, and systematically quality controlled before being adoptively transferred to multiple patients in allogeneic settings. In addition, novel
attributes are in test to enhance and control CAR-T-cell function [3,4]. These novel approaches rely on our ability to efficiently and precisely control gene expression. Several strategies have been developed to allow for specific gene manipulations in tissue culture cells, colloquially known as ‘genome editing’. These strategies involve the use of molecular tools such as designer nuclease and base editors [5]. In particular, designer nucleases, including meganuclease, zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR) nucleases can be engineered to create a double-stranded break (DSB) at specific genomic target sequences [6].

In mammalian cells, two major pathways exist to repair DSBs—nonhomologous end-joining (NHEJ) and homology-directed repair (HDR) [7]. During NHEJ repair, the rejoining of broken DNA ends, using little or no sequence homology, involves the processing of ends such that nucleotides could be deleted or inserted at the break site prior to ligation and hence potentially create mutations and disruptions to the locus [8]. Scientists have long harnessed this process to create gene knock-outs (KO) of functional genes in various cell types [9] including primary cells such as human T cells [10]. In contrast, the repair of a DSB through HDR requires substantial lengths of sequence homology so that a DNA end from one molecule can invade an homologous sequence and prime repair synthesis [8]. The HDR pathway has been used to introduce (‘Knock-In’, KI) exogenous sequences at particular genomic loci allowing reprogramming of cellular functions for therapeutic applications. Viral vectors such as adeno-associated viruses (AAV) are widely used for many applications to deliver repair templates [3,11,12]. However, in the current GMP manufacturing landscape, the time and cost of AAV vectorization efficiencies and toxicity [14–16], especially in primary cells [17]. To mitigate these limitations, several strategies such as incorporating chemical modifications into the DNA repair templates to prolong half-life [18], increasing homology arm length [19], and manipulating cell cycle [20] have been investigated.

Here, we show that a better understanding of the cleavage kinetic of designer nucleases (demonstrated for TALEN) can help improve nuclease-mediated gene knock-in when using naked DNA as repair template. We found that the TALEN protein expression peaked around 20 h post-transfection and diminished after 48 h. Concomitantly, unlinked DNA DSBs accumulated until around 20 h post-TALEN transfection.

In a separate experiment by western blot analysis. Our findings showed that the TALEN protein accumulation, we sought to deliver naked DNA repair templates 20 h post-TALEN transfection: the time when maximum number of cells have unlinked DSBs and are susceptible to DNA repair. We showed that this timed delivery of the DNA repair template (16- to 20-h delay) is a simple and robust strategy to enhance nuclease-based genome engineering, allowing substantial increase of targeted insertion rates of a short sequence (20 bp) as well as a large (2.3 kb) sequence containing a chimeric antigen receptor. We achieved up to 46% and 29% of targeted gene insertion, respectively. We demonstrate that this two-step transfection strategy provides a flexible, efficient, rapid, and cost-effective nonviral avenue to improve targeted genome reprograming of universal CAR-T cells.

Results and Discussion

Kinetics of TALEN expression and DSB creation

To understand the kinetics of TALEN expression, we transfected into primary human T cells an mRNA encoding a TALEN (TRAC TALEN) that targets the first exon of the T-cell receptor alpha constant region (TRAC) gene [21]. The TALEN mRNA was vectorized using the BTX PulseAgile electroporation system, which was previously optimized for efficient delivery (gene editing frequencies and cell viability) of mRNA into primary T cells [3,21–23]. Transfected cells were harvested at different timepoints up to 24 h postelectroporation, and the total mRNA was extracted for RNA-seq analysis (Fig. 1A). Our results showed that the TALEN mRNA level gradually declined over time, reaching an average of 31% of its initial amount 24 h postelectroporation (Fig. 1B). The TALEN protein, produced from the transfected mRNA, was monitored in a separate experiment by western blot analysis. Our findings showed that the TALEN protein was detectable 4 h postelectroporation, accumulated until 20 h, and declined below detectable level at 48 h (Fig. 1C).

To quantitate the level of genomic DNA cleavage in response to the TALEN protein accumulation, we designed a qPCR assay to assess the relative abundance of intact genomic DNA at the TRAC TALEN target site. For this purpose, two sets of primer pair were used: The first set was designed to amplify a
164 bp amplicon encompassing a region from −102 to +62 bp (‘cross’ amplicon) across the TRAC TALEN cutting site which is defined as the center of the targeted sequence. For internal control, the second set was targeted to amplify a sequence from −292 to −120 bp (‘upstream’ amplicon) upstream of the TRAC TALEN cutting site (Fig. 1D) [24]. We expected the cross amplicon to inversely correlate with the abundance of TALEN-driven unlinked DNA ends. Following TALEN mRNA electroporation, our data showed that the relative level of ‘cross’ amplicon decreased constantly, when normalized to the ‘upstream’ amplicon. The relative amount of ‘cross’ amplicon reached its lowest level 20 h postelectroporation (Fig. 1E), indicating that unlinked DSBs were most abundant 20 h post-TALEN mRNA delivery. This finding was confirmed using a second TALEN targeting the first exon of the beta-2 microglobulin gene (B2M TALEN). As previously, we quantified the abundance of a ‘cross’ amplicon that extended from −48 to +48 bp across the B2M TALEN cutting site and compared it to the abundance of a ‘downstream’ amplicon that spans from +112 to +323 bp downstream of the cut site. In accordance with the results obtained with the TRAC TALEN, this second data set showed that the largest portion of alleles harboring unlinked DNA ends at the TALEN target site also peaked at 20 h postelectroporation (Fig. 1F). As expected, the time point at which unlinked DSBs reached its highest proportion corresponded to the time when TALEN

Fig. 1. Kinetics of TALEN expression and DSB creation. (A) T-cell activation and transfection protocol. Primary human PBMCs were thawed 1 day before CD3/CD28 bead activation. Three days postactivation, cells were de-beaded and transfected with TALEN mRNA. The cells were then cultured for various length of time before being harvested for mRNA, DNA, or protein analysis. (B) Relative abundance of TRAC TALEN mRNA at different time points (15 min (0), 4, 16, 20, and 24 h) post-transfection. The total RNA was extracted from cells from three different donors and subject to NGS-based quantitation (N = 3 independent donors, 3 technical replicates, mean and SD). (C) The total cell lysate was extracted from nontransfected or TALEN mRNA-transfected cells harvested at different time points. The cell lysate was resolved by SDS/PAGE and blotted with an an-RVD antibody, which detects the expression of the TALEN protein, or an actin antibody for loading control. (D) Scheme of qPCR strategy to measure unlinked DSBs created by the TALEN. (E) Fold change of unlinked DSBs at the TRAC TALEN target site (N = 3 independent donors, mean and SD). (F) Fold change of unlinked DSB at the B2M TALEN target site (N = 3 independent donors, mean and SD).
protein level peaked, showing the DNA cleavage event is directly related to the level of TALEN protein expression.

**Indel creation kinetics and Indel signature**

To better understand the different steps of TALEN genome editing, we then decided to further investigate the DNA repair events following the genomic DNA cleavage. Genomic DNA DSBs are mainly repaired through either the NHEJ or HDR pathways, although several others have also been described [5]. The NHEJ pathway, which could lead to the introduction of small insertions or deletions (indels), is usually favored over the more precise HDR pathway [25], especially when no exogenous HDR template is provided [26]. To further characterize the indel creation kinetics at the TALEN-induced DSB sites (TRAC and B2M loci), we extracted genomic DNA from transfected cells at various timepoints up to 72 h post-TALEN mRNA transfection. A region of 300-bp across the TALEN target sites was amplified by PCR, and the resulting amplicons were subjected to next-generation sequencing (NGS) to determine the percentage of indels. The results showed a rapid accumulation of indels over time, indicating that DSBs were created and eventually repaired with mutations (Fig. 2A,B). The indel frequencies reached plateau at 48 h post-TALEN electroporation. For the TRAC TALEN, the plateau of indels frequency was 89.2% on average (SD: 3.2%), and for the B2M TALEN, the average was 89.0% (SD: 6.2%). The time to reach the indel plateau was in line with the kinetics of TALEN protein expression which fell below the detectable limit 48 h post-transfection.

To further characterize the outcome of genomic DNA cleavage by the TALEN, we examined the pattern of deletion sizes over time. Our data showed that at early timepoints (< 8 h), with both TRAC and B2M TALEN, small deletions (< 5 bp) were the major species. At later time points, the abundance of small deletion decreased, and larger deletions started to accumulate (Fig. 2C,D). We have previously shown that, with the TALEN scaffold used in the current study, the TALEN activity was optimal on targets having 15 bp spacers (nucleotide sequence between the two DNA binding sequences of the two TALEN arms). The cleavage activity was strongly reduced when the spacer length decreases below 10 bp [27]. Thus, large deletions are likely to be a result of successive cleavage-ligation cycles until the deletions are large enough to severely impact the TALEN cleavage. As expected, the indel size profile was stable between the 48-h and 72-h time points (Fig. 2C,D) which correlates with the disappearance of the TALEN protein 48 h post-mRNA delivery (Fig. 1C). Interestingly, we also observed enrichment of specific deletion sizes in the TRAC TALEN (6, 12, 24, and 34 bp) and B2M TALEN (2, 9, 11, and 34 bp)-treated samples. Detailed analysis of the genomic sequences flanking the deletions identified putative microhomology sequences (Fig. 2C,D and Table 1). These biases in deletion patterns were likely the outcome of the repair via the microhomology-mediated end-joining (MMEJ) mechanism [28].

**Optimized gene modification with ssODN**

In addition to NHEJ-mediated DNA repair, DSBs can also be repaired via the HDR pathway. Cells can incorporate an exogenous sequence at DSB sites, especially when provided with DNA repair templates harboring homologies to the flanking sequences of the DSB sites. Single- and double-stranded oligodeoxynucleotides (ssODN and dsODN) can be used as repair templates to introduce sequences into a genome [29]. Compared to double-stranded DNA (dsDNA), ssODNs are less toxic and less inclined to insert randomly into the genome [30]. The target insertion of a short DNA sequence can be used to correct mutated genes [1] or to introduce stop codons [31] and epitope tags [24].

However, ssODNs have been shown to have a short (1.5 h) in cellulo half-life postelectroporation [32], limiting their temporal availability to direct precise insertion via HDR. To overcome this limitation, we hypothesized that the optimal timing to deliver an ssODN repair template would be around the time when most of the cells have nuclease-driven unlinked DNA breaks available for DNA repair.

To test this hypothesis, we used a 170 bp ssODN, containing a 20 bp exogenous sequence designed to insert a premature stop codon within the TRAC gene (at the TALEN cut site). The 20 bp sequence was flanked by 70 bp sequences homologous to the TRAC locus (homology arms, Fig. 3A). Following the TALEN mRNA transfection, cells were incubated for different lengths of time (0 h (15 min), 3, 6, 16, 20, and 24 h) before a second transfection for ssODN delivery (200 pmol ssODN per million cells). The ssODN was delivered with a second electroporator using a program adapted for DNA delivery [14]. Cells were harvested at Day 3 post-TALEN transfection for NGS analysis to quantify ssODN insertion frequencies at the TRAC locus.

The results showed that the insertion frequency of 20 bp sequence significantly increased as we delayed the ssODN transfection (~3-fold, $P = 0.0079$ for 0 vs
16 h). We observed the maximum integration frequency (30% to 46%, mean = 40%, SD = 9%) at 16 h post-TALEN electroporation (Fig. 3B,C). This observation confirmed our hypothesis that a timed delivery of the ssODN repair template can improve target insertion frequencies.

**Efficient nonviral CAR-T-cell engineering**

Because the length of ssODN (typically < 200 bp) limits its potential for large cDNA insertions, we explored whether our previous finding could be applied to the delivery of large (2.3 kb) coding gene (dsDNA). Toward this end, we designed a 2.3 kb dsDNA repair template aiming to insert a chimeric antigen receptor (anti-CD22CAR) [3] at the TRAC TALEN target site. The CAR dsDNA repair template was designed to contain a ‘left’ TRAC homology arm (300 bp), a self-cleaving P2A peptide coding sequence fused to a cDNA (1.4 kb) encoding the chimeric antigen receptor followed by a bovine growth hormone polyadenylation (BGH PolyA) signal (200 bp) and a ‘right’ TRAC homology arm (300 bp). When appropriately integrated at the TRAC locus, this promotorless DNA cassette will lead to the surface expression of a CD22CAR under the control of the endogenous TRAC gene regulatory elements (Fig. 4A). The DNA repair template was prepared by PCR amplification.

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![Fig. 2](https://example.com/fig2.png)

Fig. 2. TALEN-mediated Indel creation kinetics and Indel signature. (A, B) Time course experiment showing gradual accumulation of indels. Cells transfected with either TRAC or B2M TALEN mRNA were harvested at different time points after transfection for gDNA extraction. The genomic regions of TALEN target sites (TRAC or B2M) were PCR amplified and subjected to NGS analysis. A sigmoid curve was fitted to the data to show the indel accumulation. The dotted line represents the 95% confidence interval (N = 3 independent donors, mean and SD). (C, D) Size of deletions over time. The abundance of deletions at TALEN target sites was determined by NGS. Diamond points indicate enrichment of deletions with particular sizes/sequences (Table 1).
from a plasmid encoding the desired construct, which produced the linear dsDNA repair template (2.3 kb).

We first examined the half-life of the dsDNA repair template in primary T cells. The cells, electroporated with the dsDNA, were harvested at different time points, and the abundance of the dsDNA was determined by qPCR. Consistent with observation from other groups [32], the qPCR analysis showed that the linear dsDNA template had a relatively short half-life (T1/2 = 54 mins) in cellulo (Fig. 4B). As the dsDNA repair template showed a similar half-life with reported half-life of ssODN [32], we hypothesized that delaying the transfection of the long dsDNA repair template would also improve the integration efficiency.

To test this hypothesis, we transfected 5E6 of cells with 2 µg (2.3 pmol) of dsDNA template encoding the CD22CAR at different time points post-TRAC TALEN mRNA transfection (Fig. 3B). The cells were then cultured for three more days before performing flow cytometry analysis to quantify the CD22CAR surface expression within the cell population. Our results demonstrated that transfecting the linear dsDNA repair template immediately after TALEN mRNA transfection led only to low frequencies of CAR surface expression (mean = 1.18%, SD = 0.49%; Fig. 4C). In contrast, the dsDNA transfection carried out 20 h post-TALEN electroporation produced the highest CD22CAR surface expression, with a CAR+
cell population reaching up to 29% (mean = 18.16%, SD = 7.3%). These results demonstrated that delaying the dsDNA repair template transfection by 20 h significantly improved CAR+ population frequency by 16-fold (SD = 4.5, P = 0.006; Fig. 4D) comparing to the 0-h time point. We further noticed that, as expected, CAR+ cells were not present within the cdT-cell population (Fig. S1) as the cdT cells do not express TCRA

Fig. 4. Two-step electroporation improved CAR-T cells engineering with dsDNA. (A) Scheme of the CD22CAR dsDNA repair template. (B) Half-life of dsDNA repair template. Cells transfected with the CD22CAR dsDNA (PCR product) were harvested at different time points. qPCR was performed to quantify the amount of CD22CAR dsDNA within the cells. Data are normalized to 0 h. The curve was fitted using one phase decay. Half-life = 54 min (N = 3 independent donors, mean and SD). (C) Total number of viable cells at Day 1 post-dsDNA transfection. In one set of experiments, transfections were performed for three donors (blue, red, and purple dots) for all time points (N = 3-7 independent donors, mean and SD). (D) Percentage of CD22CAR expressing cells in the total cell population. In one set of experiments, transfections were performed for three donors (blue, red, and purple dots) for all time points (N = 3 to 7 independent donors, mean and SD). (E) Fold change in CAR+ frequencies. CAR+ frequencies were normalized within donors to the 0 h (N = 3 independent donors, mean and SD). (F) Total viable cells at Day 3 post-dsDNA transfection (N = 3 independent donors, mean and SD). (G) Total number of CAR+ cells at Day 3 post-dsDNA transfection. The total numbers of the CAR+ cell were calculated by multiplying the total number of CAR+ cells at Day 3 by the percentage of CAR+ cells in respective samples (N = 3 independent donors, mean and SD). (H) Luciferase assay showing the tumor cell luminescence after 24 h of incubation with T cells (engineered T cells or parental cells). Single donor in technical triplicates (mean and SD; **P < 0.01, *P < 0.05, Student’s t-test).
gene and the CD22CAR cassette was targeted at the
TCRA locus and thus controlled by the expression of the
TCRA gene.

Since the electroporation process, as well as the
presence of exogenous polynucleotides (mRNA and
naked DNA), might affect the cell viability and
induce innate immune responses [29], we monitored
cell number, growth, and the release of key cytokines
postelectroporation. Day 1 post-DNA electroporation,
the 0-h samples maintained an average of 3.5E6
viable cells from the 5E6 starting cells (SD = 1.4E6;
Fig. 4E). In contrast, the 20-h samples displayed a
40% reduction of the number of viable cells, with a
mean of 2.1E6 viable cells (SD = 1.6E6). However,
the decrease was not statistically significant (P = 0.14,
0-h vs 20-h samples). At Day 3 post-DNA electropo-
ration, the number of viable cells in 0-h sample
recovered to an average of 13E6 (SD = 5.8E6)
representing a 3.7-fold proliferation from Day 1 (Fig. 4F).
The 20-h sample cells recovered to an average of
7.5E6 (SD = 5.2E6), demonstrating proliferating
capacities (3.5-fold from Day 1) similar to the 0-h
samples. However, because the KI frequencies in the
20-h delay sample were considerable higher (16-fold;
Fig. 4D) than for the 0-h sample, we obtained a sig-
nificant (P = 0.01) higher absolute number of CAR+
cells by delaying the CAR repair template transfec-
tion (0 h: 0.14E6, SD = 0.088E6; 20 h: 1.2E6
SD = 0.9E6; Fig. 4G).

In addition, at Day 2 post-dsDNA transfection, we
measured the release of cytokines (IL6, IL10, IFNγ,
TNF-α) from the cells undergoing the different trans-
fec tion steps. We observed a general trend toward an
increase in these cytokines with the different electropo-
rat ion steps and in presence of mRNA and dsDNA
(Fig. S2A). The secretion of TNF-α increased signifi-
cantly in the samples transfected with both the
TALEN mRNA and the dsDNA, compared to the
parental cells, suggesting that transfection steps indeed
trigger a cellular immune response.

Three days post-DNA electroporation, the samples
from the 20-h delayed dsDNA electroporation and the
corresponding parental cells (no electroporation, no
exogenous polynucleotides) were seeded in G-REX gas
permeable expansion devices for a large-scale cell
expansion. The cells were kept in G-Rex for 13 days
before harvest and cryopreservation. Edited cells from
two independent donors were thawed and further anal-
alyzed for two key characteristics: the T-cell differentia-
tion (CD62L and CD45RA) and the exhaustion
(LAG3 and PD1) marker profiles. We did not find major
differences between the parental cells and edited
cells (Fig. S2B,C).

In a separate experiment, we assessed the functional-
ity of cells edited with the 20-h delayed electropo-
ration. Engineered cells (13% CAR+ cells at the end of
13 days of expansion process) or their parental un-
engineered control were incubated for 24 h with
decreasing amounts of target Raji tumor cells (CD22+
and stably expressing luciferase). At the end of the co-
incubation, the remaining luciferase signal was used to
assess the tumor cell survival. The result demonstrated
that the engineered T cells promoted target cell killing,
when compared to the matching parental un-
engineered T cells (Fig. 4H).

It is also worthy to mention that in our experimental
conditions, ssODN and linear dsDNA do not require
the same delivery time post-TALEN mRNA electropo-
rat ion to achieve the best performance and do not
achieve similar integration frequencies. We assume that
these differences are probably due to the amounts of
DNA material (ssODN: 200 pm vs dsDNA: 2.3 pmol)
we could deliver without causing significant level of tox-
icity. We also noticed that for both, short ssODN and
long dsDNA, the integration frequencies declined
slightly at their latest delivery timepoint (24 h), consist-
ent with the decrease in TALEN protein which trans-
lates to lower DNA cleavage activity and thus reduced the
loci availability for integration.

Nuclease-driven targeted gene insertion offers great
promise to precisely reprogram human cells, especially
for adoptive cellular therapies. Within the last few
years, viral vectors such as adeno-associated virus
(AAV) have been used to generate engineered T cells
with a chimeric antigen receptor (CAR) genetically
encoded at the endogenous TRAC locus (45%–65% of
CAR+ cell within the population) [3,11,33]. More
recently, nonviral (naked DNA) reprograming of T
cells has been reported as an alternative to viral vec-
tors. Targeted (TRAC locus) replacement of the
endogenous TCR with an engineered TCR was
explored (~ 10% integration frequencies) [14,34,35].
Based on the understanding of nuclease-driven geno-
mic DNA cleavage and DSB repair kinetic parameters,
we showed that a two-step transfection process in
which the nuclease mRNA is introduced first into the
cells, followed by a second transfection (16–20 h later)
of the repair template allows to significantly improve
the nonviral targeted insertion of a short (20 bp, 3-
fold increase) and long (1.7 kb encoding a CAR, 16-
fold increase) sequence. Taken together, this study
describes a simple and robust protocol to enhance
nuclease-based gene integration efficiency. We envision
that this two-step procedure could also be adapted to
other designer nucleases such as ZFN or CRISPR/
Cas9. However, depending on the format of the
delivered nucleases (i.e., mRNA, RNP, or plasmid) and the kinetics of the nuclease expression and DSB creation, further optimization of the second transfection timing might be required. It was estimated in a Dox-inducible Cas9 expression system, that the DSB formation peaked around 17 h after induction of the nuclease expression [36], a timing close to what we found with TALEN mRNA transfections. We anticipate that the KI efficiency in such system would also benefit from a delayed vectorization of repair templates. In contrast, if the CRISPR/Cas9 is to be delivered as an RNP complex, which would not require the nuclease expression step, creation of the DSB might be faster and a shorter delay of the second transfection should be considered.

The target insertion of a CAR at the TRAC locus strategy would not produce CAR-expressing γδ T cells. The γδ T cells are thought to possess less allergenicity and more antitumor activity [37] and thus missing out on producing CAR γδ T cells can be a drawback. Targeting the CAR to another locus that is expressed in both αβ and γδ T cells could be a solution to this issue. Although the two-step transfection protocol was performed in this study on primary T cells for universal CAR-T-cell generation, we anticipate that this strategy is broadly applicable to various cell types. Additional work, such as the evaluation of the level of random integration events [14], will nevertheless be required to fully assess the potential of this strategy before clinical applications. Taken together, our results expand the possibilities of nuclease-mediated genome engineering in human primary cells and provide a foundation for further advances using nonviral gene editing methods.

Materials and methods

T-cell culture

Cryopreserved human PBMCs were acquired from ALLCELLS. PBMCs were cultured in X-vivo-15 media (Lonza Group, Basel, Switzerland), containing 20 ng·mL⁻¹ human IL-2 (Miltenyi Biotech, Somerville, MA, USA), and 5% human serum AB (Seralab, New York, NY, USA). Human T-cell activator CD3/CD28 Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) was used to activate T cells at 7 µL per million CD3+ cells.

TALEN mRNA

Plasmids encoding the TRAC and B2M TALEN were produced in-house. The TALEN mRNA was produced by in vitro transcription using the NEB HiScribe ARCA (NEB, Ipswich, MA, USA) kit. The sequences targeted by the two TALEN are the following (17-bp recognition sites, upper case letters, separated by a 15-bp spacer):

**TRAC:** (TTCTCTCTACTACCATGcgcctcactcTCTCTTTCTGCGG)

**B2M:** (TCCGTGGCCTTAGCTGTgcgcctcactcTCTCTTTCTGCGG)

ssODN repair template

A plasmid containing the CD22CAR repair template with sequences homologous to the TRAC target site was used as the PCR template [3]. Phosphorothioate-modified primers (IDT) were used to amplify the target region (Table S1). PCRs were performed using the PrimeSTAR Max Premix (TaKaRa, Mabuchi, Japan) system. In a 100 µL reaction, 40ng plasmid template and 4 µL of each of the 10 nM primers were used according to the manufacturer protocol. PCRs were set to 98 °C 10 s, 55 °C 5 s, 72 °C 12 s, 40 cycles. The PCR products were then purified with AMPure XP beads (1: 1 ratio; Beckman Coulter, Brea, CA, USA) and eluted into ddH2O.

Production of dsDNA repair template

A plasmid containing the CD22CAR repair template with sequences homologous to the TRAC target site was used as the PCR template [3]. Phosphorothioate-modified primers (IDT) were used to amplify the target region (Table S1). PCRs were performed using the PrimeSTAR Max Premix (TaKaRa, Mabuchi, Japan) system. In a 100 µL reaction, 40ng plasmid template and 4 µL of each of the 10 nM primers were used according to the manufacturer protocol. PCRs were set to 98 °C 10 s, 55 °C 5 s, 72 °C 12 s, 40 cycles. The PCR products were then purified with AMPure XP beads (1: 1 ratio; Beckman Coulter, Brea, CA, USA) and eluted into ddH2O.

TALEN electroportation

T cells activated with CD3/CD28 beads (Life Technologies/Thermo Fisher Scientific) for 3 days were passaged into fresh complete media containing 20 ng·mL⁻¹ human IL-2 (Miltenyi Biotech) and 5% human serum AB (Seralab) 10-12 h before transfection.

For TALEN mRNA transfection, the cells were first bead-dephased by magnetic separation with EasySep (Stemcell Technology, Cambridge, MA, USA), washed twice in Cytoperonation buffer T (BTX Harvard Apparatus, Holliston, MA, USA), and 5E6 cells were then resuspended in 180 µL of Cytoperonation buffer T. This cellular suspension was mixed with mRNA encoding TALEN at 1 µg mRNA per TALEN arm per million cells in a final volume of 200 µL. Transfection was performed using Pulse Agile technology by applying two 0.1 mS pulses at 3000 V·cm⁻¹ followed by four 0.2 mS pulses at 325 V·cm⁻¹ in 0.4-cm gap cuvettes. The electrooporated cells were then immediately transferred to a 12-well plate containing 2 mL of prewarmed X-vivo-15 complete media and allowed to recover at 37 °C for 15 min before transfer to 30 °C.

Target insertion with ssODN

For ssODN transfection, TALEN mRNA-transfected cells were incubated at 30 °C for various length of time (15 mins
(0, 4, 6, 16, 20, or 24 h) before harvesting. The harvested cells were washed once with warm PBS. 1E6 PBS-washed cells were pelleted and resuspended in 20 µL Lonza P3 primary cell buffer (Lonza). 200 pmol ssODN repair template was mixed with the cell, and then, the cell mixture was electroporated using the Lonza 4D-Nucleofector under the EO115 program for stimulated human T cells. After electroporation, 80 µL warm complete media was added to the cuvette to dilute the electroporation buffer, and the mixture was then carefully transferred to 400 µL prewarmed complete media in 48-well plates. Finally, 5 unit·mL⁻¹ benzonase (Sigma, St. Louis, MO, USA) was added to the cell culture to remove extracellular DNA. Cells transfected with ssODN were then incubated at 30 °C until 24 h post-TALEN transfection before transfer back to 37 °C. 48 h after TALEN transfection, the cells were passaged to fresh complete media at 1E6 per ml.

**Target insertion of CD22CAR at the TRAC locus**

For dsDNA transfection for target integration, the TALEN mRNA-transfected cells were incubated at 30 °C for various length of time (15 min (0), 4, 6, 16, 20, or 24 h) before harvesting. The harvested cells were washed once with warm PBS. 5E6 PBS-washed cells were resuspended in 100 µL Lonza Human T-cell buffer (Lonza, 82 µL Human T-cell buffer + 18 µL Supplement). Two micrograms dsDNA repair template (PCR product) was mixed with the cells, and the mixture was electroporated using the Lonza Nucleofector II under the T23 program for stimulated human T cells. After electroporation, 500 µL warm complete media was added to the cuvette to dilute the electroporation buffer, and the mixture was then carefully transferred to 2 mL of prewarmed complete media in 12-well plates. Finally, 5 unit·mL⁻¹ benzonase (Sigma) was added to the cell culture to remove extracellular DNA. Cells transfected with dsDNA were then incubated at 30 °C until 24 h post-TALEN transfection before transfer back to 37 °C. 48 h after TALEN transfection, the cells were passaged to fresh complete media at 1E6 per mL.

**DNA extraction and qPCR**

Cells were harvested and washed once with PBS. Genomic DNA extraction was performed using Mag-Bind Blood & Tissue DNA HDQ kits (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer’s instructions.

For DSB detection, qPCR primers (Table S1) were designed to amplify the genomic sequence containing either the TALEN target sites or upstream (or downstream) of the TALEN target sites as controls. The qPCR was set up with the PowerUp SYBR Green Master Mix (Thermo Fisher) and analyzed on Bio-Rad CFX. qPCR annealing temperature was 60 °C for all primers.

To determine the exogenous dsDNA half-life, qPCR primers were designed to specifically amplify the CAR sequence. Primer pairs amplifying part of the genomic DNA of actin were used as loading control (Table S1). The qPCR was set up with the PowerUp SYBR Green Master Mix (Thermo Fisher) and analyzed on Bio-Rad CFX. qPCR annealing temperature was 60 °C for all primers.

**Western blot analysis**

To detect the expression of TALEN by western blots, cells were harvested at different time points after TALEN transfection. Cell lysate was prepared with 1x RIPA buffer (Milipore, Burlington, MA, USA) supplemented with 1x Halt Protease inhibitor cocktail (Thermo Scientific). Protein concentration was quantified with Pierce BCA protein assay (Thermo Scientific). 25 µg of total protein lysate from each sample was then resolved on an acrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. The expression of TALEN was detected by an anti-RVD antibody (Table S2). An antiactin antibody (Table S2) was used to detect the level of actin in the lysate as loading control. The ECL (Thermo Scientific) signal was detected on Li-COR (Li-COR, Lincoln, NE, USA). See Table S2 for the list of antibodies used in this study.

**Flow cytometry**

The information concerning antibodies used in this study is listed in Table S2. To detect the CD22CAR expression at the surface of the edited T cells, the cells transfected with TALEN mRNA and CD22CAR dsDNA were kept in culture for 96 h before harvesting and stained with a CD22Fc recombinant protein (Lake Pharma, Belmont, CA, USA) and an anti-Fcγ secondary antibody. The CD3 antibody was used to detect the loss of CD3 as a result of TRAC knock-out. Flow cytometry was performed on MACS-Quant (Miltenyi Biotech) and data analysis processed with FlowJo. Cell population was first gated for lymphocytes (SSC-A vs FSC-A) and singlets (FSC-H vs FSC-A). The lymphocyte gate was further analyzed for expression of CD22CAR from this gated population.

To detect CD22CAR expression in γδT cells, the cells transfected with TALEN mRNA and CD22CAR dsDNA were kept in culture 3 additional days before harvesting and stained with a CD22Fc recombinant protein and TCR γδ antibody.

The cells transfected with the TALEN and CD22CAR dsDNA were cultured for 3 days after dsDNA transfection. At Day 3 post-dsDNA transfection, the cells, as well as the untouched parental cells, were counted. 5E6 cells were seeded into a G-Rex 10 gas permeable culture device (Wilson Wolf) for expansion in 40 ml of complete media. IL-2 was replenished to the culture every 4 days to a final
concentration of 20 ng·mL\(^{-1}\). 13 days after expansion, the cells were harvested and frozen in complete media with 10% DMSO in 10E6 cell aliquots.

Frozen vials of CAR-T cells or parental cells were thawed and passaged in fresh complete media, at 1E6 per ml and kept in culture at 37 °C for 24 h before the measurement of T-cell differentiation marker and exhaustion markers. To measure T-cell exhaustion, cells were stained with anti-PD1-BV510 antibody and anti-LAG3-PerCP-eFlour710 antibody. To measure T-cell differentiation, cells were stained with an anti-CD45RA-VioBlue antibody (Miltenyi Biotec) and an anti-CD62L-PE antibody (Miltenyi) for flow cytometry analysis.

**CAR-T-cell activity assay**

Parental cells or cells edited using the two-step transfection protocol (20-h delay) were seeded into G-Rex10 for expansion for 13 days. Cells were then harvested. 1E6 T cells were then seeded with different amount of Raji cells (1E6, 5E5, 2E5, 1E5) which stably express luciferase in 24-well plate. The cell mixtures were incubated at 37 °C for 24 h. Five microlitre cell suspension was collected and mixed with 25 μL of ONE-glo buffer (Promega, Madison, WI, USA) for luciferase activity quantification.

**LEGENDplex cytokine releasing assay**

Cells from different steps of the engineering procedure were cultured for an additional 48 h after the DNA transfection step. The cell culture supernatants were collected, and 15 μL of the supernatant was used in the LEGENDplex assay with BioLegend’s LEGENDplex Human T helper cytokine panel version 2 (BioLegend, San Diego, CA, USA) Cytokine release assay according to manufacturer’s protocol. The data were analyzed using BioLegend’s software Qognit.

**Deep-sequencing indel analysis**

At different time points after TALEN transfection, cells were harvested, washed once in PBS and subject to genomic DNA extraction by Mag-Bind Blood & Tissue DNA HDQ kits (Omega Bio-Tek).

For indel analysis, PCR amplifications spanning TRAC or B2M targets were performed using primers described in the Supplemental Materials. 1 μg genomic DNA was used per reaction in a 50 μL reaction with Phusion High-Fidelity PCR Master Mix (NEB). The PCR condition was set to 1 cycle of 30 s at 98 °C; 8 cycles of 10 s at 98 °C, 30 s at 60 °C, 30 s at 72 °C; 1 cycle of 5 min at 72 °C; hold at 4 °C. The PCR product was then purified with Omega NGS beads (1 : 1.2 ratio) and eluted into 30 μL of 10 mM Tris buffer pH7.4. The second PCR which incorporates NGS indices to the sample was then performed on the purified product from the first PCR. 15 μL of the first PCR product was set in a 50 μL reaction with Phusion High-Fidelity PCR Master Mix (NEB). The PCR condition was set to 1 cycle of 30 s at 98 °C; 8 cycles of 10 s at 98 °C, 30 s at 62 °C, 30 s at 72 °C; 1 cycle of 5 min at 72 °C; hold at 4 °C. Purified PCR products were sequenced on MiSeq (Illumina, San Diego, CA, USA) on a 2 x 250 Nano-V2 cartridge. At least 150 000 sequences were obtained per PCR product, and sequences were analyzed for the presence of site-specific indels.

**RNA-seq**

Cells were harvested at different time points after TALEN mRNA transfection. The RNA was extracted by TRIzol (Thermo Fisher). The total RNA QC was performed on Fragment Analyzer (Agilent, Santa Clara, CA, USA). PolyA pull-down was then perform using NEBNext Poly (A) isolation module (NEB) to enrich for mRNAs. The RNA library was prepared with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB) following manufacturer’s instruction. The resulting RNA-seq library was sequenced with Illumina NextSeq Hi-Output cartridge. Data analysis was performed as follow: Read pairs were aligned using Bowtie 2 with local alignment on the N-terminal sequence upstream of TALE repeat sequence, common to both TALEN arms. Reads mapping to the N-terminal sequence were counted, and these counts were normalized to the number of total reads of the sample. Then, for each donor, normalized counts were re-renormalized to the average value at time 0 for this donor.

**Statistical analysis**

Unpaired Student’s t tests were used to analyze the data in Fig. 4C,E because the data points were collected from multiple independent experiments. Ratio paired Student’s t-test was used to analyze the data presented in Fig. 3C,D, and Fig. 4D–G because the experiments were performed with three donors and the data for each time points were collected for each of the donors.

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**Conflict of interest**

All authors were Cellectis employees at the time of paper submission. TALEN® is a Cellectis’ patented technology.
Data accessibility

All data generated or analyzed during this study are included in the article.

Author contributions

MY, AJ, and PD designed experiments. MY and DT performed the experiments. AB designed ssODN oligos, and SK performed NGS sequencing. AD analyzed NGS data. MY AJ, PD, and LP analyzed the results.

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**Supporting information**

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

**Table S1.** Primers and Oligos

**Table S2.** Information for antibodies used in this study.

**Fig. S1.** Expression of the CD22CAR in γδT cells. Six days after transfection, the expression of TCRαβ, TCRγδ and CD22CAR was measured by flow cytometry. To the right, the CD22CAR expression was gated on the TCRγδ+ population (red box). The TCRγδ−population (blue box) was further divided by their expression of CD22CAR and TCRαβ.

**Fig. S2.** T cell activation, differentiation and exhaustion after the two-step electroporation. (A) Cytokine (IL6, IL10, IFNγ and TNFα) profile after electroporation of mRNA and/or dsDNA. Cells were cultured for 2 additional days before supernatant collection and analysis of cytokine expression by LEGENDPlex (*N = 3 indep. donors, median and range*). (B) Expression of T cell differentiation markers after G-Rex for expansion and cryopreservation. Thawed cells from two donors were culture for 24 h before staining for CD62L and CD45RA (*N = 2 indep. donors*). (C) The same cells were stained for LAG3 and PD1 to detect the exhaustion markers (*N = 2 indep. donors*).