

# Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell Immunotherapies

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## Abstract

Adoptive immunotherapy using autologous T cells endowed with chimeric antigen receptors (CAR) has emerged as a powerful means of treating cancer. However, a limitation of this approach is that autologous CAR T cells must be generated on a custom-made basis. Here we show that electroporation of transcription activator–like effector nuclease (TALEN) mRNA allows highly efficient multiplex gene editing in primary human T cells. We use this TALEN-mediated editing approach to develop a process for the large-scale manufacturing of T cells deficient in expression of both their  $\alpha\beta$  T-cell receptor (TCR) and CD52, a protein targeted by alemtuzumab, a chemotherapeutic agent. Functionally, T cells manufactured with this process do not mediate graft-versus-host

reactions and are rendered resistant to destruction by alemtuzumab. These characteristics enable the administration of alemtuzumab concurrently or prior to engineered T cells, supporting their engraftment. Furthermore, endowing the TALEN-engineered cells with a CD19 CAR led to efficient destruction of CD19<sup>+</sup> tumor targets even in the presence of the chemotherapeutic agent. These results demonstrate the applicability of TALEN-mediated genome editing to a scalable process, which enables the manufacturing of third-party CAR T-cell immunotherapies against arbitrary targets. As such, CAR T-cell immunotherapies can therefore be used in an "off-the-shelf" manner akin to other biologic immunopharmaceuticals. *Cancer Res*; 75(18); 3853–64. ©2015 AACR.

## Introduction

Chimeric antigen receptors (CAR) are generated by fusing the antigen-binding region of a monoclonal antibody (mAb) or other ligands to membrane-spanning and intracellular-signaling domains derived from lymphocyte activation receptors (1–11). When expressed in primary human T cells, CARs take advantage of potent cellular effector mechanisms without the limitations of major histocompatibility complex (MHC) restriction. The potential of this approach has recently been demonstrated in clinical studies where T cells endowed with CARs were administered to adult and pediatric cancer patients (11–18). The achievement of durable remissions and significant objective responses in patients

with refractory disease, at unprecedented frequencies, has raised hopes that the wider application of CAR technology may lead to a revolution in cancer treatment.

At present, CAR technology is administered through the custom-made manufacturing of therapeutic products from each patient's own T cells. However, this patient-specific autologous paradigm is a significant limiting factor in the large-scale deployment of CAR technology as it requires either execution by a skilled team, with dedicated access to a Good Manufacturing Practice (GMP)–compliant facility or substantial investment in a centralized processing infrastructure. In addition, delays inherent to the generation of a therapeutic product preclude immediate administration, thus prejudicing favorable outcomes for the most critically ill patients. Also, custom-made autologous product generation may not be feasible for patients who are profoundly lymphopenic owing to previous chemotherapy.

Here, we describe a platform for the mass production of "off-the-shelf" CAR T cells from unrelated third-party donor T cells. We overcome the key barriers to the adoptive transfer of third-party CAR T cells via the application of transcription activator–like effector nuclease (TALEN) gene-editing technology. We therefore eliminate the potential for T cells bearing alloreactive  $\alpha\beta$  T-cell receptors (TCR) to mediate graft-versus-host disease (GvHD) by disrupting the TCR $\alpha$  constant (*TRAC*) gene and we exploit the requirement of lymphodepletion for engraftment of third-party CAR T cells by the disruption of the target of a chemotherapeutic agent. For proof of concept, we chose to target the *CD52* gene that codes for the protein targeted by alemtuzumab. Simultaneous

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editing of the *TRAC* and *CD52* genes results in TCR/CD52-deficient T cells, which can be administered with, or following, alemtuzumab treatment. Alemtuzumab mediates lymphodepletion/immunosuppression thereby promoting engraftment. We provide proof of concept for the general application of this approach by manufacturing a TCR/CD52-deficient CD19 CAR T cell (dKO-CART19). We demonstrate that this product does not mediate alloreactivity and that it can be selectively engrafted in the presence of alemtuzumab, while maintaining antitumor activity indistinguishable from standard CD19 CAR T cells in an orthotopic CD19<sup>+</sup> lymphoma murine model. The manufacturing platform is highly reproducible, thus suitable for use in the large-scale manufacturing of CAR T-cell products directed against arbitrary targets for administration as "off-the-shelf" immunopharmaceuticals.

## Materials and Methods

### Cells

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteer donors. T-lymphocytes were purified using the EasySep human T-cell enrichment kit (Stemcell Technologies) or directly activated from PBMCs.

Lymphoma cells for bioluminescent imaging were generated by transfection of Raji cells (ATCC-CCL86) or lentiviral transduction of Daudi cells (ATCC-CCL213) with an expression cassette for firefly luciferase (Raji-ffluc, Daudi-ffluc). All cell lines were used less than 6 months after resuscitation or receipt from ATCC, where short tandem repeat analysis is used to authenticate human cell lines.

### TALENs

CD52 and TRAC TALEN were obtained from Collectis Bioresearch. The target sequence for TRAC and CD52 TALENs are TTGTCCACAGATATCCGagaccctgacccgCCGTGTACCAGCTGAGA and TTCCTCTACTCACCATcagcctctggttatGGTACAGGTAAGAGCAA, respectively, where two 17-bp recognition sites (upper case letters) are separated by a 15-bp spacer.

### mRNA synthesis

mRNAs were synthesized using the mMessage mMachine T7 Ultra kit (Life Technologies). RNAs were purified with RNeasy columns (Qiagen) and eluted in water or cytoporation medium T (Harvard Apparatus). Following process optimization, TALEN mRNAs were produced by a commercial manufacturer (Trilink Biotechnologies).

### CARs

Second-generation CD19 CARs were constructed using single-chain antibody fragments derived from antibody clones fmc63 (19) or 4g7 (20), hinge and transmembrane regions from CD8A, intracellular domain from TNFRSF9 (CD137), and intracellular domain from CD247 (CD3-zeta). CD19 CARs were cloned into a commercial lentiviral vector backbone downstream from an EF1 $\alpha$  promoter and concentrated lentiviral vectors were produced by Vectalys.

### Animal studies

NSG mice (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ) were obtained from Charles River. NOG mice (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Sug</sup>/JicTac) were obtained from TACONIC. *In vivo* experiments were

performed either at Oncodesign or at UCL. All animal model experiments were carried out in accordance with experimental protocols explicitly approved for compliance with all institutional policies.

All injections were via tail vein. For antitumor activity, 6- to 8-week old NSG mice were injected with tumor cells and later, at the indicated time, with engineered or control T cells. For bioluminescence imaging, mice were anesthetized and imaged using a Xenogen IVIS Imaging System (Perkin Elmer Life Sciences). In the xenogeneic GvHD model, TCR-negative or TCR-positive T cells were injected in 8- to 10-week-old NOG mice 1 day after whole-body irradiation (2.5 Gy). Mortality, clinical signs, behavior, animal body weights, and GvHD signs were monitored and recorded daily. Animals were euthanized if they were observed to be suffering (cachexia, weakening, difficulty moving, or eating), have compound toxicity (hunching, convulsions), have 15% body weight loss over a period of 3 consecutive days, or 20% body weight loss from randomization day. An autopsy was performed in each case.

### Statistical analysis of *in vivo* data

A log-rank test was used to compare survival data. The Student *t* test was used to compare tumor burdens measured by bioluminescence. A one-tailed Fisher exact probability test was used to compare the incidence of GvHD.

### Mutagenesis analysis

PCR amplifications spanning *TRAC* or *CD52* targets or 15 *in silico* predicted potential offsite targets (named dKOsite1-15) were performed from gDNA using primers described in Online Supplementary Methods. Purified PCR products were sequenced using a 454 sequencing system (454 Life Sciences). Approximately 10,000 sequences were obtained per PCR product and analyzed for the presence of site-specific mutations.

### Translocation analysis

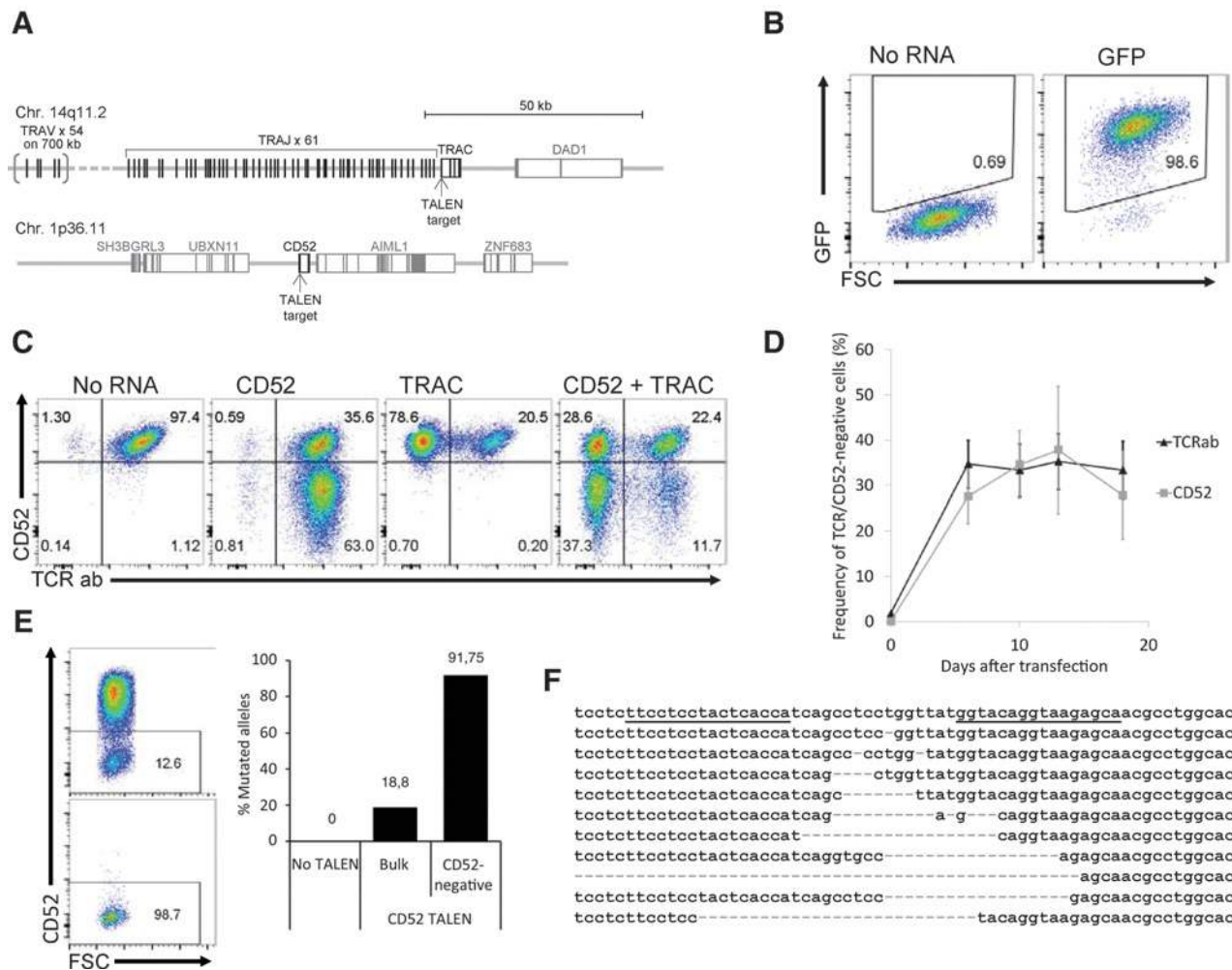
Translocation events between *CD52* and *TRAC* loci were detected by quantitative PCR (qPCR) using primers described in Online Supplementary Methods. Amplification efficiencies and copy numbers were determined using plasmids containing the expected translocation events.

## Results

### Functional inactivation of human *TRAC* and *CD52* genes by TALENs

TALENs were designed to recognize and cleave the coding sequences of human *TRAC* and *CD52* genes (Fig. 1A), and their activity was validated in HEK293 cells (Supplementary Fig. S1). As plasmid DNA transfection induces toxicity in primary T cells, we developed an mRNA electrotransfer method to express TALENs in T-lymphocytes for the purpose of genome editing. We used mRNA encoding GFP to assess transfection efficiency and obtained homogenous GFP expression in more than 95% of cells (Fig. 1B). Five days after TALEN transfection, we analyzed surface expression of CD52 and TCR $\alpha\beta$  and observed that 63% and 78% of the T cells had lost surface expression of the molecule targeted by CD52 and TRAC TALEN, respectively (Fig. 1C).

When combining TALEN treatments by cotransfection with the 4 mRNAs encoding the CD52 and TRAC TALENs, we achieved high levels of CD52/TCR-negative double knockout

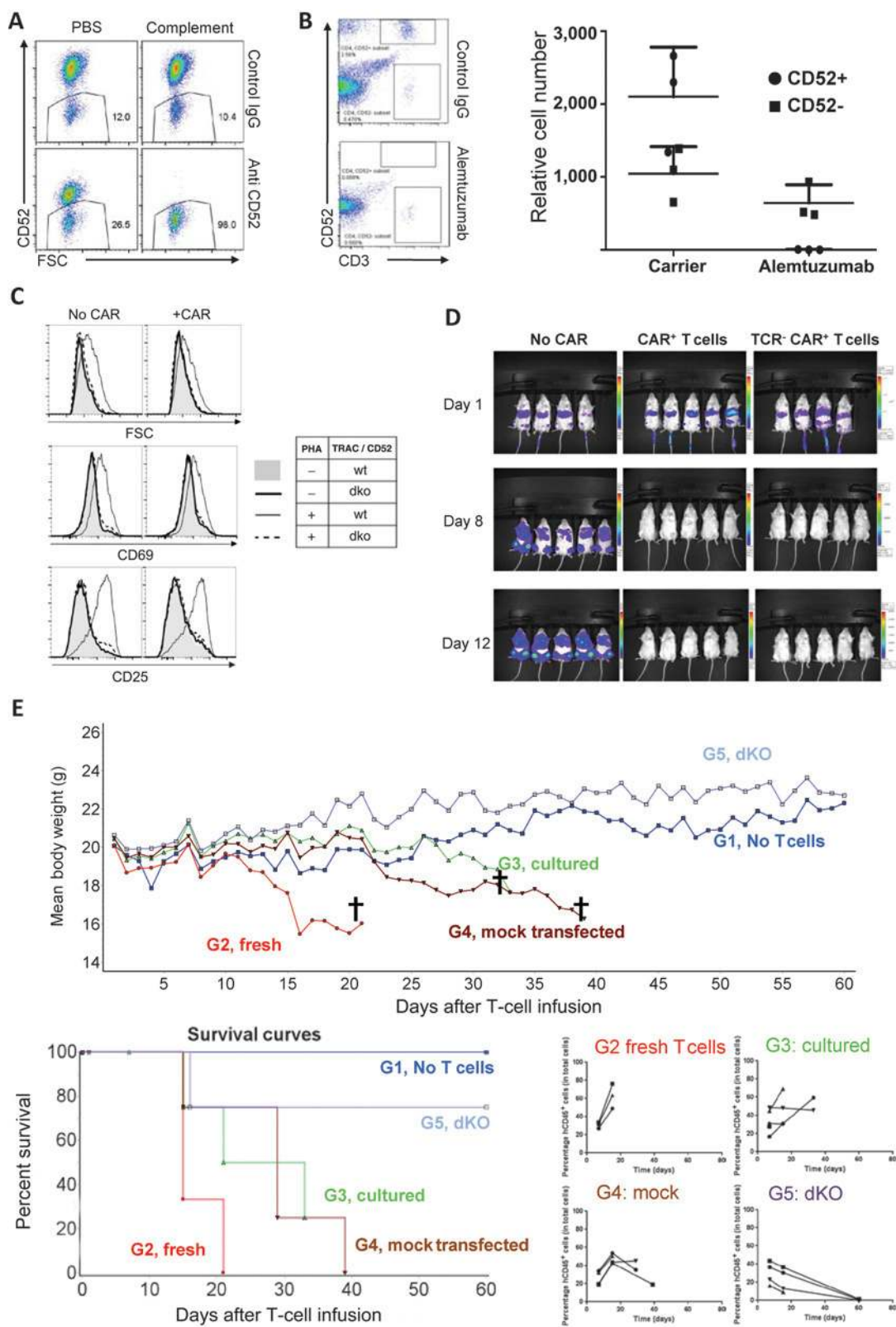
**Figure 1.**

Inactivation of human *TRAC* and *CD52* genes by TALENs. **A**, genomic representation of human *TRAC* and *CD52* genes. **B**, transfection efficiency of T-lymphocytes 24 hours after electroporation with GFP-encoding mRNA. **C**, CD52 and TCRαβ surface expression in human T-lymphocytes 5 days after CD52 and/or TRAC TALEN mRNA electroporation. **D**, flow cytometric analysis of the frequency of TCR/CD52-negative cells in TALEN-transfected T-lymphocytes maintained in culture up to 18 days posttransfection. Represented are data averaged over 5 donors. **E**, genomic analysis of TALEN inactivation of the *CD52* gene. Left, purity analysis of CD52-negative TALEN-transfected cells before (top) and after (bottom) magnetic depletion. Right, mutation frequency at the *CD52* gene in TALEN-transfected cells (or mock transfected as control) before and after depletion of remaining CD52-positive cells. **F**, representative sequences of mutated *CD52* alleles in TALEN-transfected cells compared with wild-type sequence (top). Binding sites for the nuclease are underlined.

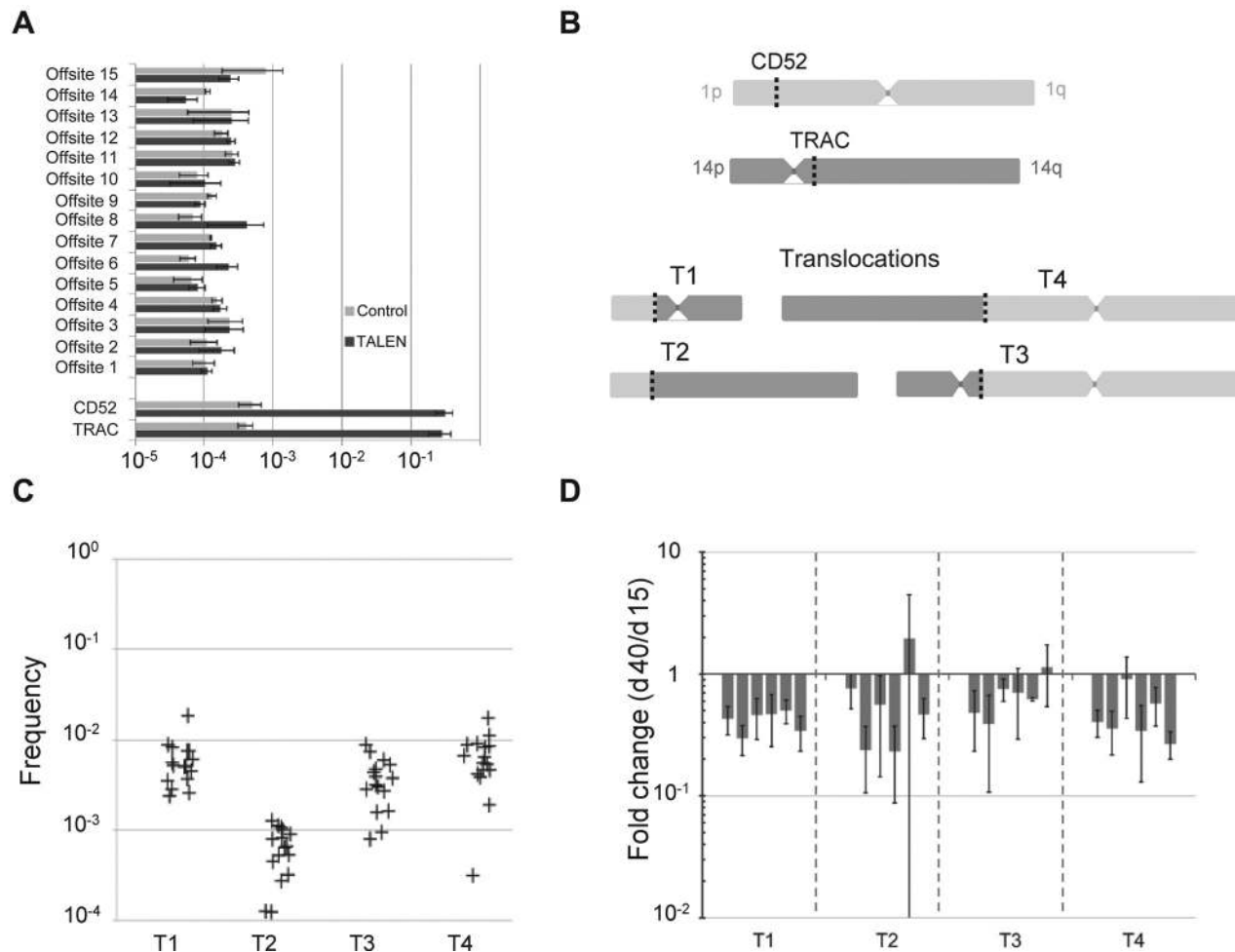
(dKO) cells (Fig. 1C). Over the duration of this study, we found that the variation in the efficiency of gene targeting was mostly a result of TALEN mRNA format or quality, cell handling, and—to a lesser degree—donor-to-donor variation. We obtained on average 12.2% ( $\pm 2.96\%$ ) of dKO cells when using RNA from small batch preparations, polyadenylated with the bacterial enzyme *Escherichia coli* poly(A) polymerase. We modified the cell handling process by optimizing the timing of media changes and the cellular density during electroporation. The mRNAs were optimized by using a plasmid template containing the 3'-untranslated region of the mouse *Hba* gene between the TALEN coding sequence and a 120-base long poly-A tail. RNAs were produced in large batches, dissolved in electroporation buffer, and stored in single-use aliquots. After the optimization process, we observed an average of 53.7% ( $\pm 12.9\%$ ) of dKO cells over a series of 8 independent experiments (Supplementary Fig. S2).

As TALENs possess high-affinity DNA-binding domains (21–26), we demonstrated that loss of expression following TALEN transfection was not due to transient transcriptional inhibition via RNA polymerase blockade: Whereas mRNA-mediated protein expression is transient (GFP expression is undetectable at 7 days posttransfection), we observed that the loss of TCRαβ/CD52 surface expression was stable over 2 weeks of culture (Fig. 1D). These data are consistent with a stable gene inactivation event and also suggest that TCR/CD52-deficient T cells are not at a disadvantage in terms of proliferation. To confirm that the loss of CD52 expression could be entirely accounted for by gene inactivation events, CD52 genomic sequences from purified TALEN-treated CD52-negative cells were analyzed using high-throughput sequencing. We found that 92% of the 2,000 sequences obtained exhibited insertions and/or deletions predicted to result in the loss of the extracellular domain of CD52 located downstream from the TALEN target site (compared with 19% in the unsorted cells, Fig. 1E





**Figure 2.** Functional characterization of human T-lymphocytes after TALEN-mediated *CD52* or *TRAC* inactivation. **A**, *in vitro* functional characterization of *CD52*-deficient cells. Five to 10 days following *CD52* TALEN transfection, T-lymphocytes were treated with 50  $\mu$ g/mL of a rat anti-*CD52* antibody from which alemtuzumab is derived or control rat IgG with or without rabbit complement for 2 hours at 37°C and analyzed by flow cytometry. (Continued on the following page.)



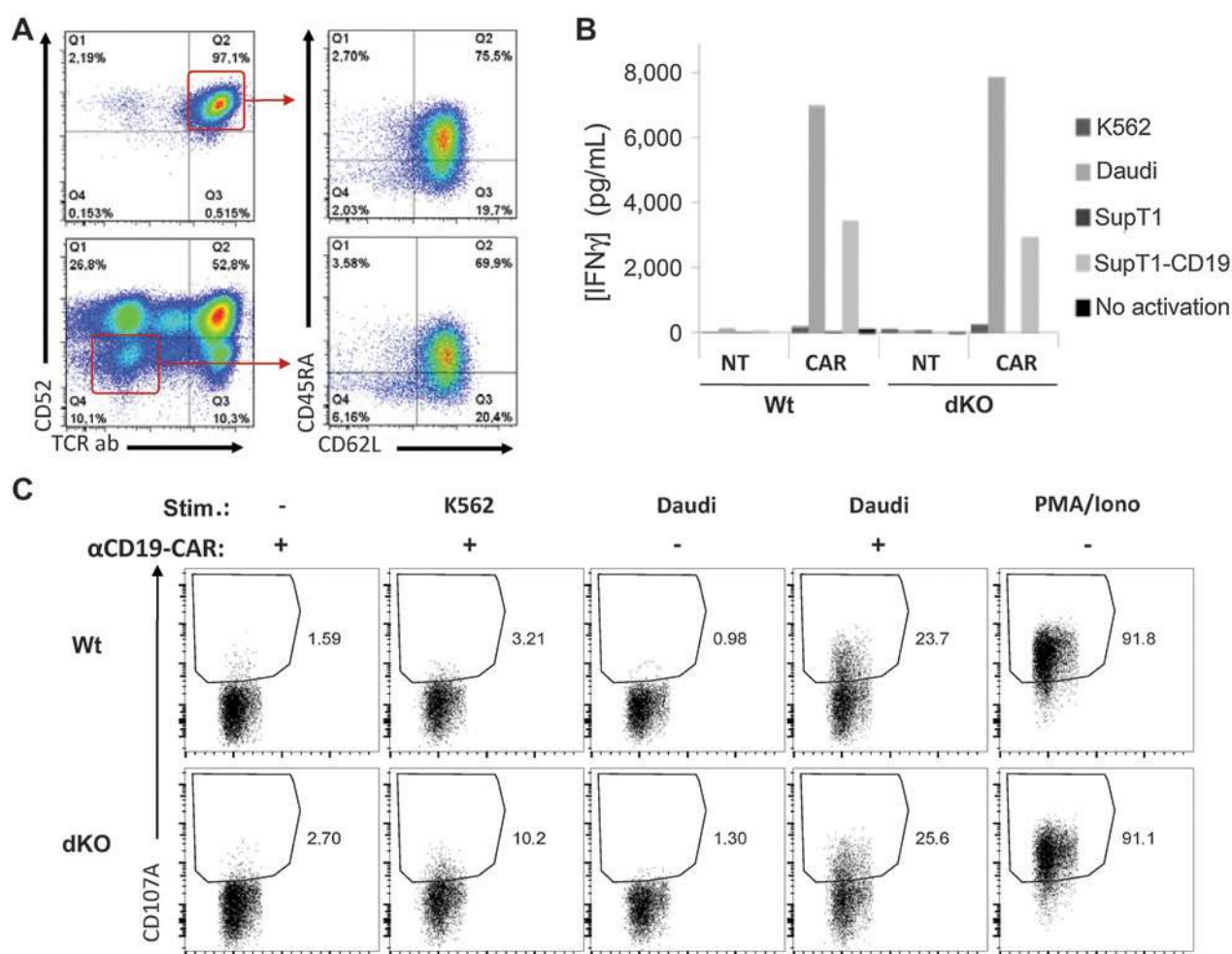
**Figure 3.** Specificity of TALEN gene editing. A, mutation frequency at the 15 predicted offsite targets in T cells treated with TRAC and CD52 TALENs averaged over two experiments compared with mutation rates at *CD52* and *TRAC* targets averaged over 8 experiments. B, schematic representation of expected translocations. C, translocation frequency in T cells treated with CD52 and TRAC TALENs from 8 independent experiments. D, kinetic evolution of translocation frequency in 6 different samples of purified CD52<sup>+</sup>/TCR $\alpha\beta$ <sup>+</sup> cells 15 or 38 days after TALEN electroporation.

and F). Thus, loss of surface CD52 following electrotransfer of TALEN mRNA can be accounted for by stable mutations in the *CD52* gene. For the *TRAC* gene, 49% of alleles were mutated in TCR-negative enriched cells (Supplementary Tables S1 and S2 and Supplementary Figs. S3 and S4). As the vast majority of mature T-lymphocytes only express one TCR $\alpha$  chain at their cell surface (allelic exclusion), they can become functionally TCR-negative while only having the productive allele inactivated by TALEN.

#### Functional characterization of human T-lymphocytes after TALEN-mediated *CD52* or *TRAC* inactivation

Complement-dependent lysis of CD52-deficient cells and TCR stimulation of TCR-deficient cells were used to evaluate the functional consequence of *CD52* and *TRAC* gene inactivation, respectively (Fig. 2A–E). *In vitro*, we demonstrated that, following TALEN transfection, CD52-negative cells are resistant to anti-CD52-dependent complement-mediated cytotoxicity (Fig. 2A). To

(Continued.) Plots are gated on viable cells. B, *in vivo* functional characterization of CD52-deficient cells. CD52 TALEN-treated cells ( $20 \times 10^6$  T cells, 22% CD52-deficient) were intravenously injected in NSG mice. After 1 week, mice were treated with alemtuzumab (10 mg/kg) or vehicle and sacrificed 1 week later. Flow cytometric analysis of the spleen is presented as flow plots (left) or relative cell counts (right). C, TCR-deficient cells are resistant to TCR stimulation. CAR<sup>+</sup> CD52<sup>+</sup>/TCR $\alpha\beta$ <sup>+</sup> cells (>95% TCR $\alpha\beta$ <sup>+</sup>) or CAR<sup>+</sup> mock-transfected control cells were treated for 2 days with 0.5  $\mu$ g/mL PHA and analyzed by flow cytometry for size and CD25/CD69 expression. D, CAR-transduced TCR-deficient cells maintained antitumor activity similar to TCR-expressing CAR T cells. At day 0, NSG mice were injected with  $2.5 \times 10^5$  Raji-ffluc cells, and 1 day later, were injected with  $4 \times 10^6$  CAR<sup>+</sup> T cells or  $4 \times 10^6$  TCR $\alpha\beta$ <sup>+</sup> CAR<sup>+</sup> T cells (>99.9% TCR $\alpha\beta$ <sup>+</sup>). Luciferase activity was measured at days 1, 8, and 12 after Raji-ffluc cell injection. E, disruption of the *TRAC* gene prevents GvH reaction in a xenogeneic model. Following irradiation, NOG mice (four per group) were injected with PBS (group G1) or  $30 \times 10^6$  human T cells after 3-day expansion (G2), 15-day expansion (G3), mock electroporation, and 15-day expansion (G4), or CD52 and TRAC TALEN electroporation, 15-day expansion, and TCR depletion (>99.9% TCR $\alpha\beta$ <sup>+</sup>; G5). CD45-positive cells in the peripheral blood were measured by flow cytometry at days 7, 15, and at sacrifice (bottom right). Animals were sacrificed at day 60 (earlier if ill); weight loss is plotted in the top right panel, survival curves in the bottom left. One mouse from group G5 was found dead at day 16 but no sign of disease was apparent.  $P < 0.02$  for incidence of GvHD when comparing group 5 to other groups. Results are representative of two independent experiments.



**Figure 4.**

Functional and antitumor properties of TCR/CD52-deficient T cells endowed with CD19 CAR. **A**, flow cytometric analysis of naïve/memory markers in TALEN-treated cells 18 days posttransfection. **B** and **C**, IFN $\gamma$  secretion (**B**) and degranulation (**C**) analysis in CAR-transduced, purified CD52<sup>-</sup>/TCR $\alpha\beta$ <sup>-</sup> T cells or mock-transfected controls after coculture with CD19-expressing cells.

demonstrate that the TALEN-mediated CD52 KO was compatible with cell engraftment, as well as sufficient to render T cells resistant to alemtuzumab *in vivo*, we administered unsorted CD52 "TALENized" T cells to NSG mice and, 1 week later, treated the mice with either alemtuzumab or vehicle. In control mice, CD52-negative and -positive T cells were engrafted equally, in proportion to the input T-cell population. However, in alemtuzumab-treated mice, the CD52-positive T cells were completely depleted in the spleen (Fig. 2B), blood, and bone marrow (not shown). TALEN-mediated inactivation of the *TRAC* gene was also shown to prevent responses to TCR stimulation, as TCR-negative cells failed to blast or upregulate the activation markers CD69 and CD25 upon phytohemagglutinin (PHA)-mediated TCR stimulation (Fig. 2C). Abrogation of TCR-mediated cell activation via *TRAC*-KO was confirmed *in vivo* where T cells treated with both *TRAC* and CD52 TALENs, followed by magnetic depletion of remaining TCR-positive cells, were unable to initiate xenogeneic GvHD after infusion into irradiated NOG immunodeficient mice (Fig. 2E). In contrast, all mice injected with mock-transfected T cells and maintained in culture for the same time developed GvHD. Furthermore, the antitumor activity of TCR-deficient T cells was not compromised,

as CD19 CAR-transduced TCR-deficient T cells exhibited antitumor activity indistinguishable from standard CD19 CAR T cells in an orthotopic human CD19<sup>+</sup> Raji-ffluc lymphoma model in NSG immunodeficient mice (Fig. 2D; ref. 27). This suggests that the loss of TCR $\alpha\beta$  expression does not grossly diminish the antitumor activity of CAR T cells. Together, these results demonstrate that TALEN mRNA electroporation is an efficient means to inactivate genes for the purpose of creating engineered T cells with stable, functional phenotypes.

#### Genomic integrity of TALEN-treated cells

As TALENs use a FokI domain that is activated upon homodimerization, an important question for the utilization of multiplex transfection of TALEN constructs is whether multiplexing engenders significant genotoxicity through off-target cleavage at "close match" sequences in the genome or at sequences recognized by mispaired half-TALENs. To address the potential for genotoxicity of our *TRAC* and CD52 TALEN reagents, we used recent data quantifying the impact of a substitution in a target sequence on the efficiency of TALEN cleavage (28) to find potential off-target sequences (Online Supplementary Methods). We

identified 173 unique sequences with a score reflecting an estimation of the likelihood of cleavage. As recent data suggest that TALENs are unlikely to cleave any noncognate target with >3 mismatches per half site (28), we selected the 15 top scores as the most likely targets. Using deep sequencing analysis, we then examined the frequency of mutations found at these loci in TCR/CD52-deficient T cells (Fig. 3A, Supplementary Table S3). The highest observed frequency of insertion/deletion at any of the offsite targets was 1 in  $8 \times 10^{-4}$  (black bars)—a rate that is statistically indistinguishable from what we observed in control amplicons (gray bars). These results demonstrate an exceptionally low rate of offsite target cleavage—at least 600 times less likely than the intended targets. We attribute this high level of specificity to the long recognition sequences of TALEN reagents, the selection of TALEN target sites to avoid close offsite targets, and the homogeneous expression provided by mRNA electroporation.

The genomic integrity of TALEN-treated cells was also considered with respect to the occurrence of translocations in cells treated with two TALENs simultaneously. We designed qPCR assays to detect the four possible translocations between chromosomes 1 and 14 (Fig. 3B). We measured the frequency of translocation in gDNA from cells transfected with CD52 and TRAC TALENs. Translocation frequencies ranged from  $10^{-4}$  to  $2 \times 10^{-2}$ , with translocations resulting in acentromeric or dicentric chromosomes occurring the least frequently (Fig. 3C, left, note that the acentric T2 translocation occurred at a very low frequency approaching the detection limit of our assay). To understand whether these translocations conferred a proliferative advantage, we used purified CD52<sup>+</sup>/TCR $\alpha\beta$ <sup>+</sup> cells and isolated gDNA after 15 and 38 days ( $\pm 2$  days) in culture—approximating the period of time when these cells would be most abundantly engrafted in a patient. Despite the fact that the cells expanded more than 4,000-fold between the two culture samplings (not shown), frequencies of all of the translocations were found to be stable or reduced (Fig. 3D), providing strong evidence that TRAC/CD52 translocations do not provide a proliferative advantage.

### In vitro functional characterization of TCR/CD52-deficient T cells endowed with CD19 CAR

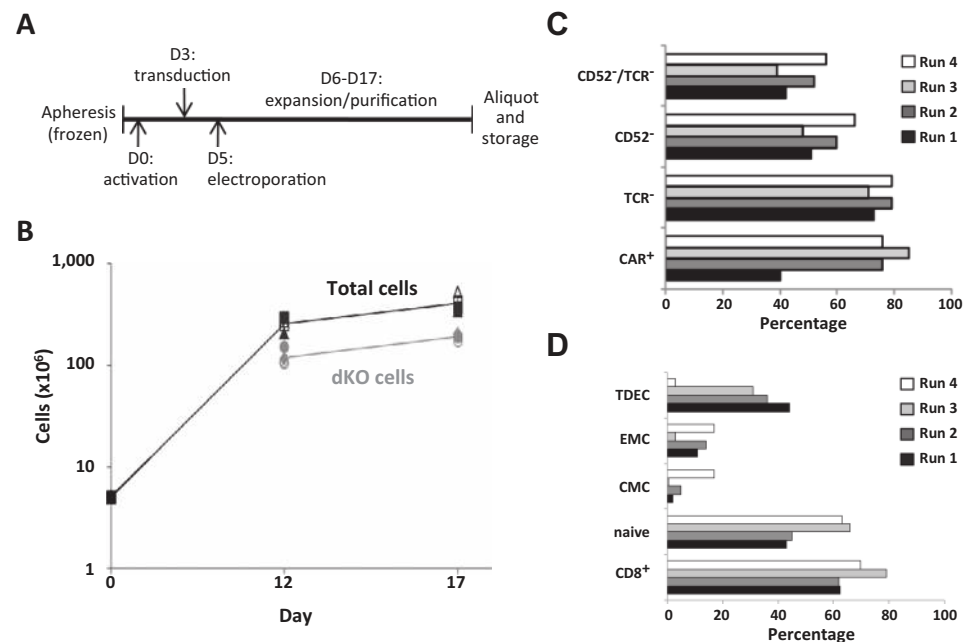
To evaluate the functional properties of gene-edited T cells, we used CAR transduction and TALEN electroporation to generate TCR/CD52-deficient CD19 CAR T cells (these double-knockout CAR19 T cells are herein referred to as dKO-CART19 cells), and characterized their surface phenotype and cytotoxic function. dKO-CART19 cells were indistinguishable from unmodified cells in terms of their distribution between naïve, effector, and memory subsets as indicated by CD62L/CD45RA expression (Fig. 4A). To determine dKO-CART19 responses to a tumor antigen, we evaluated CD19-mediated T-cell activation by monitoring surface expression of CD107a (indicative of the release of cytotoxic granules or degranulation; ref. 29) following coculture of dKO-CART19 cells with cells with or without the CD19 antigen. As shown in Fig. 4C, we observed an increase in the frequency of CD107a<sup>+</sup> dKO-CART19 cells only when CAR<sup>+</sup> cells were cocultured with CD19-expressing Daudi cells. This degranulation was dependent on the presence of the CAR, as nontransduced T cells did not degranulate in the presence of Daudi cells. Furthermore, degranulation of CART19 cells was quantitatively indistinguishable between unmodified T cells and dKO cells. The same was true when using other cellular targets, that is, SupT1 cells engineered to express CD19 compared with control parental SupT1 cells (not shown). These results were confirmed by quantification of IFN $\gamma$  secretion by ELISA after a 24-hour incubation of the same coculture (Fig. 4B).

### TALEN-mediated TRAC/CD52 inactivation is compatible with a 17-day CAR T-cell manufacturing process

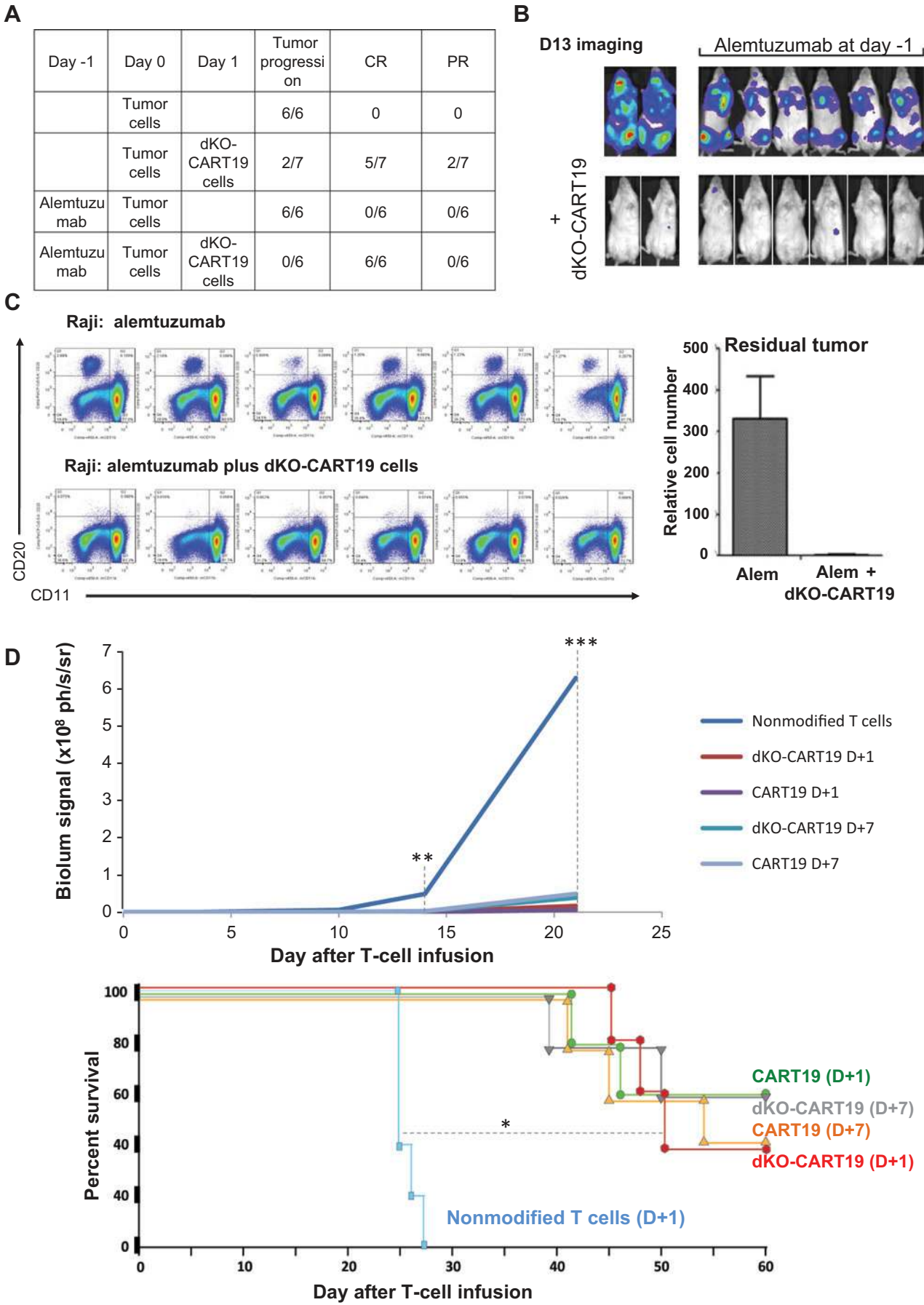
To extend our laboratory method of TALEN-mediated genome editing into a general platform for the engineering of third-party CAR T cells for "off-the-shelf" use, we developed and optimized a 17-day manufacturing process for gene-edited CAR T cells (Fig. 5A). The current, optimized version of this process is initiated by CD3-dependent T-cell activation at time 0, incorporates a lentiviral

**Figure 5.**

dKO-CART19 cell manufacturing process. A, dKO-CART19 manufacturing process. B, total cell number (black open square, run 1; filled square, run 2; open triangle, run 3; filled triangle, run 4) determined by Trypan blue exclusion and CD52<sup>+</sup>/TCR $\alpha\beta$ <sup>+</sup> T-cell number extrapolated from frequency measured by flow cytometry (gray open circle, run 1; filled circle, run 2; open diamond, run 3; filled diamond, run 4) at days 12 and 17 for 4 dKO-CART19 runs. Lines represent averages. C, frequency of CAR, TCR $\alpha\beta$ , and CD52 surface expression of T-lymphocytes at day 17 of 4 dKO-CART19 runs, determined by flow cytometry. D, frequency of CD8<sup>+</sup> cells, naïve-like cells, central memory cells (CMC), effector memory cells (EMC), and terminally differentiated cells (TDEC), determined by flow cytometry (CD45RA and CD62L, gating strategy in Supplementary Fig. S6) at day 17 of 4 dKO-CART19 runs.









transduction step at 72 hours to allow for the expression of a CAR, and finally a TALEN mRNA electroporation step for *TRAC/CD52* gene disruption at 120 hours. Subsequent to the TALEN electroporation step, the cells are cultured in a closed system for 10 to 12 days followed by magnetic depletion of remaining TCR $\alpha\beta$ -positive cells. Using this process, TCR/CD52-deficient CAR T cells were manufactured with highly reproducible yields (Fig. 5B), levels of genome modification (Fig. 5C), and cell phenotype (Fig. 5D).

#### **dKO-CART19 T cells are functional in the presence of alemtuzumab and maintain antitumor activity equivalent to standard CAR T cells**

The functional and antitumor properties of cells manufactured with the aforementioned optimized process were evaluated using orthotopic human CD19<sup>+</sup> lymphoma xenograft models. In the first experiment, dKO-CART19 cells (CAR-transduced T cells electroporated with CD52 and TRAC-TALENs followed by depletion of remaining TCR-positive cells) were assessed for their performance in the presence and absence of alemtuzumab (Fig. 6A). For this experiment, a Raji-ffluc cell line was treated with the TALEN targeting *CD52* (CD52-edited Raji-ffluc); the cell line derived from TALEN treatment was maintained as a bulk cellular population where not all *CD52* alleles were inactivated. The CD52-edited Raji-ffluc cells therefore consisted in a heterogeneous mixture of CD52-deficient and CD52-sufficient cells to recapitulate situations where alemtuzumab only results in a partial response and tumor growth is only delayed by treatment. Alemtuzumab was injected 1 day prior to CD52-edited Raji-ffluc cell injection, and dKO-CART19 cells were infused the following day. All of the mice that received tumor cells but no dKO-CART19 cells showed tumor progression, leading to their sacrifice by 13 days postinjection. In 5 of the 7 mice that received tumor and dKO-CART19 cells, the tumor was completely controlled at day 13 and in the remaining two mice, partial responses were observed. In contrast, alemtuzumab treatment was observed to only delay progression of the CD52-edited Raji-ffluc tumor without T-cell infusion (6 of 6 mice)—a result analogous to those observed when alemtuzumab is used to treat high-risk chronic lymphocytic leukemia in humans (30). Strikingly, in mice treated with alemtuzumab combined with dKO-CART19 cells, the tumor was completely eliminated from their preferred engraftment niche, bone marrow [assessed by luminescence (Fig. 6B) or flow cytometry from bone marrow cells 13 days postinjection (Fig. 6C)]. To confirm antitumor activity against another tumor cell line and obtain a relative assessment of potency of dKO-CART19 cells in comparison to standard CART19 cells, we used a Daudi-ffluc tumor cell xenograft model without concomitant alemtuzumab treatment (as this would eliminate the CART19 cells) and extended the periods of monitoring (Fig. 6D). For this experiment, Daudi-ffluc tumor cells were injected either 7 days or 1 day before injection of CD19 CAR T cells. Tumor burdens were

consecutively monitored via luminescence imaging for the first 3 weeks, and mice were monitored over an 8-week period for clinical signs of tumor evolution and survival. Tumor progression resulting in death occurred around day 25 in all animals that received nonmodified T cells. However, animals that received dKO-CART19 cells and CART19 cells either 1 day or 7 days post-tumor cell injection survived significantly longer (40 days or more), with no difference in survival curves between mice that received dKO-CART19 or CART19 cells. The differences between the group receiving nonmodified T cells and all other groups were statistically significant. From these data, we conclude that the manufacturing method used to generate TCR/CD52-deficient CAR T cells results in little or no compromise of their function in the presence of alemtuzumab or in their CD19-directed antitumor potency relative to non-"TALENized" CD19 CAR T cells.

## **Discussion**

Here we demonstrate the development of a practical platform for the manufacture of T cells from third-party donors for use in adoptive T-cell therapies. Our platform involves the use of TALEN-mediated genome editing to simultaneously knock out two genes in the third-party T cells. The first KO—the disruption of the *TRAC* gene—eliminates TCR $\alpha\beta$  expression and abrogates the third-party T-cell's potential for GvHD, whereas a second KO is used to render the third-party T cells resistant to a lymphodepleting/immunosuppressive agent and prevent their rejection by the patient. For an initial proof of concept, we chose to knock out *CD52*, a target of the lymphodepleting monoclonal antibody alemtuzumab, based on the rationale that alemtuzumab could be used for selective host lymphodepletion to create a receptive environment for TCR/CD52-deficient T-cell engraftment.

To achieve the simultaneous KO of two genes in primary human T cells, we developed and optimized a method for the electroporation of TALEN mRNAs into T cells. The optimized method results in no immediate visible toxic effects on the cells and achieves double KO rates consistently greater than 40% at the targeted loci. Furthermore, the transduction of a CAR gene cassette and the TALEN electroporation step were incorporated into a 2-week, large-scale process for the GMP manufacturing of TCR $\alpha\beta$ /chemotherapy-resistant CAR T cells. The specificity properties of our TALENs were associated with rates of off-target cleavage below the detection limits of our deep sequencing assay ( $<1$  in  $10^3$ ), a rate lower than that reported for other genome-editing platforms (26, 30–33). We attribute this observation to the inherent specificity properties of TALENs coupled with careful selection of target sites to avoid other genomic sites potentially susceptible to cleavage (21). While the simultaneous dual gene-editing step of our process led to the expected generation of translocations between the two double-strand break sites, we detected no evidence of growth advantage for

**Figure 6.**

dKO-CART19 cells eradicate CD19<sup>+</sup> tumors in the presence of alemtuzumab and exhibit antitumor indistinguishable from standard CART19 cells. A, antitumor activity of dKO-CART19 cells in the presence of alemtuzumab. NSG mice were treated with alemtuzumab or vehicle on day  $-1$ ,  $2.5 \times 10^5$  CD52-edited Raji-ffluc cells on day 0, and 1 day later either  $4 \times 10^6$  CAR-positive dKO-CART19 T cells or left untreated. B, bioluminescence imaging 13 days after Raji-ffluc cell inoculation. C, detection of tumor cells in bone marrow of alemtuzumab-treated mice with or without dKO-CART19 cells. Results from flow cytometric analysis are presented as flow plots (left) or as relative cell counts (right). D, evaluation of antitumor activity of dKO-CART19 and CART19 T cells. A total of  $4 \times 10^6$  CAR-positive T cells (dKO-CART19 or CART19 cells) were intravenously injected either 1 day after tumor cell injection ( $5 \times 10^5$  Daudi-ffluc cells) or 7 days after ( $2.5 \times 10^5$  Daudi-ffluc cells). Mice were monitored for 3 weeks via luminescence imaging to follow tumor progression, and over 8 weeks for weight, activity, clinical signs of tumor development, and survival. \*,  $P < 0.02$ ; \*\*,  $P < 10^{-3}$ ; \*\*\*,  $P < 10^{-4}$ . This study is representative of at least three independent experiments yielding similar results.

cells harboring any of the 4 possible translocations over 12 generations of *in vitro* culture. On the basis of these analyses, we conclude that TALEN-mediated genome editing can be incorporated into a large-scale T-cell culture process as a practical and safe approach to the manufacturing of third-party CAR T cells for use in adoptive T-cell immunotherapies.

Zinc finger nucleases have previously been applied for gene editing in human T cells, including inactivation of both the *TCRα* and *TCRβ* genes for enabling allogeneic "off-the-shelf" and TCR replacement strategies and inactivation of an HLA class I gene (34–36). Our observations, regarding the functional capabilities of TRAC KO T cells, are consistent with the results described in the aforementioned publications; T cells lacking a TCR component lose CD3 expression and all capacity for activation via either the TCR or through the CD3 complex. Our results also significantly extend previous results, as we have demonstrated the potential of the simultaneous inactivation of a second gene without deleterious effects on T-cell function. We have also shown that "double KO" CD19 CAR T cells can be efficiently and consistently manufactured and that the *in vivo* antitumor activity of manufactured TCR/CD52-deficient CD19 CAR T cells, in the presence or absence of alemtuzumab, is indistinguishable from that of a standard CD19 CAR T cell in an orthotopic lymphoma model.

A fundamental aspect of our approach is the use of TALEN gene-editing technology to simultaneously inactivate the TCR and a target of a lymphodepleting immunosuppressive chemotherapeutic as a potential means of supporting third-party T-cell engraftment across MHC barriers. For an initial proof of concept, we chose to generate T cells deficient in the pan-lymphoid surface marker protein CD52. The KO of CD52 is easily assessed by flow cytometry, and elimination of CD52 renders cells resistant to the lytic properties of alemtuzumab (37–40)—a therapeutic monoclonal antibody that binds to CD52. Alemtuzumab has been used extensively for the treatment of lymphoid malignancies, including chronic lymphocytic leukemia, where it has been shown to result in a profound reduction of B cells, T cells, as well as natural killer (NK) cells (41). In addition, use of alemtuzumab to deplete T cells in hematopoietic stem cell transplants ("Campath in the bag") potently suppresses alloreactivity, highlighting its potential for use in engraftment of T cells across MHC barriers (37–40). While its long *in vivo* half-life precludes its use as a lymphodepleting agent for standard adoptive immunotherapies due to targeting of both host and adoptively transferred cells, disruption of CD52 expression, as demonstrated here, would allow T cells to be used following, or in conjunction with, alemtuzumab to simultaneously achieve both host cell depletion and adoptive T-cell engraftment. This approach would be of particular interest for the treatment of patients with chronic lymphocytic leukemia, in particular relapsed patients, where alemtuzumab is a treatment option. However, it should be noted that the limited availability of this antibody may prevent its systematic use in all patients. Thus, an alternative approach to allow engraftment of the cells described in this work would be to use standard lymphodepletion regimens, similar to those in autologous trials, such as cyclophosphamide and fludarabine.

The use of allogeneic CAR T cells following lymphodepletion by alemtuzumab or another agent would result in their transient engraftment, the cells being eliminated upon the reconstitution of the patient's immune system. Although the importance of sustained persistence of CAR<sup>+</sup> T cells in the blood is unknown,

previous autologous CD19 CAR studies have shown that significant proliferation occurs rapidly with maximum CAR<sup>+</sup> cell numbers observed 7 to 17 days after injection (18, 42). In these studies, the clinical response is evaluated 28 days after treatment with a large fraction of complete responses occurring within this time frame. Thus, ideally the duration of lymphodepletion in an allogeneic approach would be limited: sufficient to provide CAR T cells time to proliferate and eliminate cancer cells while minimizing potential secondary effects due to prolonged lymphodepletion.

A particularly significant aspect of our results is the demonstration of a GMP-compatible scalable process for manufacturing third-party T cells deficient in two gene products. We propose that this process could serve as a general platform to enable the production of "off-the-shelf" T-cell immunotherapies—we estimate that a single production run, starting from an apheresis product containing 10<sup>9</sup> T cells, would allow the production of 10<sup>10</sup> CAR dKO T cells—sufficient to treat in the range of more than 500 patients at 1 to 2 × 10<sup>7</sup> cells per dose (e.g., the lowest biologically effective dose defined by Kalos and colleagues; ref. 13). As the same manufacturing method can be used to generate T-cells expressing different CARs or be endowed with alternative chemotherapy resistance (e.g., HPRT KO for resistance to 6-MP, GR KO for resistance to glucocorticoids; ref. 43), it has significant potential to enable CAR therapies to be extended to a much wider range of patients than present custom-made CAR T-cell products.

In conclusion, we demonstrated that TALEN-mediated gene editing can be used in a large-scale process for manufacturing third-party CAR T-cell therapies—engineered T cells can be pre-manufactured from healthy third-party donors. These T cells can be endowed with CAR or engineered TCR, of an arbitrary specificity, that enables their tumor cell killing capabilities. Furthermore, the engraftment of these engineered T cells, into any patient, can be further supported when administered with a chemotherapeutic agent (following or concomitantly with treatment) to which they have been engineered for resistance. Our results also suggest that we are just scratching the surface of the potential for genome editing to enhance CAR T-cell therapies. With an increasingly detailed knowledge of tumor evasion mechanisms and T-cell checkpoints (44, 45), it appears that there may be multiple avenues through which genome editing could be applied to render CAR T-cell tumor evasion resistant and increase their potency.

## Disclosure of Potential Conflicts of Interest

J. Eyquem is an employee of Collectis SA as a PhD student. M. Pule is an employee of Autolus Ltd. as the CSO; reports receiving commercial research grant from Collectis; speakers bureau honoraria from Amgen; and has ownership interest (including patents) in Patents. A.M. Scharenberg is an employee of Bluebird bio as the Scientific Advisory Board member; has ownership interest (including patents) in Bluebird bio; and is a consultant/advisory board member of Bluebird bio. No potential conflicts of interest were disclosed by the other authors.

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