A Multidrug-resistant Engineered CAR T Cell for Allogeneic Combination Immunotherapy

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The adoptive transfer of chimeric antigen receptor (CAR) T cells represents a highly promising strategy to fight against multiple cancers. The clinical outcome of such therapies is intimately linked to the ability of effector cells to engraft, proliferate, and specifically kill tumor cells within patients. When allogeneic CAR T-cell infusion is considered, host versus graft and graft versus host reactions must be avoided to prevent rejection of adoptively transferred cells, host tissue damages and to elicit significant antitumoral outcome. This work proposes to address these three requirements through the development of multidrug-resistant T cell receptor αβ-deficient CAR T cells. We demonstrate that these engineered T cells displayed efficient antitumor activity and proliferated in the presence of purine and pyrimidine nucleoside analogues, currently used in clinic as preconditioning lymphodepleting regimens. The absence of TCRαβ at their cell surface along with their purine nucleotide analogues-resistant properties could prevent their alloreactivity and enable them to resist to lymphodepleting regimens that may be required to avoid their ablation via HvG reaction. By providing a basic framework to develop a universal T cell compatible with allogeneic adoptive transfer, this work is laying the foundation stone of the large-scale utilization of CAR T-cell immunotherapies.

INTRODUCTION
The adoptive transfer of chimeric antigen receptor (CAR) T cells represents a highly promising strategy to fight against multiple cancer indications. This strategy relies on the engineering of T cells to redirect their cytolytic activity toward malignant cells via transgenic expression of a tumor antigen-specific receptor at their cell surface. Today, the current protocols of treatment consist in autologous adoptive cell transfer (ACT). In this approach, T lymphocytes recovered from patients, are genetically modified and expanded in vitro before infusion back into patients. This process requires precise logistics, proximity between dedicated production facilities and the bedside and more importantly, delays the availability of genetically engineered T cells for patient treatment. Recent reports proposed to address these issues by developing a CAR T cell compatible with allogeneic adoptive transfer. This alternative approach consists in generating from a third-party donor, a bulk population of CAR T cells that can be injected into multiple patients, a strategy likely to unleash the full potential of CAR T-cell therapies by bringing them to the industrial level. When allogeneic CAR T-cell adoptive transfer is considered, host versus graft (HvG) and graft versus host (GvH) reactions must be avoided to safely allow effector cells to engraft, proliferate, and specifically kill given tumor cells in patients. While a GvH reaction can be tackled by sequestration of lymphocytes in lymph nodes or by targeted gene knockout of T cell receptor (TCRαβ) within CAR T-cell genome, controlling their rejection via HvG remains a technological hurdle that need to be addressed. It has been proposed that HvG reaction, involving host T-cell activation after direct or indirect allorecognition, could be prevented by lymphodepleting regimens. Such regimens, usually consisting of alkylating agents and/or purine nucleotide analogues (PNA) compounds, are known to deplete the host immune system for weeks to month periods, depending on the dose being used. They could thus theoretically create a therapeutic window during which allogeneic CAR T cell could eradicate tumors before being rejected via HvG reaction. If this scenario can be envisioned for the treatment of some hematological tumors reported to be rapidly eradicated by ACT (< 1 month), it may not be applicable to other type of malignancies including solid tumors that may require an extended period of treatment. Thus, developing strategies to control the extent of therapeutic window for allogeneic ACT treatments is highly desired. One way to address this challenge would be to prolong lymphodepleting regimens during adoptive T-cell transfer. However, because such regimens are also highly likely to deplete adoptively transferred CAR T cells, this strategy requires to use regimen resistant-CAR T cells.

This report describes the genetic engineering and characterization of CAR T cells resistant to three different PNAs currently used in clinic as preconditioning lymphodepleting regimens. Our engineering process includes a lentiviral transduction for CAR expression followed by the simultaneous TALEN-mediated gene processing of TCR constant region (TRAC) and deoxy-cytidine kinase (dCK) respectively responsible for TCRαβ surface expression and PNA toxicity. It enables expansion as well as recovery of a homogeneous population of engineered CAR T cells that retain their proliferative capacity and cytolytic activity toward...
tumor cells in the presence of lymphodepleting dose of different PNAs. We envision that these engineered CAR T cells could be generated from third party healthy donors and used in any patients as antitumor allogeneic immunotherapy without generating TCRαβ-dependent GvH reaction. Their drug resistance properties could enable them to resist to simultaneous infusion of lymphodepleting regimens to inhibit the host immune system and control their rate of ablation via HvG reaction.

RESULTS

TALEN-mediated TRAC/dCK dual gene processing is highly efficient in primary T cells

PNAs used as lymphodepleting regimens or as antineoplastic drugs are usually delivered as nucleoside prodrugs. They become toxic after being metabolized to their respective triphosphate forms through sequential phosphorylations catalyzed by deoxycytidine kinase (dCK). To prevent such metabolism from occurring in T cells and to endow them with PNA resistance properties, we sought to inactivate the gene encoding dCK. For this purpose, a TALEN targeting the second exon of the DCK gene (Figure 1a, upper panel) was designed, assembled and its corresponding mRNA was used to electroporate primary T cells. Because our goal was to inactivate TCRα concomitantly with dCK, we coelectroporated mRNA encoding TRAC TALEN, reported earlier to efficiently inactivate TCRα gene and prevent TCRα surface expression.12 The molecular events generated by both TALEN at their respective locus, were characterized in depth by high-throughput DNA sequencing (Figure 1a). Our sequencing results revealed nucleotide insertions or deletions (Indels) at frequencies of ~80 and 76% at the TRAC and dCK loci respectively. Thus, TALEN-mediated processing of these two genes was highly efficient under our experimental conditions. Deconvolution of sequencing results allowed us to observe that the vast majority of processing events obtained with both TALEN was due to deletions, a pattern consistent with several reports regarding TALEN activity.13 For both loci, deletion events ranged from 2 to 20 bp with a minority of events > 20 bp. Regarding insertions, most of them consisted of two to four nucleotides inserted within the target spacer and generally corresponded to sequence duplication (data not shown).

TALEN-mediated TRAC/dCK dual gene processing does not promote genetic adverse event

Potential off-site targets were carefully evaluated in silico and in vitro for both loci. We defined off-site targets as genomic sequences bearing any combinations of TALEN half binding sites containing ≤ 4 mismatches with respect to the sequence to target and separated form one another by 9 to 30 bp. Because we intended to perform two simultaneous gene inactivations, all combinations of TRAC and dCK TALEN half binding sites were considered (Table 1) as well as how close they were to coding sequences. The vast majority of potential offsite targets identified in silico contained a total ≥ 5 mismatches when combining the two half-sites, a number reported to abrogate TALEN activity.14,15 From this list, 16 different off-sites located inside or less than 30 bp away from exonic regions were selected and studied by high-through DNA sequencing (Supplementary Material and Supplementary Figure S1). As expected, we were not able to detect indels events above the threshold set by the control experiment performed with mock transfected primary T cells (Table 1). In addition, the presence and stability of translocations induced by two simultaneous TALEN treatment were also checked by nested polymerase chain reaction (PCR). Our results showed that TALEN treatment induced translocations that were selected against during a prolonged 30-day expansion (Supplementary Materials and Supplementary Figure S2). Together these results are consistent with the high specificity of TALEN and their suitability for therapeutic applications.14,15

Figure 1 TALEN treatment of primary T cells enables simultaneous and highly efficient processing of TRAC and dCK genes. (a) Overall gene architectures of dCK and TRAC open reading frames. Exons (dark blue) and introns (light blue) are indicated. dCK and TRAC TALEN targets are displayed and underlined. The TALEN binding sites and spacer sequences are respectively displayed in upper and lower cases letters. (b) Genotypic characterization of TALEN-mediated inactivation of dCK and TRAC genes by high throughputs DNA sequencing. T cells treated with dCK and TRAC TALEN were allowed to grow for 6 days, their genomic DNA was extracted, amplified using TRAC or dCK specific PCR amplicons and analyzed by high throughput DNA sequencing. The frequencies of indels generated at TRAC and dCK locus are indicated.
| Locus name | In exon | In coding exon | Gene | Position chromosome | Mismatch # left | Left sequence with mismatches | Mismatch # right | Right sequence with mismatches | Number of indels | Number of sequence analyzed | TALEN mRNA Number of indels | Number of sequence analyzed | Frequency of off-site processing ≤ to |
|------------|---------|----------------|------|--------------------|----------------|-------------------------------|----------------|-------------------------------|----------------|-----------------------------|-----------------------------|-------------------------------|-------------------------------|
| TRAC       | Yes     | Yes            | TRAC | Chr14:23016437-23016484 | 0 | ttgccccacagatctcc | 0 | ccgtgtacactagaga | 2 | 16,963 | 4,597 | 6,605 | — |
| dCK        | Yes     | Yes            | dCK  | Chr7:1863841-71863889 | 0 | ttgtctgaacctgtt | 0 | gttaagtaactcaaga | 1 | 4,548 | 3,867 | 5,641 | — |
| Offsite1   | No      | No             | SLTM | Chr15:59914112-59914166 | 3 | ttgtcAcaTaTtcC | 3 | gTaaTtggTgaca | 0 | 7,486 | 0 | 2,567 | 3.9E-04 |
| Offsite2   | No      | No             | TBC1D2B | Chr15:78329275-78329328 | 3 | ttgtagGatCtGagaa | 3 | tCaaaAtacTtaga | 0 | 20,771 | 0 | 16,401 | 6.1E-05 |
| Offsite3   | No      | No             | SMAD2 | Chr18:45426619-45426679 | 3 | tGttagatatCaTcA | 3 | tTCaagTactcaTa | 0 | 14,038 | 1 | 4,681 | 2.1E-04 |
| Offsite4   | No      | No             | FAM123C | Chr2:131516718-131516782 | 3 | ttgtctGATCTTtgt | 3 | tGcaagtGctcaTa | 0 | 9,324 | 0 | 3,290 | 3.0E-04 |
| Offsite5   | No      | No             | EYA2  | Chr20:45578659-45578708 | 3 | ttgtctATGctgtt | 3 | tGcaagtGctcaCa | 0 | 4,291 | 0 | 3,082 | 3.2E-04 |
| Offsite6   | No      | No             | KCNMB2 | Chr3:17850875-178508809 | 3 | ttgtaAtacCGagaa | 3 | tAcaaAtacCtCaga | 0 | 1,516 | 0 | 35,669 | 2.8E-05 |
| Offsite7   | No      | No             | CACNA2D1 | Chr7:81965814-81965859 | 3 | ttCttgTaAcctttga | 2 | tCtaagTactcaAG | 0 | 15,839 | 1 | 15,183 | 6.6E-05 |
| Offsite8   | No      | No             | SLC2A8 | Chr9:130164310-130164352 | 3 | ttCttgTaAttcttga | 3 | tTcaaaTGTcaCa | 0 | 24,597 | 0 | 3,043 | 3.3E-04 |
| Offsite9   | No      | No             | GPC4  | ChrX:132495536-132495596 | 3 | ttgtgaaTctCtCaa | 3 | tCaaaACactCtCaga | 0 | 19,253 | 0 | 18,876 | 5.3E-05 |
| Offsite10  | No      | No             | C10orf11 | Chr10:77735642-77735704 | 3 | ttGtctgtaaTctCtCt | 3 | tATAACttGggcCa | 0 | 23,186 | 0 | 28,361 | 3.5E-05 |
| Offsite11  | Yes     | Yes            | VEPH1 | Chr13:15703477-157035140 | 3 | ttCttcTtacGctcC | 3 | aGggtttcaaggAAca | 0 | 10,851 | 0 | 25,011 | 4.0E-05 |
| Offsite12  | No      | No             | NOX4  | Chr11:89237228-89237288 | 3 | ttgtgattGcatTgGa | 3 | tGacGtctGAAgaa | 0 | 9,541 | 0 | 3,965 | 2.5E-04 |
| Offsite13  | No      | No             | BRCA2 | Chr13:82990178-32960238 | 3 | ttCtgaaGactGGGagaa | 3 | tActgtggtcGgGgaa | 0 | 7,645 | 0 | 16,677 | 6.0E-05 |
| Offsite14  | No      | No             | TRPC4 | Chr13:83279111-83279169 | 3 | AtgtccAaGagataCt | 3 | ttcTaAtaAtacaaga | 0 | 1,018 | 0 | 4,526 | 2.2E-04 |
| Offsite15  | No      | No             | RPL39L | Chr13:186841908-186841964 | 3 | tGctcTtacacGatCtCt | 3 | tGcaattTTrtcaaga | 0 | 18,335 | 1 | 18,232 | 5.5E-05 |
| Offsite16  | No      | No             | FAM190A | Chr4:91469558-91469611 | 3 | ttCgactcGgcatCacaG | 3 | tCtaagGacGcaagG | 0 | 23,392 | 0 | 7,200 | 1.4E-04 |

The 16 offsite targets, identified in silico, are displayed along with the dCK and TRAC targets. Their genomic coordinates (obtained from GRCH37/hg19 genome assembly) are indicated. Their relative position with respect to coding and noncoding exon as well as with respect to the closest gene is also documented. The number of mismatch present in the predicted TALEN left and right half binding sites are indicated along with their position and nature (upper case red letters). Six days after being treated by T solution (mock, negative control) or by TCR constant region (TRAC) and deoxyribosyl kinase (dCK) TALEN, primary T cells were recovered and their genomic DNA was extracted for further high-throughput DNA sequencing analysis. The number of indels detected over the total number of sequence analyzed are displayed for each targets.
After purification, clofarabine and displayed IC50 values of about 100 µmol/l (Table 1), suggesting engineered T cells viability remained constant up to 10 µmol/l of clofarabine. Our results showed that the viability of wt T cells was not impaired by the simultaneous inactivation of TRAC, as indicated by similar IC50 values obtained for single and double knock-out T cells (Table 1 and Supplementary Figure S3, KO-D and KO-D/T, compare open triangles and open squares).

Because TALEN-mediated dCK gene inactivation was not total (Figure 1b), we hypothesized that depleting the remaining wt T cells bearing active dCK from the bulk T-cell population would improve the overall resistance properties the cells. To test this assumption, we implemented a 5-day selection process in the presence of 1 µmol/l clofarabine, a dose previously determined to be lethal for wt T cells (1 µmol/l, Figure 3a, bottom). These T cells were then cultured for five additional days in the absence of clofarabine before testing their clofarabine resistance at D13 as described above (Figure 3b, closed symbols). Our results showed that the 5-day selection barely increased IC50 values (Table 2, Figure 3b, compare close and open symbols), indicating that such a selection process did not markedly improve clofarabine resistance of the TALEN-treated T-cell population. Therefore, our results indicated that TALEN-mediated inactivation of dCK gene, even when combined with TRAC inactivation, is efficient enough to generate clofarabine resistant T cells without requiring any chemical selection or purification process.

**TALEN-mediated TRAC/dCK dual gene processing disrupts TCRαβ expression at the surface of primary T cells and increased their resistance to clofarabine**

To verify that TRAC gene processing prevented surface expression of the functional TCRαβ receptor in primary T cells, cells were analyzed by flow cytometry, 6 days postelectroporation with mRNAs encoding TRAC and dCK TALEN. As expected from previous results obtained in jurkat cells, we found that 81% of TALEN-treated T cells had lost TCRαβ surface expression (Figure 2a, lower left panel). TCRαβ-deficient T cells were purified using anti-CD3 magnetic beads (Figure 2a, lower right panel), enabling the recovery of a highly homogenous preparation of TCRαβ-deficient T cells (>99% of the bulk population). Further analysis of their genomic DNA allowed us to determine that they displayed 89 and 93% of indels at TRAC and dCK loci respectively (Figure 2b).

To determine if dCK gene inactivation resulted in PNA resistance, T cells treated with dCK and TRAC TALEN were analyzed for their in vitro resistance to the purine nucleotide analogue clofarabine. T cells were treated with dCK and TRAC TALEN and allowed to grow for 13 days before assessing their resistance property to clofarabine (Figure 3a, upper part). Resistance was determined by comparing the viability of wild-type (wt) and TALEN-treated T cells after 48 hours incubation in the presence of increasing amounts of clofarabine. Our results showed that the viability of wt T cells dropped at low doses of clofarabine with an IC50~100 nmol/l, while engineered T cells viability remained constant up to 10 µmol/l of clofarabine and displayed IC50 values of about 100 µmol/l (Figure 3b open symbols and Table 2). Such IC50 differences, spanning about 3 logs of clofarabine concentration, indicated that TALEN-mediated processing of the dCK gene enabled increased resistance of T cells to clofarabine. In addition, this resistance property was not impaired by the simultaneous inactivation of TRAC, as indicated by similar IC50 values obtained for single and double knock-out T cells (Table 1 and Supplementary Figure S3, KO-D and KO-D/T, compare open triangles and open squares).

**TALEN-treated CAR T cells are resistant to multiple PNA**

Having demonstrated that treatment of primary T cells by TRAC and dCK TALEN could eliminate cell surface expression
of TCRαβ and endow them with clofarabine resistance, we next evaluated their potential as an antitumor immunotherapy agent toward Daudi cells, a CD19+ lymphoblast cell line used here as an convenient tumor model. To do so, we transduced T cells with a lentiviral vector containing a polycistronic DNA expression cassette encoding a membrane exposed rituximab-dependent depletion system (RQR8 (ref. 16)), followed by a 2A cis-acting hydrolase element and a CD19-specific CAR7 (Figure 4a). Such a polycistronic coding sequence was designed to endow T cells with cytolytic properties toward the CD19+ tumor cells and to allow to grow for 3 days (D0-D3) and then cultured for 5 days in the presence or in the absence of 1 µmol/l clofarabine (D3-D8). After 5 additional days of culture (D8-D13), cells were plated in a 96-well plate at 10^4 cells per well in presence of increasing concentration of clofarabine (from 0 to 100 µmol/l) and in a total volume of 100 µl. Two days after plating, viability of cells was determined by flow cytometry and plotted as function of clofarabine concentration. Resulting curves were fitted with the exponential function described in Material and Methods to determine IC_{50} values.

Table 2 Assessment of IC_{50} values of different PNAs toward wt and engineered T cells

| PNA          | Cell               | IC_{50} (µmol/l) | SD  | # of exp |
|--------------|--------------------|------------------|-----|----------|
| Clofarabine  | T cell             | 0.10             | -   | n = 1    |
| T cell KO D  | 63.4               |                  |     |          |
| T cell KO D/T| 96.1               |                  |     |          |
| T cell KO D/T + Clo | 191.6       |                  |     |          |
| Clofarabine  | CAR T cell         | 0.14             | 0.03| n = 3    |
| Fludarabine  | CAR T cell         | 10.0             | 3.7 | n = 3    |
| Cytarabine   | CAR T cell         | 11.4             | 10.0| n = 2    |
| CAR T cell KO D/T | 1,211.4       |                  |     |          |

To determine purine nucleotide analogues (PNAs) IC_{50} values toward wild-type (wt) or engineered T cells, T cells were plated in a 96-well plate at 10^4 cells per well in presence of increasing concentration of either clofarabine, fludarabine or cytarabine (from 0 to 1,000 µmol/l) in a total volume of 100 µl. Two days after plating, viability of cells was determined by flow cytometry and plotted as function of PNA concentration. Resulting curves were fitted with the exponential function described in Material and Methods to determine IC_{50} values.

The ability to proliferate in the presence of clinically relevant doses of PNA was then evaluated. Toward this aim, untreated and TALEN-treated CAR T cells were grown for 10 days in the presence or in the absence of 1 µmol/l clofarabine, 5 µmol/l fludarabine, or 10 µmol/l cytarabine, the doses previously reported as their respective average C_{50} values after their uptake in human patients.\(^{17–19}\) Viable cell numbers were determined every 2–3 days and plotted as a function of time (Figure 4g,h). Our results demonstrated that the proliferation of untreated and TALEN-treated CAR T cells were similar in the absence of any PNA. Thus, the dual inactivation of TCR and dCK did not impair the rate of T cells division. In addition, the presence of PNAs in the culture media, strongly impaired or completely abolished proliferation of untreated CAR T cells while being harmless with respect to TALEN-treated CAR T cells (Figure 4g,h). Similar conclusions could be drawn out of identical experiments performed with three other blood donors (Supplementary Figure S5). Such difference between the proliferation pattern of untreated and TALEN-treated CAR T cells were consistent with their respective drug resistance curves. Indeed, the presence of either 1 µmol/l clofarabine, 5 µmol/l fludarabine, and 10 µmol/l cytarabine decreased the cellular viability of untreated CAR T cells by about 80, 30, and
Figure 4 TALEN-treated CAR T cells are resistant to lethal dose of different purine and pyrimidine nucleotide analogues. (a) Scheme of the polycistronic DNA expression cassette encoding the membrane exposed rituximab-dependent depletion system (RQR8), followed by a 2A cis-acting hydrolase element and the anti-CD19 CAR. (b) Scheme of the experimental workflow used to engineer and characterize T cells proliferative capacity and resistance properties toward clofarabine, fludarabine and cytarabine. (c, d, e) Clofarabine, fludarabine, and cytarabine IC₅₀ determination. 10⁵ CAR T cells were incubated in the presence of increasing concentration of clofarabine, fludarabine or cytarabine for 48 hours before being analyzed by flow cytometry to determine their viability. Experimental viability values obtained were normalized with respect to the one obtained in the absence of drug and then plotted as a function of drug concentration. The resistance properties of untreated (CAR T cell) and TALEN-treated CAR T cells (CAR T cell KO D/T) are displayed. (f–h) Proliferation capacity of CAR T cells in the presence or in the absence of clofarabine, fludarabine and cytarabine. The number of viable untreated (CAR T cell) and TALEN-treated CAR T cells (CAR T cell KO D/T) counted at each passage are plotted as a function of time. Vertical arrows indicate each passage step where cells were diluted to 10⁶ cell/ml in the presence or in the absence of drug.

50% respectively while only a minimal impact was observed with TALEN-treated CAR T cells (Figure 4c–e). Noteworthy, TALEN-treated CAR T cells were also able to proliferate in the presence of a combination of clofarabine and cytarabine (1 and 10 µmol/l respectively, Supplementary Materials, Supplementary Figure S6). Together, these results indicated that TALEN-treated CAR T cells were able to resist and proliferate in the presence of clinically relevant doses of different PNAs.

Clofarabine prevents alloreactivity of PBMC toward CAR T cells in vitro

To evaluate the ability of PNA to prevent alloreactivity of peripheral blood mononuclear cells (PBMCs) toward CAR T cells, we performed allogeneic mixed lymphocyte reaction in the presence or in the absence of clofarabine. Briefly, PBMC freshly purified from a donor B (PBMC B) and labeled with carboxy fluorescein succinimidyl ester (CFSE) were incubated with either T cells purified from the same blood donor (T cells B) or with CAR T cells engineered out of a different blood donor (CAR T cell A). After 6 days of incubation, we evaluated the extent of PBMC viability, activation, and proliferation by flow cytometry and anti IFNγ ELISA (Supplementary Figure S7). Our results showed that when mixed with CAR T cells A, PBMC B showed a typical pattern of activation/proliferation that was similar to the one observed in the positive control experiment performed with phytohaemagglutinin (PHA) (Supplementary Figure S7a). This pattern consisted in a
activities of TALEN-treated CAR T cells cultured with or without their cytolytic activity was assessed. Using the flow cytometry-toured for 2–9 days in the presence of 1 µmol/l clofarabine and the presence of lethal dose of PNA. To this end, cells were cul-

dual inactivation of TRAC and dCK genes did not markedly affect CAR T cells cytolytic activity and specificity.

Figure 5 CAR T-cell cytolytic activity is not affected by dual inactivation of dCK and TCRαβ. To assess the impact of TALEN treatment on the cytolytic activity and specificity of CAR T cells toward Daudi cells the flow-based cytotoxicity assay reported in ref. 20 was used. T0 Daudi (CD19+) and 100 K562 (CD19 negative control) cells labeled respectively with CellTrace CFSE and CellTrace violet were coincubated with 100 effector CAR T cells (E/T ratio of 10:1), for 5 hours at 37 °C. Cells were then recovered and their viability was determined by flow cytometry. Viability of Daudi and K562 cells was used to calculate the frequencies of targeted cytotoxicity using the formula decribed in ref. 20. Untransduced T cells, untreated or treated by dCK and TRAC TALEN (Lanes 1 and 2), were used as negative controls. Error bars represent the mean of two experiments ± SD.

significant dilution of CFSE dye, an increase of cellular viability and size along with a high level of IFNγ release (Supplementary Figure S7b), typically associated with T cells activation and blasting. As expected, this phenomenon was not observed in the presence of T cells B. Interestingly addition of 1 µmol/l clofarabine to the reaction media, was able to deplete about than 90 % of PBMC (Supplementary Figure S7c) and impair their alloreactivity toward CAR T cells, as illustrated by the low level of IFNγ release (Figure 7b). Together, our results indicated that clinically relevant dose of clofarabine prevents the alloreaction between PBMC and CAR T cells in vitro.

TALEN-treated CAR T cells display similar antitumor cytotoxicity than wt CAR T cells

The cytolytic properties of TALEN-treated CAR T cells toward CD19+ tumor cells were then evaluated. For that purpose, we performed a flow cytometry-based cytotoxicity assay to assess their killing potency and evaluate their level of specificity toward CD19+ cells. Our results showed that untreated and TALEN-treated CAR T cell displayed similar specific cell lysis activity toward Daudi cells (35% ± 2 and 39% ± 2 respectively, Figure 5 and Supplementary Figure S8). These results indicated that dual inactivation of TRAC and dCK genes did not markedly affect CAR T cells cytolytic activity and specificity.

We then investigated the ability of TALEN-treated CAR T cells to retain their antitumor activity after being expanded in the presence of lethal dose of PNA. To this end, cells were cultured for 2–9 days in the presence of 1 µmol/l clofarabine and their cytolytic activity was assessed. Using the flow cytometry-based cytotoxicity assay described earlier, the specific cell lysis activities of TALEN-treated CAR T cells cultured with or without
clofarabine were compared to those obtained for untreated CAR T cells or T cells cultured without clofarabine (Figure 6 and Supplementary Figure S9). Our results showed that the presence of 1 µmol/l clofarabine, while lethal for untreated CAR T cells (Figure 4c, closed circles and Figure 4f, open triangles), did not impair the cytolytic properties of TALEN-treated CAR T cells even after 2–9 days of culture (Figure 6, compare lanes 1 and 2 with 3 and 4). Indeed, they display similar activity to those cultured in the absence of drug, whatever the length of culture of our experimental conditions. In addition, our results confirmed that the dual inactivation of dCK and TRAC did not impair T-cell cytolytic activity as demonstrated by the similar specific cell lysis activities observed for the TALEN-treated and untreated CAR T cells (Figure 6, compare lanes 2 with lane 6).

TALEN-treated CAR T cells can pair up with PNA chemotherapy for better antitumor activity

Clofarabine is currently used in clinic as cytotoxic agent to treat patients with relapsed or refractory acute lymphoblastic leukemia and displays potent cellular toxicity toward multiple tumor cell lines, including Daudi cells.24 We thus sought to investigate whether a combination of CAR T-cell immunotherapy with clofarabine chemotherapy would enhance the antitumor activity toward Daudi cells. To do so, TALEN-treated CAR T cells were used to perform a flow cytometry based cytotoxicity assay with CFSE-labeled Daudi cells preincubated for 24 hours in the presence of 1 µmol/l clofarabine (C_{50}). To effectively assess the potential benefit of combination therapy, two control experiments were performed to determine the cytotoxicity elicited by clofarabine or

Figure 6 TALEN-treated CAR T cells retain their cytolytic activity after 2 to 9 days of culture in the presence of clinically relevant dose of clofarabine. 100 Daudi (CD19+) and 100 K562 (CD19 negative control) cells labeled respectively with CellTrace CFSE and CellTrace violet were coincubated for 5 hours at 37 °C with 100 of TALEN-treated CAR T cells grown for 2 or 9 days in the presence or in the absence of 1 µmol/l clo-

farabine. Cells were then recovered and their viability was determined by flow cytometry. Viability of Daudi and K562 cells were used to calculate the frequencies of specific cell lysis using the formula decribed in ref. 20 (lanes 1 to 4). T cells and CAR T cells were cultivated in the absence of clofarabine for 9 days were respectively used as negative and positive controls of specific cell lysis activity (lanes 5 and 6). Results are displayed as specific cell lysis activities relative to the control experiment performed with CAR T cells. Error bars represent the mean of two experiments ± SD.
CAR T cell alone toward Daudi cells. An additional experiment performed in the absence of both treatments was performed and used as reference for Daudi cells maximal viability. Our results showed that combination therapy led to more than a 50% drop of Daudi cells viability (Figure 7a, lane 4 and Supplementary Figure S10a). Such decrease was greater than those obtained with TALEN-treated CAR T cells alone (Figure 7a, lane 3) or with clofarabine alone (Figure 7a, lane 2). Similar results were obtained when clofarabine was replaced by 10 µmol/l cytarabine (Cmax, Figure 7b and Supplementary Figure S10b). Therefore, altogether our results indicated that when provided simultaneously, both treatments elicited a better antitumor activity compared with either treatment alone.

DISCUSSION
The clinical outcome of CAR-T-cell adoptive therapies is intimately linked to the ability of effector cells to engraft, proliferate, and specifically kill given tumor cells. When allogeneic CAR T-cell infusion is considered, HvG and GvH reactions must be avoided to prevent, (i) rejection of adoptively transferred cells, (ii) host tissue damage, and (iii), to elicit significant antitumor activity. In this study, we have engineered primary T cells so that they could potentially address these three requirements. Using the TALEN-mediated gene editing method, we generated a homogeneous population of TCRαβ-deficient CAR T cells resistant toward clofarabine, fludarabine, and cytarabine, three nucleotide analogues used in the clinic as lymphodepleting preconditioning regimens for ACT. TALEN-treated CAR T cells were able to proliferate in vitro in the presence of drugs and displayed similar antitumor properties compared to untreated CAR T cells. In addition, we showed that when combined, chemotherapy and CAR T cells immunotherapy resulted in a better antitumor activity compared with either treatment alone. Due to the absence of TCRαβ at the surface, such engineered T cells could be generated from third party donors and used in patients as an antitumor allogeneic immunotherapy treatment without generating a GvH reaction. In addition, their broad PNAs resistance properties could enable them to resist to multiple lymphodepleting regimens used to inhibit the host immune system and prevent their ablation via HvG reaction.

A haute couture editing method to process primary T-cell genome in a highly precise and efficient manner: a prerequisite for therapeutic applications
The efficiency of TALEN-mediated dual inactivation of dCK and TCRα gene in primary T cells was unprecedented. Both loci were shown to be processed at Indels frequencies of ~90%, a value exceeding former studies describing gene inactivation in primary T cells. Such high indels frequencies are likely due to multiple parameters that were chosen in an educated manner. Among the most important ones are the choice of TALEN target and the method used to vectorize TALEN into T cells.

Regarding the choice of TRAC and dCK TALEN targets, we made sure to carefully consider their epigenetic status in T cells, their nucleotide composition, and predict their potential off-site targets using the TALEN specificity matrices reported in ref. 14. Regarding their position, both TALEN targets were chosen to be at, or within the immediate vicinity of an exon/intro border (Figure 1a). Processing of this type of region is expected to disrupt donor or acceptor splice sites in addition to the exonic regions, a strategy highly likely to promote aberrant splice variants and result in the production of inactive truncated proteins. Concerning the vectorization method, we chose to use RNA encoding TALEN and the Agilpulse electroporation method. This combination was shown to have two main advantages to engineer primary T cells. On the one hand, using mRNA instead of DNA prevents any unexpected random insertion of TALEN encoding sequence and improves the gene processing efficiency (Poirot et al., submitted). On the other hand, this electroporation method, optimized in-house for T cells transfection, was shown to decrease cellular stress and mortality usually associated with this step. Highly efficient on-target processing did not imply off-site target activity and downstream genetic adverse events. Indeed,
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we showed that while about 90% of both TRAC and dCK loci were processed, no off-site activity could be detected above the background measured with mock transfected T cells, at the most probable off-site targets predicted in silico (see Table 1 and Supplementary Material). Regarding translocation events, they could be detected in the double TALEN-treated CAR T cells 10 days after being treated with TALEN. However, after an additional 20 days of culture, corresponding to the cellular expansion step needed for a standard CAR T-cell production process, most of these events were selected against. This likely indicated that the translocations were unstable and did not confer any proliferative advantages to engineer CAR T cells. Untreated and TALEN-treated CAR T cells consistently displayed similar proliferation rates (Figure 4f–h), indicating that simultaneous processing of TRAC and dCK genes did not generate durable adverse events in T-cell genome under our experimental conditions. Regarding that matter, it is important to consider that the frequency of translocation induced by two simultaneous TALEN treatments are loci-dependent.26,27 When clear evidence of a stable translocation is observed, one may consider sequential gene inactivation using short lifespan mRNA encoding engineered nuclease.4 However, although it has yet to be demonstrated, such sequential process could impact the antitumor efficacy of CAR T cell by increasing the cellular stress associated with consecutive transfection steps and by extending the length of the production process.

Engineered CAR T cells display stable, robust and multi drug resistant phenotype allowing prevention of HvG alloreaction

We have demonstrated that TALEN-mediated inactivation of dCK translated into stable and efficient purine and pyrimidine nucleotide analogue resistance phenotype of CAR T cells. We were able to recover PNA-resistant CAR T cells without requiring any PNA-dependent selection process. In addition, inactivation of dCK neither altered CAR T cells cytotoxicity toward tumor cells nor their capacity to proliferate. This latter property was expected because rather than being involved in the pivotal de novo synthesis of dNTP, dCK is implicated in the nonessential nucleotide salvage pathway.24 Thus, TALEN-mediated inactivation of dCK represents two major advantages for the production of PNA-resistant CAR T cells: (i) it allows to expand and recover large quantities of CAR T cells in a short time frame and (ii) it does not expose them to genotoxic selection agents, a harmful procedure that would jeopardize their utilization in adoptive transfer therapies.

The PNA resistance properties of CAR T cells allowed them to resist to lymphodepleting treatment (Figure 4) necessary to prevent HvG reaction in an allogeneic context (Supplementary Figure S7). Thus, we envisioned that this treatment could modulate the extent of the therapeutic window during which allogeneic CAR T cells could engraft and eradicate tumors before being rejected by the patient immune system. The benefit of sustained engraftment of CAR T cells is still unclear. Indeed, previous autologous ACT studies showed that 15 days was sufficient to elicit a complete response in multiple instances.8 Nonetheless, a longer persistence could benefit other patients. Thus, ideally, controlling the duration of lymphodepletion in allogeneic settings could provide CAR T cells with enough time to eliminate tumor cells while minimizing risks associated with prolonged lymphodepletion.

The broad PNA resistance of engineered CAR T cells generated in this work offers different options for lymphodepletion regimen. Today, fludarabine alone or in combination with cyclophosphamide, is frequently used as lymphodepleting agent prior to adoptive T-cell transfer.50,29 Utilization of either one or both chemotherapies was shown to improve antitumor efficacy by removing endogenous cellular elements (cytokine sinks) that compete with transferred T cells for supportive cytokines.30 Because of their similar chemical structure and physiological properties, clofarabine and cytarabine could also be used for the same purpose as demonstrated recently for adoptive CAR T-cell immunotherapy of acute lymphoblastic leukemia.8

A multidrug resistant CAR T cell allowing combination therapy for better therapeutic outcomes

Endowing CAR T cell with resistance to PNA is not the only functionality allowed by our gene editing approach as it could also enable combination therapy. Combination therapy using chemo- and cell-based therapies represents an appealing strategy to improve the efficiency and persistence of treatment as demonstrated for stem cell adoptive therapy.31–33 The potential advantage of this approach for CAR T-cell immunotherapies relies on the well-known ability of certain chemotherapies to increase the persistence and activity of effector T cells,34,35 increase their tumor trafficking,34,35 modulate immunosuppressive factors36 and for some of them including PNAs, directly affect tumor cell viability.6 While highly promising, the development of combination therapies have been dampened by the cytotoxic properties of chemo-therapy toward immunotherapeutic cellular agents. However, during the past ten years, development of genome engineering techniques through transgene expression or shRNA transfection have enabled development of cells resistant to methotrexate,27 trimetrexate and temolozide,38 cyclophosphamide derivative,37 micofenolate mofetil,39 6-thioguanine,31 and rapamycin40 as non-exhaustive examples. While our work extended the portfolio of genome engineering approaches enabling permanent and stable cellular drug resistance without relying on transgene expression, it also established the in vitro proof of principle that combining CAR T cells with PNAs lead to better antitumor outcome than either treatment alone. Noteworthy, because all the experiments delineated in this work were performed in vitro, we could not assess the therapeutic benefit of PNA-dependent immunomodulation including the depletion of regulatory T cells (Treg)41 and myeloid-derived suppressor cells (MDSC),42 two main cellular protagonists of T-cell inhibition. We envision that inhibition of such cellular subsets including others, could potentize CAR T-cell cytolytic activity in vivo and thus significantly improve the overall therapeutic outcome. It will be now interesting to challenge this system in a more complex environment such as humanized xenograft mouse models.

A dual activity lock for better safety of adoptive CAR T-cell therapy

CAR T cells therapy has been reported to be highly efficient at killing tumor cells. However, one of the downsides of this therapeutic
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Materials and Methods

Materials. Clofarabine, fludarabine (fludarabine-phosphate), and cytarabine were obtained from Sigma (St Louis, MO, cat #C7495, # F9813, and #C3350000 respectively), diluted according to the manufacturer protocol. The concentrations of diluted PNA solutions were accurately determined by spectrophotometry. dCK and TRAC TALEN were produced in-house using the solid phase assembly method described in ref. 49. Regarding cell culture reagents, X-vivo-15 was obtained from Lonza (Basel, Switzerland cat#BE04-418Q), IL-2 from Miltenyi Biotech (Bergisch Gladbach, Germany, cat#130-097-748), human serum AB from Seralab (West Sussex, UK cat#GEM-100–318), human T activator CD3/CD28 from Life Technologies (Beverly, MA, cat#11132D), MACS-LD column from Miltenyi Biotech (cat#130-042-901), fixable viability dye eFluor780 from eBioscience (San Diego, CA, cat#65-0865-14). CFSE dye was obtained from Life Technologies (cat#C34354) and anti-IFNγ ELISA kit was obtained from R&D Systems (Minneapolis, MN, cat#DIF50).

Generation of TCR/dCK-deficient primary T cells. To generate TCRαβ/dCK-deficient T cells, primary T cells were first purified fromuffy-coats, activated and transfected according to the procedure described in ref. 12. Briefly regarding transfection, 4 days after their activation by Dynabeads human T activator CD3/CD28, 5 million of T cells were simultaneously transfected with 5 μg of each mRNA encoding left and right arm of TALEN targeting the TCRα constant chain and dCK exon 2 (see Supplementary Material). Transfection was performed using Agilipulse technology, by applying two 0.1 ms pulses at 3,000 V/cm followed by four 0.2 ms pulses at 325 V/cm in 0.4 cm gap cuvettes and a final volume of 200 μl of Cytoporation buffer T (BTX Harvard Apparatus, Holliston, MA). Cells were then immediately diluted in X-Vivo-15 media supplemented by 20 ng/ml IL-2 (final concentration) and 5% human serum AB. Transfected T cells were eventually diluted at 1 × 10^5/ml and kept in culture at 37 °C in the presence of 5% CO₂ and 20 ng/ml IL-2 (final concentration) and 5% human AB serum for further characterization.

A similar procedure was used to generate TCRαβ/dCK-deficient primary T cells expressing anti-CD19 CAR (FMC63 construct), given the few difference described in the following. Freshly purified primary T cells were activated for 3 days according to the procedure described above and in ref. 12, and then transduced with lentiviral vectors harboring a RQR8-2A-FMC63 CAR expression cassette under the control of the Ef1α promoter (see Supplementary Material), at the multiplicity of infection (MOI) of 5. Two days after transduction, T cells were transfected with mRNA encoding TRAC and dCK TALEN using the procedure described above.

Isolation of TCRαβ-deficient T cells using magnetic separation. TCRαβ-deficient cells were purified according to the protocol described in refs. 12,25. Briefly, about 10^7 T cells recovered 6 days after TALEN treatment were labeled with biotin conjugated anti-TCRαβ antibody MicroBeads before being loaded onto a MACS LD-Column placed in the magnetic field of a MACS Separator. Using this procedure, the magnetically labeled CD3-positive cells were retained in the column while the unlabeled TCRαβ-deficient cells could be recovered in the flow through. One round of purification was usually necessary to obtain a homogeneous population of TCRαβ-deficient cells (purity > 99%).

Detection of translocation event by nested-PCR. The nested-PCR protocol used to detect translocation event after treating T cells with TRAC and dCK TALEN has been adapted from the protocol described in ref. 26.
Briefly, it consists of amplifying the genomic DNA, extracted from a bulk population of TALEN-treated T cells, via a first PCR specific for the four translocations named TR1, TR2, TR3, and TR4 expected to occur between TRAC and dCK loci. This PCR is then amplified using a second set of oligonucleotides designed to anneal inside the first PCR amplicons. The first and second PCR were performed with the Herculase II Fusion DNA Polymerases kit (Agilent Technologies) using 25 cycles of amplification and a 1 °C = 50 °C. A comprehensive list of oligonucleotides and translocation matrix sequences used to set up and optimize the nested-PCR protocol is documented in the Supplementary Material section.

**IC50 determination.** IC50 of a given drug is defined as the concentration of drug need to decrease the cellular viability by 50%. To determine IC50 of a clorafarine, fludarabine and cytarabine, 100 × 103 T cells were incubated in the presence of increasing concentration of drugs (from 0 to 100 μmol/l typically) for 48 hours and in a total volume of 100 μl X-Vivo-15 media supplemented by 20 ng/ml IL-2 and 5% human AB serum. Cells were washed with 100 μl of phosphate buffer saline (PBS) and then labeled by eFluor 780 for 15 minutes at 4 °C according to the manufacturer protocol. Labeling was stopped by addition of PBS 2% fetal seal serum (SVF) and cells were eventually fixed by 4% paraformaldehyde (PFA) before being analyzed by flow cytometry to determine their viability. Evolution of cell viability as a function of PNA concentration was fitted with the drift R package software using the formula: y = 1/(1 + EXP((LOG10(x) - LOG10(0(IC50) / x))^100 with y, x, and z corresponding to viability frequency, the concentration of drug, and the scale parameter.

**Flow-based cytotoxicity assay.** The cytolytic activity and specificity of CAR T cell was assessed according to the flow cytometry-based cytotoxicity assay described in ref. 20. This assay consisted of labeling 105 Daudi (CD19+) and 105 K562 (CD19- negative control) cells with 1 μmol/l CellTrace CFSE and 1 μmol/l CellTrace violet respectively (Life Technology) and coincubate them with 105 effector CAR T cells (E/T ratio of 10:1) in a final volume of 100 μl X-Vivo-15 media, for 5 hours at 37 °C. Cells were then recovered and labeled with eFluor780 viability marker before being fixed by 4% PFA as described above. Fixed cells were then analyzed by flow cytometry to determine their viability. The frequency of specific cell lysis was calculated using the formula described in ref. 20 and displayed in the following:

\[
\text{Frequency of specific cell lysis} = \frac{\text{Via Daudi-T} \times \text{Via K562-T}}{\text{Via Daudi} \times \text{Via K562}}
\]

where Via Daudi-T and Via K562-T correspond respectively to the % of viable Daudi and K562 cells obtained after 5 H in the presence of CAR T cells and where Via Daudi and Via K562 correspond respectively to the % of Daudi and K562 cells obtained after 5 H in the absence of CAR T cells.

**Allogeneic mixed lymphocyte reaction.** 20 × 10⁴ PBMC freshly purified from a donor B (PBMC B) were labeled by a 10-minute incubation with 2 nmol/l CFSE in the dark, at 37 °C in a final volume of 5 ml. CFSE-labeled PBMC B were then diluted two times in fetal bovine serum, and washed with X-Vivo-15 media supplemented by 20 ng/ml IL-2 and 5% human AB serum. Cell number and viability was then determined and their concentration was adjusted to 1 × 10⁶ viable cells/ml. To perform mixed lymphocyte reaction, CFSE-labeled PBMC B were incubated with either T cells purified from the same blood donor (T cells B) or with CAR T cells engineered out of a different blood donor (CAR T cell A) in X-Vivo-15 media supplemented by 20 ng/ml IL-2 and 5% human AB serum. CFSE labeled PBMC viability, activation and proliferation were determined after 6 days of incubation, by flow cytometry and anti IFNy ELISA.

**Statistical analysis.** Viability values obtained on Daudi cells were averaged for each condition in each experiment (n = 2 with two different donors and n = 3 with three different donors for experiments performed with cytarabine and clofarabine respectively), and these averages were used in a paired t-test (pairing by experiment) to compare two different conditions and determine P values indicated in Figure 7.

**SUPPLEMENTARY MATERIAL**

**Figure S1.** High-throughput DNA sequencing analysis method

**Figure S2.** Simultaneous treatment of primary T cells with dCK and TRAC TALEN does not generate long-standing genetic adverse events.

**Figure S3.** TALEN-mediated dCK processing increased primary T cells resistance to clofarabine without needing time consuming and potentially harmful drug selection process.

**Figure S4.** Efficient expression of RQ8 depletion system at the surface CAR T.

**Figure S5.** TALEN-treated CAR T cells can proliferate in the presence of lethal dose of different purine and pyrimidine nucleotide analogues (PNAs).

**Figure S6.** TALEN-treated CAR T cells can proliferate in the presence of a combination of purine and pyrimidine nucleotide analogues (PNAs).

**Figure S7.** PNA treatment prevents allorreactivity of PBMC toward CAR T cells in vitro.

**Figure S8.** CAR T cell cytolytic activity is not affected by dual inactivation of dCK and TCRβ.

**Figure S9.** TALEN-treated CAR T cells retain their cytolytic activity after 2 to 9 days of culture in the presence of clinically relevant dose of clofarabine.

**Figure S10.** TALEN-treated CAR T cells can pair up with chemotherap- y for better antitumor activity.

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