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Research Article

Cell biology

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# TCR $\alpha\beta$ /CD3 disruption enables CD3-specific antileukemic T cell immunotherapy

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T cells engineered to express chimeric antigen receptors (CARs) against B cell antigens are being investigated as cellular immunotherapies. Similar approaches designed to target T cell malignancies have been hampered by the critical issue of T-on-T cytotoxicity, whereby fratricide or self-destruction of healthy T cells prohibits cell product manufacture. To date, there have been no reports of T cells engineered to target the definitive T cell marker, CD3 (3CAR). Recent improvements in gene editing now provide access to efficient disruption of such molecules on T cells, and this has provided a route to generation of 3CAR, CD3-specific CAR T cells. T cells were transduced with a lentiviral vector incorporating an anti-CD3 $\epsilon$  CAR derived from OKT3, either before or after TALEN-mediated disruption of the endogenous TCR $\alpha\beta$ /CD3 complex. Only transduction after disrupting assembly of TCR $\alpha\beta$ /CD3 yielded viable 3CAR T cells, and these cultures were found to undergo self-enrichment for 3CAR<sup>+</sup>TCR<sup>-</sup>CD3<sup>-</sup> T cells without any further processing. Specific cytotoxicity against CD3 $\epsilon$  was demonstrated against primary T cells and against childhood T cell acute lymphoblastic leukemia (T-ALL). 3CAR T cells mediated potent antileukemic effects in a human/murine chimeric model, supporting the application of cellular immunotherapy strategies against T cell malignancies. 3CAR provides a bridging strategy to achieve T cell eradication and leukemic remission ahead of conditioned allogeneic stem cell transplantation.

## Introduction

T cell acute lymphoblastic leukemias (T-ALL) and T cell lymphoblastic lymphoma account for around 15% of pediatric and 25% of adult ALL. These are heterogeneous conditions and often exhibit extramedullary and CNS involvement. Outcome for childhood T-ALL has improved over time with advances in pediatric ALL therapy, and long-term survival is over 75% (1). Nonetheless, most relapsing subjects face high levels of morbidity and mortality, and survival in adults is less favorable, highlighting a need for innovative interventions.

There has been notable progress in the development of engineered T cell therapies against B cell malignancies, and chimeric antigen receptors (CARs) against B cell antigens including CD19, CD20, CD22, and other targets have proven highly effective against refractory B cell leukemia (2). CARs usually comprise an extracellular domain derived from an antibody single-chain variable fragment (scFv), linker, transmembrane moiety, and activation domains derived from combinations of CD28, 41BB, and CD3 (3). Most of these therapies have been generated in a bespoke manner using autologous or HLA-matched allogeneic peripheral blood mononuclear cell (PBMC) harvests, although recently nonmatched universal donor CAR19 T cells manufactured using TALEN (transcription activator-like effector nuclease) gene editing have been reported (4, 5). The development of similar approaches against T cell malignancies has been extremely challenging, not least because of fratricidal T-on-T cytotoxicity during cell manufacture. Certain cell surface antigens such as CD5 (6) have been targeted using specific CARs, and downregulation of their expression in activated T cells has allowed effector cells to expand and yield a product. Similarly, CD4 has been targeted by CD8<sup>+</sup> T cells expressing a CD4-specific CAR (7), and CARs discriminating between T cell receptor constant regions (TRBC1 or TRBC2) have also been developed, with the aim of eradicating whichever of the 2 compartments malignant transformation may have arisen in (8). In addition, CRISPR/Cas9-mediated disruption of CD7 expression has also recently been reported in T cells transduced to express a CD7-specific CAR (9), as well as alternative strategies deploying an anti-CD7 scFv linked to

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endoplasmic reticulum retention domains, to prevent cell-surface expression of CD7 (10). Furthermore, CD7 specific universal CAR T cells depleted of both CD7 and T cell receptor expression have also been generated (11). However, to date, there have been no reports to our knowledge of CAR T cell strategies targeting the definitive T cell marker CD3. Previous attempts to generate anti-CD3 CAR cells have been limited to NK cells that did not express CD3 but were then able to specifically eliminate CD3<sup>+</sup> lymphoma lines and primary T cell targets in vitro, as well as CD3<sup>+</sup> Jurkat leukemic T cells in human/murine chimeras (12).

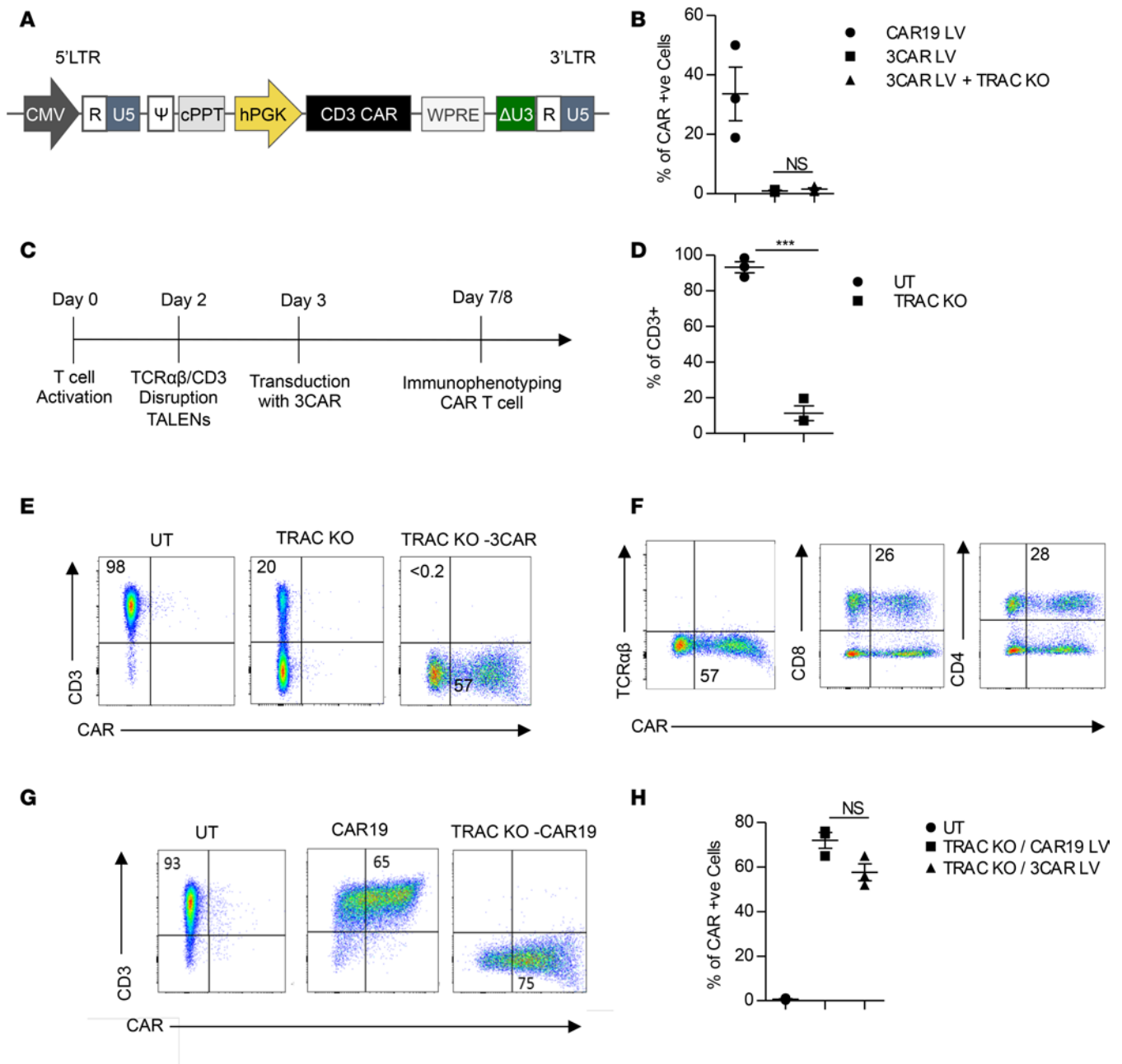
We report anti-T cell immunotherapy using 3CAR, a CAR against the CD3 $\epsilon$  chain of the T cell receptor complex, generated by fusing the scFv regions of OKT3, a widely used anti-CD3 $\epsilon$  monoclonal antibody, with a transmembrane stalk and intracellular activation domains from 41BB and CD3 $\zeta$  (Figure 1A). Effective manufacturing of anti-CD3-specific T cells was only possible following critically scheduled TALEN-mediated disruption of the TCR $\alpha\beta$ /CD3 complex; once modified, 3CAR T cells rapidly dominated cultures and self-enriched without the need for further processing to deplete residual TCR $\alpha\beta$  T cells required for other gene-edited CAR T cells (5). Anti-CD3 cellular immunotherapy could deliver valuable antileukemic effects in relapsed refractory disease and, in the first instance, would be deployed as a form of cellular conditioning to secure molecular remission ahead of allogeneic stem cell transplantation.

## Results

We generated 3CAR by fusing the scFv fragment of OKT3 (a therapeutic-use anti-CD3 $\epsilon$  monoclonal antibody) to a CD8 transmembrane region linked to 41BB and CD3 $\zeta$  signaling domains. Lentiviral expression of 3CAR (Figure 1A) in cell lines lacking expression of CD3 $\epsilon$  (HEK293T cells) was found to be stable, dose dependent, and saturable at high multiplicities of infection (MOI) (Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.99442DS1>). However, in primary CD3<sup>+</sup> T cells, lentiviral transduction of T cells activated with anti-CD3/CD28 (TransAct reagent) resulted in inefficient 3CAR gene transfer, with only around 10% modified cells present after 4 days and none detectable by day 11 (Supplemental Figure 1B) as a result of T-on-T cytotoxicity. In order to alleviate 3CAR-mediated destruction of T cells, gene editing was employed to disrupt assembly of the multimeric TCR $\alpha\beta$ /CD3 complex. We reasoned that disruption and nonhomologous end joining-mediated repair of the T cell receptor  $\alpha$  constant (*TRAC*) locus would not only prevent expression of TCR $\alpha$ , but also disrupt cell-surface CD3 $\epsilon$  expression. First, we attempted to disassemble the TCR $\alpha\beta$ /CD3 multimeric complex expression on T cells by electroporation of *TRAC*-specific TALEN mRNA immediately after activation and lentiviral transduction with 3CAR (Supplemental Figure 1C). This reflected our existing approach for the generation of universal TCR $\alpha\beta$ -CAR19<sup>+</sup> T cells (4, 5). This order of transduction followed by editing was known to support high levels of proliferation in T cells, and it was hypothesized that constitutive CAR signaling in TCR<sup>-</sup> T cells provides resilience and promotes survival through subsequent gene-editing steps. Whilst around 80% of cells were disrupted for both CD3 and TCR $\alpha\beta$ , the eventual 3CAR T cell yield was poor (Figure 1B and Supplemental Figure 1, C and D), in contrast to CAR19 vectors, and this suggested widespread targeting of CD3 $\epsilon$  antigen leading to self destruction and/or rapid fratricide of other CD3<sup>+</sup> T cells in the culture.

Next, the event sequence was reversed to first deplete endogenous CD3 $\epsilon$  expression in activated T cells, and then undertake lentiviral (LV) transduction within an 18- to 24-hour time window (Figure 1, C and D, and Supplemental Figure 2). Here, within 7 days of initiation, 57% of T cells expressed 3CAR (Figure 1E) with approximately equal proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 1F) and with comparable outcomes to vectors expressing CAR19 (Figure 1, G and H). Importantly, the cultures self-enriched for T cells devoid of CD3/TCR $\alpha\beta$ , obviating the need for any further processing to deplete residual TCR<sup>+</sup> T cells that comprised <0.2% of the product. In contrast, residual TCR<sup>+</sup> populations in TCR<sup>-</sup>CAR19<sup>+</sup> cell products required removal by bead-mediated depletion by targeting TCR $\alpha\beta$  to achieve a target of <1%TCR<sup>+</sup> cells in the final product. This is a stringent threshold that strongly influences allogeneic dosing strategies where graft versus host disease (GVHD) is considered unlikely by capping TCR $\alpha\beta$  carriage to  $<5 \times 10^4$ /kg.

To assess scalability, cells were activated, edited, and transduced, and  $2 \times 10^6$  3CAR T cells were cultured further in a gas-permeable static cell culture flask (G-Rex). Cells expanded by 100-fold over 11 days (Figure 2A) and were devoid of TCR/CD3-expressing cells with residual populations of <0.5% (Figure 2B). After electroporation of *TRAC* TALEN mRNA, CD3<sup>+</sup>TCR<sup>+</sup> T cells were selectively depleted in 3CAR T cell cultures (Figure 2B). Tracking indels by decomposition PCR(TIDE PCR) sequencing across the *TRAC* locus confirmed an allele-modification frequency of 62.9% based on molecular signatures of nonhomologous end joining repair (Figure 2C). As the *TRAC* locus exhibits allelic exclusion (13), this fre-



**Figure 1. Successful generation of 3CAR requires disruption of CD3ε expression prior to lentiviral 3CAR transduction.** (A) Vector configuration showing codon optimized single-chain variable fragment (scFv) derived from OKT3 with CD8 stalk and 41BB/CD3ζ activation domains, all under the control of an internal human phosphoglycerate kinase (hPGK) promoter in a third-generation self-inactivating (SIN) lentivirus with HIV-1-derived reverse response element (RRE), central polypurine tract (cPPT), and mutated woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). Human cytomegalovirus (CMV); Repeat region (R); Unique 5' region (U5); Psi (Ψ); and modified unique 3' region (ΔU3). (B) Percentage of CAR<sup>+</sup> cells with and without TRAC-KO prior to lentivirus (LV). Data are presented as mean ± SEM. *n* = 3 donors. (C) Schema of event precedence required for successful 3CAR T cell production. CD3/CD28 activation with TransAct for 48 hours was followed by electroporation of mRNA encoding TRAC-specific TALENs ahead of LV transduction by 72 hours. (D) Summary of CD3<sup>+</sup> cells following TRAC-KO (*n* = 3 donors), mean ± SEM. \*\*\**P* < 0.0005, by unpaired, 2-tailed Student's *t* test. (E) Representative flow cytometry plots of day 7 cells. In cultures with 3CAR expression, there were no surviving CD3<sup>+</sup> cells compared with 20% residual expression in the absence of 3CAR transduction (*n* = 3 donors). (F) Representative flow cytometry plots showing 3CAR T cells retained CD4 and CD8, but not TCRαβ, expression; *n* = 3 donors. (G) For comparison, control transductions with LV CAR19 mediated 65% transduction but required further processing by column-mediated TCRαβ depletion to yield TRAC-KO CAR19<sup>+</sup> T cells; *n* = 3 donors. (H) Summary of CAR<sup>+</sup> cells following TRAC-KO (*n* = 3 donors); mean ± SEM. Two-tailed Student's *t* test.

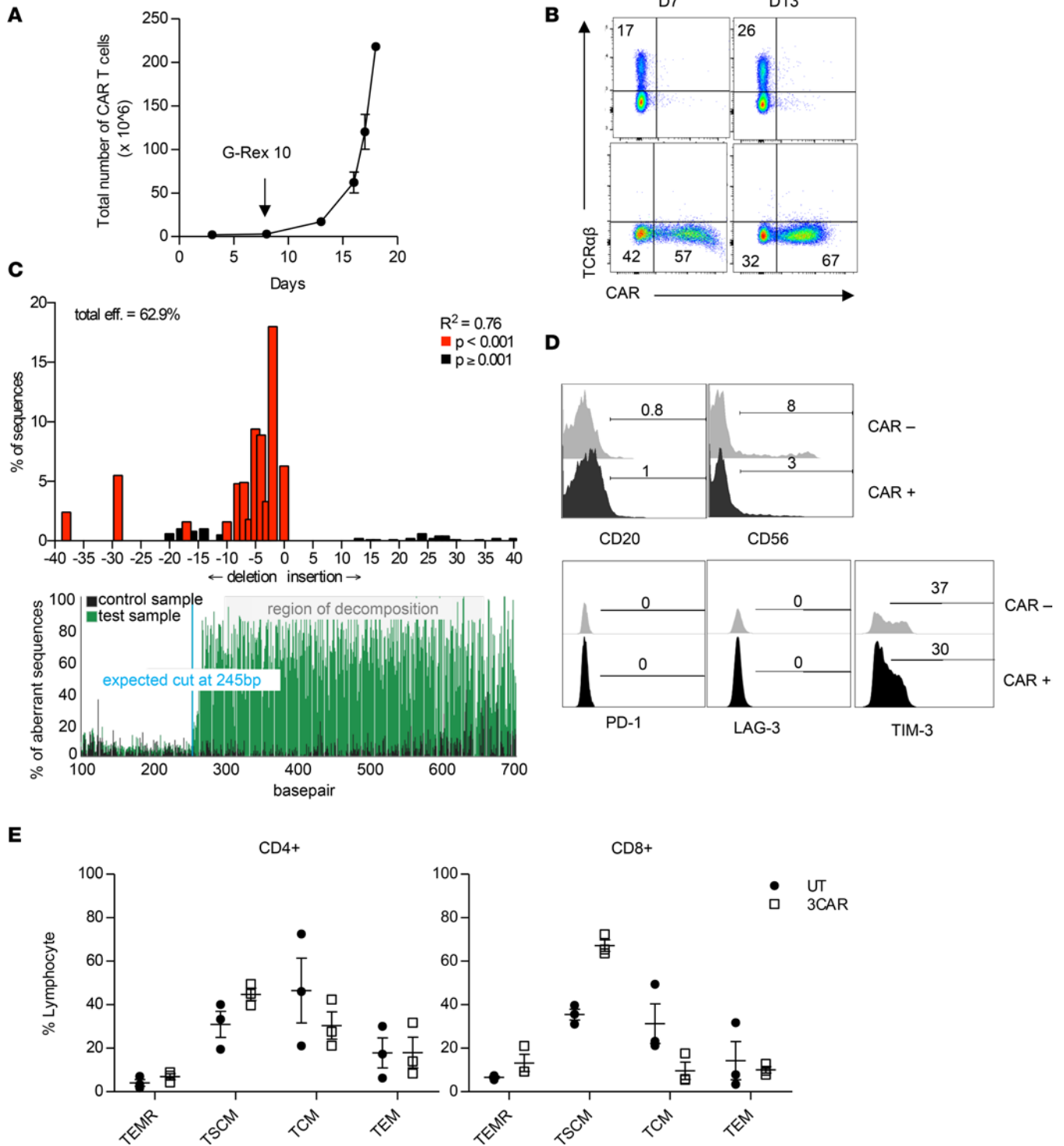
quency of modification was consistent with the near-complete TCR disruption assessed by flow analysis. Flow cytometric analysis for relevant subset markers was performed at the end of expansion (gated on CAR<sup>+</sup> and CAR<sup>-</sup> populations) and revealed enrichment of 3CAR T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) and negligible residual B cells (CD20) or NK cells (CD56). There was very little change in expression of T cell exhaustion markers PD-1, LAG-3, and TIM-3 (Figure 2D), and the majority of 3CAR T cells exhibited a central memory phenotype (Tcm CD45<sup>-</sup>CD62L<sup>+</sup>) or stem cell memory phenotype (stem memory T cell [Tscm] CD45<sup>+</sup>CD62L<sup>+</sup>CD95<sup>+</sup>) (Figure 2E and Supplemental Figure 3). 3CAR T cells exhibited IFN- $\gamma$  production responses against TCR/CD3<sup>+</sup> Jurkat leukemia cells (Supplemental Figure 4) and mediated CD3 $\epsilon$ -specific cytotoxicity in vitro. Chromium 51-labeled (<sup>51</sup>Cr-labeled) TCR/CD3<sup>+</sup> or TCR/CD3<sup>-</sup> Jurkat leukemia cells (14) were cocultured with either 3CAR T cells or nontransduced control T cells. 3CAR<sup>+</sup> T cells mediated specific high-level cytotoxicity of targets expressing TCR/CD3<sup>+</sup> but not TCR/CD3<sup>-</sup> targets (Figure 3, A and B). Next, a flow-based assay also confirmed that EGFP<sup>+</sup>TCR/CD3<sup>+</sup> Jurkat cells were almost completely eliminated by 3CAR cells (0.8%), compared with untransduced effector controls (61%). TCR/CD3<sup>-</sup> leukemia cells were unaffected by 3CAR (57%) or effector controls (54%) (Figure 3, C and D). 3CAR T cells also mediated cytotoxic effects against purified primary CD3<sup>+</sup> T cells is loaded with carboxyfluorescein succinimidyl ester (CFSE) dye. Only around 4% of these cells survived, compared with 42% in control cultures, and TCR/CD3<sup>-</sup> purified populations were not eliminated by 3CAR cells, confirming specificity against CD3 $\epsilon$  (Figure 3, C and D).

To further evaluate the function of 3CAR T cells, cultures were established against allogenic and autologous primary healthy donor PBMC. <sup>51</sup>Cr-labeled allogenic or autologous PBMC were cocultured with either 3CAR T cells or nontransduced control T cells. In both settings, 3CAR<sup>+</sup> cells mediated high-level cytotoxicity compared with untransduced effector cells (Figure 4A). On a single-cell level, flow cytometry analysis confirmed the high level of specific cytotoxicity mediated by 3CAR cells. CFSE-loaded healthy donor PBMC cultured with 3CAR T cells resulted in reduced CD3-expressing cells (8% CD3<sup>+</sup> cells) compared with untransduced T cell effectors (93% CD3<sup>+</sup> cells), without eliminating CD7<sup>+</sup>CD3<sup>-</sup> cells, nor CD19<sup>+</sup> B cells, whereas control CAR19 cells did not target T-lymphocytes (Figure 4B). 3CAR T cells cultured with allogenic PBMC or purified allogenic TCR/CD3<sup>+</sup> T cells exhibited IFN- $\gamma$  responses and low-level secretion of TNF- $\alpha$ , IL-10, IL-6, and IL-4 (Figure 4C and Supplemental Figure 4). 3CAR T cells also exhibited notable <sup>3</sup>H-thymidine proliferation responses when cocultured with irradiated allogenic CD3<sup>+</sup> T cells. Proliferation against irradiated allogenic CD3-depleted PBMC was reduced, but control T cell-mediated allorecognition was intact (Figure 4D). These data suggest that 3CAR can mediate elimination of healthy TCR $\alpha\beta$ /CD3 T cells, an effect that could be harnessed during lymphodepletion procedures ahead of allogeneic transplantation.

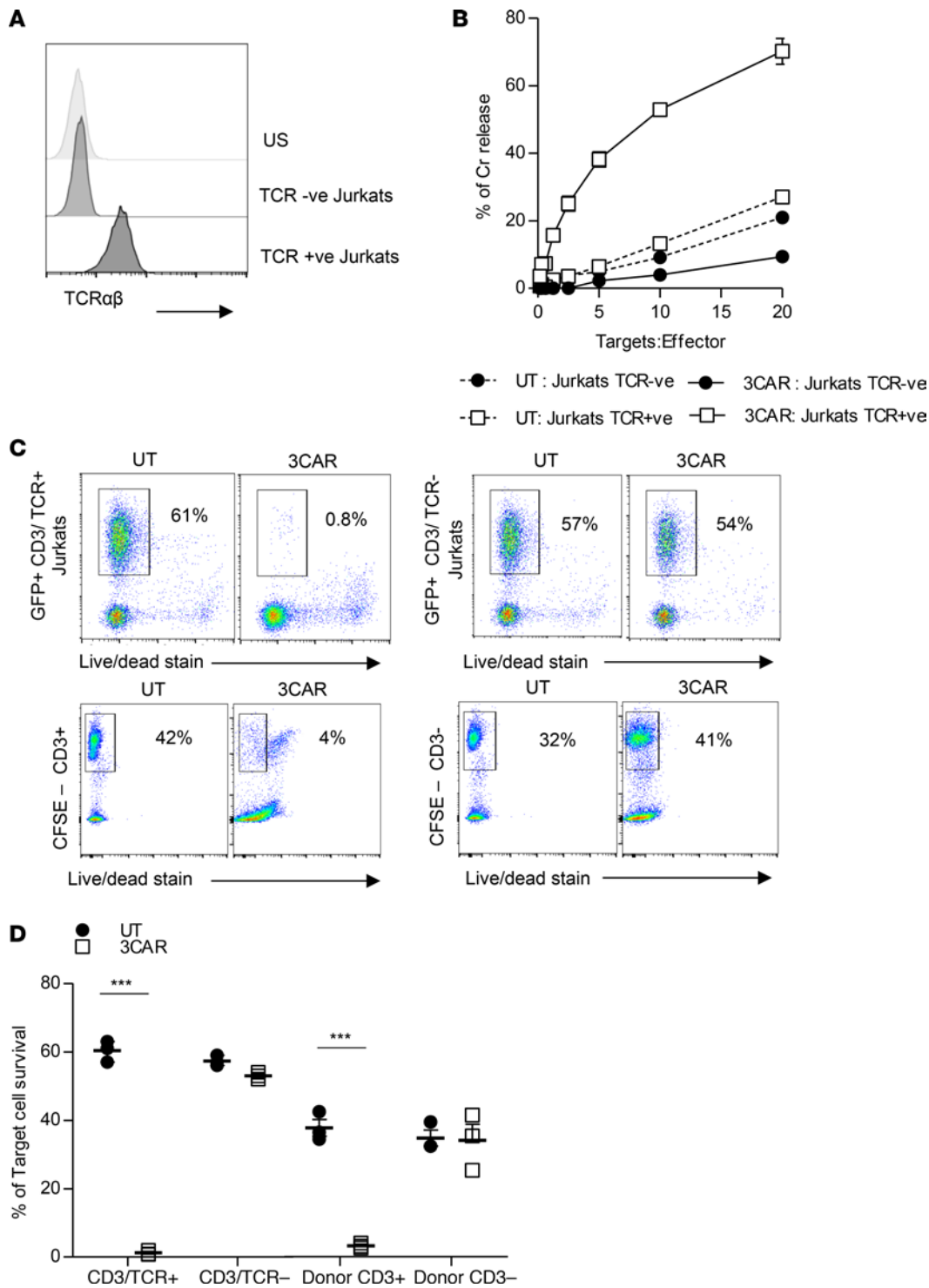
The function of 3CAR T cells was also assessed against viable tissue bank samples from children with T-ALL. Flow cytometry verified CD3, CD7, CD19, and CD34 expression upon thawing in these targets (Table 1). In the case of T-ALL#1 cells, 86% CD3-expressing cells were almost completely eliminated by 3CAR<sup>+</sup> cells (Figure 5A). Despite the phenotypic heterogeneity of the T-ALL, in 6 of 6 samples, 3CAR cells displayed highly specific cytotoxicity against CD3<sup>+</sup> but not CD3<sup>-</sup>CD19<sup>+</sup> cells, CD3<sup>-</sup>CD7<sup>+</sup> cells, and CD3<sup>-</sup>CD34<sup>-</sup> cells (Figure 5, B and C, and Supplemental Figure 5).

In vivo functionality of 3CAR T cells was assessed in a humanized murine model of leukemic T cell clearance. NOD/SCID/ $\gamma$ c<sup>-/-</sup> (NSG) mice were inoculated i.v. with 10  $\times$  10<sup>6</sup> TCR/CD3<sup>+</sup>EGFP<sup>+</sup>LUC<sup>+</sup> or TCR/CD3<sup>-</sup>EGFP<sup>+</sup>LUC<sup>+</sup> Jurkat T cell targets and imaged after 3 days to confirm leukemia establishment, and they were then injected with effector cells. These comprised either 3CAR or untransduced effector T cells. Serial bioluminescence imaging on days 3–18 showed rapid clearance of TCR/CD3<sup>+</sup> Jurkat cells in the cohort receiving 3CAR T cells, with negligible signal detected by day 11, in contrast to mice receiving nontransduced T cells or PBS where leukemia progressed (Figure 6, A and B). By day 18, disease burden had increased by >60-fold in animals receiving untransduced effectors compared with the 3CAR effector group (Figure 6C). As expected, neither 3CAR effectors nor control T cells exhibited an antileukemic effect against TCR/CD3<sup>-</sup> Jurkat cells lacking target antigen expression (Supplemental Figure 6). At necropsy, flow cytometry was used to detect EGFP<sup>+</sup> Jurkat T cells and/or CD45<sup>+</sup>CD2<sup>+</sup>EGFP<sup>-</sup> effector T cell populations in BM (Figure 6D). Analysis from day 18 samples found complete clearance of TCR/CD3<sup>+</sup> Jurkat T cells in 5 of 5 animals in 3CAR-treated animals compared with control animals receiving PBS or untransduced T cells (Figure 6D and Supplemental Figure 6A). In most animals, only very small numbers of TCR-EGFP<sup>+</sup> Jurkat cells were iden-



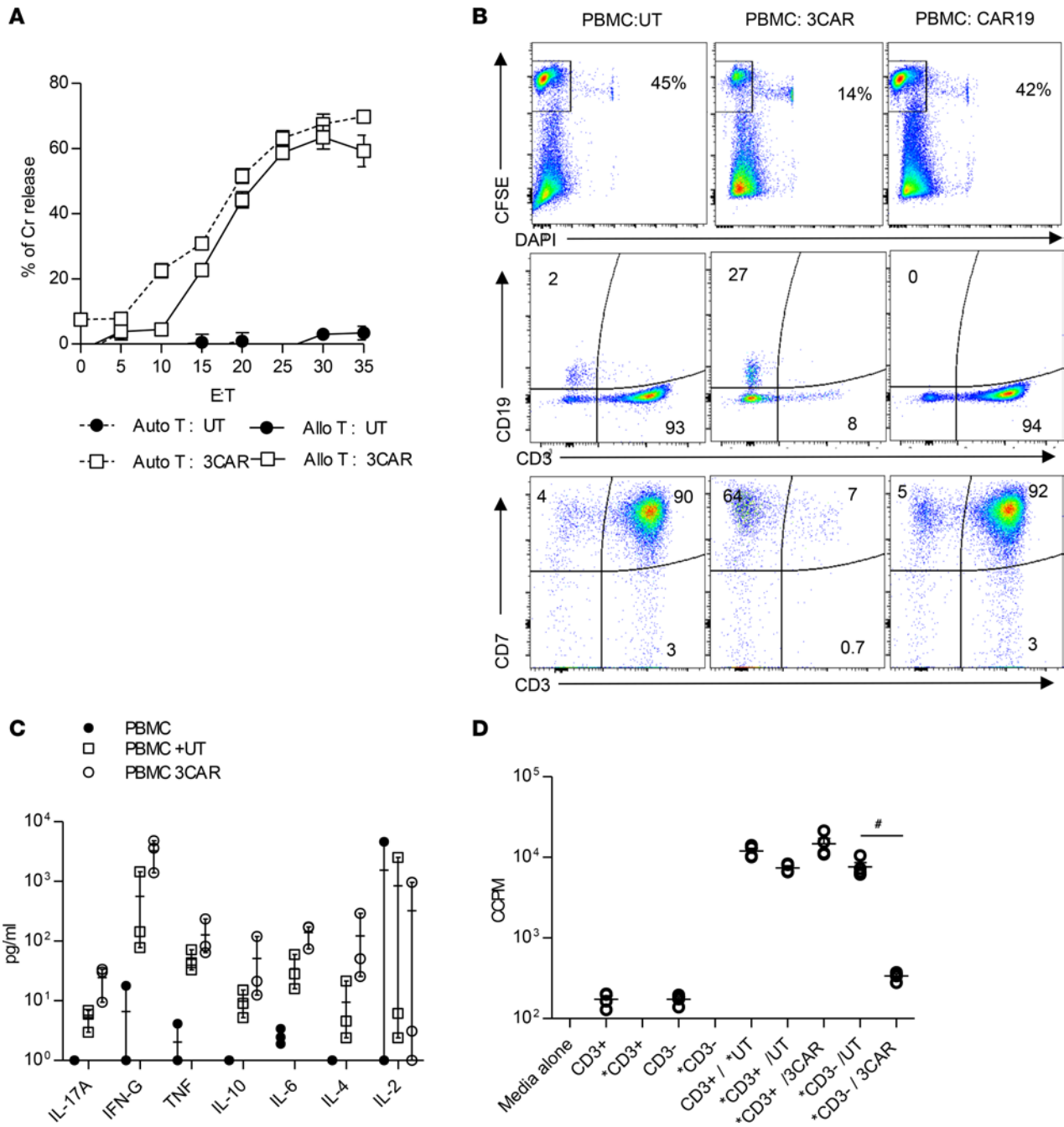


**Figure 2. 3CAR T cell expansion and self-enrichment.** (A) Primary T cells were activated, and following TALEN *TRAC* mRNA exposure,  $2 \times 10^6$  T cells were transduced and expanded in a G-Rex flask over 17 days;  $n = 2$  donors. (B) Representative expression of CD3/TCR in *TRAC*-KO untransduced (top panel) and 3CAR T cells (lower panel) on day 7 and 13, confirming the absence of CD3/TCR $\alpha\beta$  cells after 3CAR lentiviral expression;  $n = 3$  donors. (C) Tracking indels by decomposition PCR (TIDE-PCR) confirmed 62.9% of alleles harbored molecular signatures of nonhomologous end joining across the *TRAC* locus ( $n = 2$ ). (D) Representative flow cytometry plots at end of manufacture for CD20 and CD56 expression and exhaustion markers PD-1, LAG-3, and TIM-3 (gated on CAR $^+$  and CAR $^-$  populations);  $n = 3$  donors. (E) The percentage of memory T cell markers on expanded day 17 cells;  $n = 3$  donors, mean  $\pm$  SEM.



**Figure 3. 3CAR T cells mediate potent killing of CD3<sup>+</sup> cells in vitro.** (A) TCR<sup>+</sup> and TCR<sup>-</sup> Jurkat target cells were stained for TCR expression prior to coculture experiments. (B) <sup>51</sup>Cr-labeled CD3<sup>+</sup>TCR<sup>+</sup> (white symbols) or CD3<sup>-</sup>TCR<sup>-</sup> (black symbols) Jurkat leukemia cells were cocultured with either 3CAR T cells (solid lines) or untransduced controls (dotted lines); (n = 3) from 3 donors. (C) Upper panel, 3CAR T cells were cocultured with either GFP<sup>+</sup>CD3<sup>+</sup>TCR<sup>+</sup> or GFP<sup>+</sup>CD3<sup>-</sup>TCR<sup>-</sup> Jurkat leukemia cells and, in the lower panel, cocultured with either CFSE-loaded primary CD3<sup>+</sup> or CD3<sup>-</sup> cells at the effector target ratio of 1:1 for 24 hours. Representative flow cytometry plots of gated on GFP<sup>+</sup> or CFSE<sup>+</sup> cells at the end of coculture demonstrate target-specific killing. (D) Summary of 3CAR cytotoxicity as in C where data are presented as mean ± SEM; n = 3 per group. \*\*\*P < 0.0005, by unpaired, 2-tailed Student's t test.

tified, except 1 animal where there appeared to have been an outgrowth of CD3<sup>-</sup>EGFP<sup>+</sup> Jurkat cells that had presumably not been to susceptible to 3CAR-mediated killing (Figure 6E and Supplemental Figure 7). 3CAR effectors retained expression of their chimeric receptor and CD4<sup>+</sup>/CD4<sup>-</sup> marking at levels comparable with those in the original inoculum (Figure 6D and Supplemental Figures 7 and 8). Although 3CAR expression (24%) was detected in animals engrafted with CD3<sup>-</sup>TCR<sup>-</sup> Jurkat cells, the absence of suitable target antigen resulted in rapid accumulation of disease in these animals (Supplemental Figure 7).



**Figure 4. 3CAR T cell functionality against healthy donor PBMC.** (A) <sup>51</sup>Cr-labeled peripheral blood mononuclear cell (PBMC) in autologous (dotted lines) or allogenic (solid lines) coculture. PBMC were cultured with either 3CAR T cells (white symbols) or untransduced controls (black symbols) (*n* = 3). (B) 3CAR, CAR19, or untransduced T cells were cocultured with CFSE-loaded healthy donor PBMC at the effector target ratio of 1:1 for 24 hours. Top panel shows representative frequency of gated CFSE<sup>+</sup> target cells at the end of coculture. Flow cytometry plots below show representative frequency of surface antigen markers CD3, CD19, and CD7 on gated CFSE<sup>+</sup> tumor cells, displaying the specificity in mixed cell populations; *n* = 3 donors. (C) Cytokine production by 3CAR T cells when cocultured with healthy donor PBMC target cells were comparable with untransduced cells; *n* = 3 donors. (D) Mixed lymphocyte reactions of 3CAR or untransduced T cells cultured with irradiated (\*) allogeneic PBMC or CD3<sup>-</sup> cells. <sup>3</sup>H-thymidine proliferation responses are consistent with specific targeting of CD3 rather than alloreactive proliferation); mean ± SEM, 4 experimental replicates (*n* = 2 donors). \**P* < 0.05, by unpaired, 2-tailed Student's *t* test. Corrected counters per minute, CCPM.

### Discussion

Following successful elimination of B cell malignancies using CAR T cells targeting a variety of B cell antigens (most notably CD19), attempts are underway to generate similar therapies against T cell malignancies. Recent publications have reported targeting CD5 and CD7, the latter in combination with CRIS-



**Table 1. Immunophenotype of 6 T-ALL samples tested**

Percent expression of surface antigen ( $\pm$ 2%)				
	CD3	CD7	CD19	CD34
T-ALL#1	86	83	11	1.5
T-ALL#2	40	86	10	8
T-ALL#3	43	94	0	33
T-ALL#4	21	98	0	94
T-ALL#5	7	96	16	38
T-ALL#6	1.5	94	0.5	96

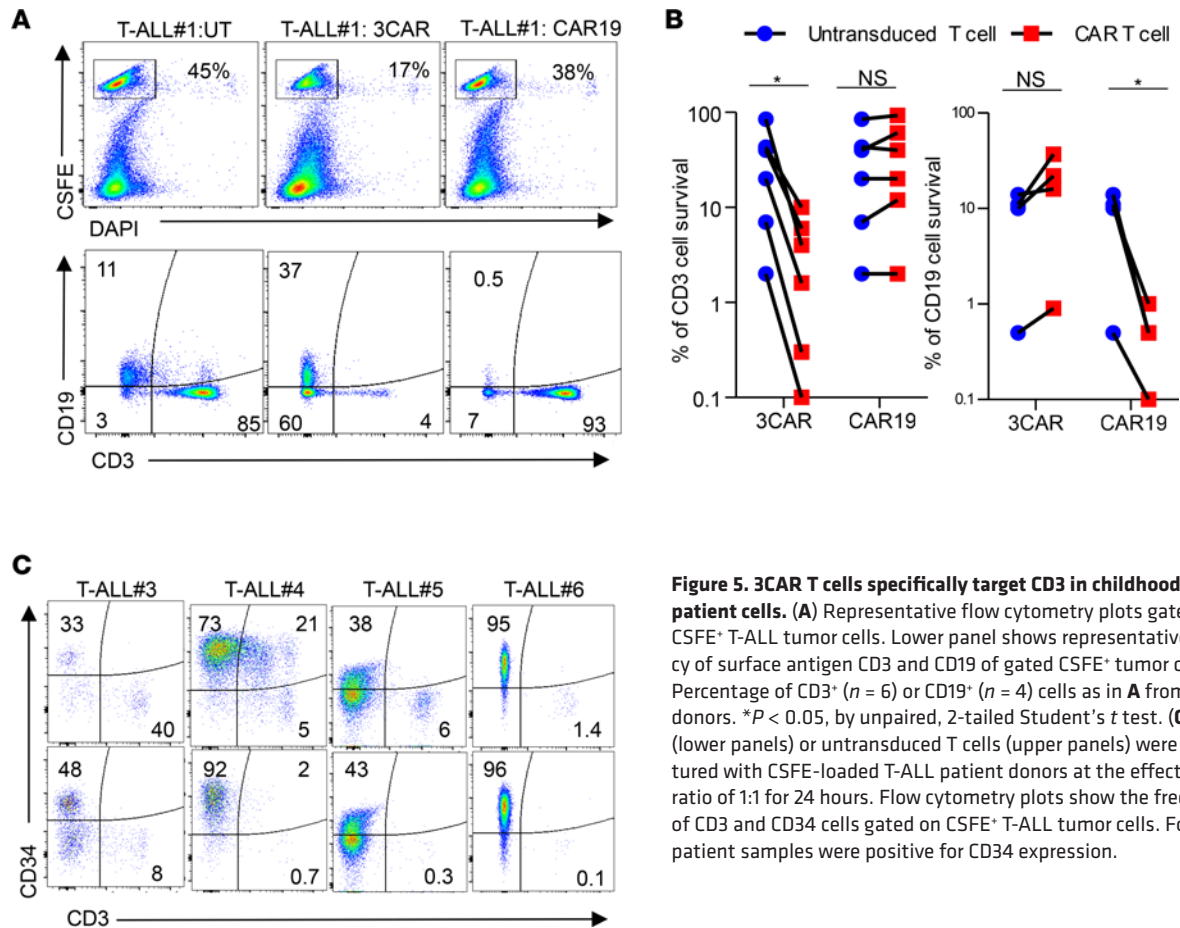
PR-mediated disruption of endogenous CD7 expression on T cells. To date, there have been no reports to our knowledge of CAR therapy targeting the definitive T cell marker CD3, which is widely expressed on T cell lymphomas and also expressed with variable levels of intensity on acute T cell leukemias. Previous attempts to generate anti-CD3 CAR cells have only been successful using NK cells that did not express CD3. CD3CAR NK-92 cells were shown to specifically eliminate CD3<sup>+</sup> lymphoma cell lines and primary T cell targets in vitro and CD3<sup>+</sup> Jurkat cells in human/murine chimeras (12).

The gene-editing platform used in this iteration of 3CAR was based on TALENs and extends our previous experience with similar reagents for CAR19 aimed at securing molecular remission ahead of allo-stem cell transplantation (allo-SCT) for relapsed/refractory B-ALL. We reported minimal evidence of off-target effects using high-throughput NGS interrogation of in silico predicted activity of *TRAC*-specific TALENs (5). Alternative editing platforms may be as effective, although are likely to need to operate within a similarly well-defined window of activity to ensure target antigen removal before 3CAR expression.

We have found that gene editing to disrupt TCR expression can be exploited to enable 3CAR T cells to be generated through avoidance of T-on-T killing and fratricide effects. Previously described TALENs specific for the *TRAC* locus (15) were delivered by electroporation of mRNA and mediated highly efficient disruption of the TCR $\alpha$  chain, which in turn resulted in failure to assemble cell-surface CD3/TCR complexes. A therapeutic monoclonal antibody OKT3 targeting CD3 $\epsilon$  has been previously used in transplant settings to lymphodeplete T cells. The scFv of this antibody was incorporated into a CAR configuration by fusion to a CD8 transmembrane region, 41BB signaling domain, and CD3 $\zeta$  elements. Delivery by lentiviral vector was combined with delivery of TALEN mRNA and conditions investigated to determine optimal order of events for effective production of TCR<sup>-</sup>3CAR<sup>+</sup> T cells. In previous campaigns, we had found that the generation of universal TCR<sup>-</sup>CAR19<sup>+</sup> T cells was most efficient when *TRAC* disruption was performed after lentiviral transduction. However, a similar schedule for 3CAR resulted in poor yields, presumably because prior expression of 3CAR mediated detection of CD3 $\epsilon$ . A strictly ordered sequence of *TRAC* disruption ahead of 3CAR expression was found to be essential for the generation of 3CAR, and this event precedence was incorporated into scaled production of effector cells.

This strictly ordered sequence of *TRAC* disruption ahead of 3CAR expression was incorporated into scaled production of effector cells. The resulting 3CAR cells were characterized in detail by flow cytometry, cytokine profiles, and functional assessments of alloreactivity and cytotoxicity. The potency of these responses was also confirmed in a human/murine chimeric model where animals infused with 3CAR had greatly reduced tumor burdens compared with control groups.

The expression of CD3 on leukemic T cells can be variable, but critically 3CAR-mediated cytotoxicity of CD3<sup>+</sup> targets was near complete in 6 of 6 samples evaluated. It is likely that, for effective elimination of heterogenous leukemias, a strategy targeting multiple T cell antigens will be required, akin to combination antibiotic therapy for mycobacterial disease or antiviral therapy against HIV. Based on our analysis, a combination of CARs targeting CD3 and CD7 could be highly effective against childhood T-ALL. Applications in patients with CD3<sup>+</sup> ALL refractory to conventional chemotherapy is envisaged, most likely as a bridge to transplant after achieving molecular remission. There are trials ongoing whereby universal CART19 (<https://clinicaltrials.gov/>; NCT02808442) for B-ALL or UCART123 (NCT03190278) or AML are being tested for their ability to secure remission ahead of allo-SCT. Similarly, premanufactured 3CAR



**Figure 5. 3CAR T cells specifically target CD3 in childhood T-ALL patient cells.** (A) Representative flow cytometry plots gated on CSFE<sup>+</sup> T-ALL tumor cells. Lower panel shows representative frequency of surface antigen CD3 and CD19 of gated CSFE<sup>+</sup> tumor cells. (B) Percentage of CD3<sup>+</sup> ( $n = 6$ ) or CD19<sup>+</sup> ( $n = 4$ ) cells as in A from T-ALL donors. \* $P < 0.05$ , by unpaired, 2-tailed Student's  $t$  test. (C) 3CAR (lower panels) or untransduced T cells (upper panels) were cocultured with CSFE-loaded T-ALL patient donors at the effector target ratio of 1:1 for 24 hours. Flow cytometry plots show the frequency of CD3 and CD34 cells gated on CSFE<sup>+</sup> T-ALL tumor cells. Four of 6 patient samples were positive for CD34 expression.

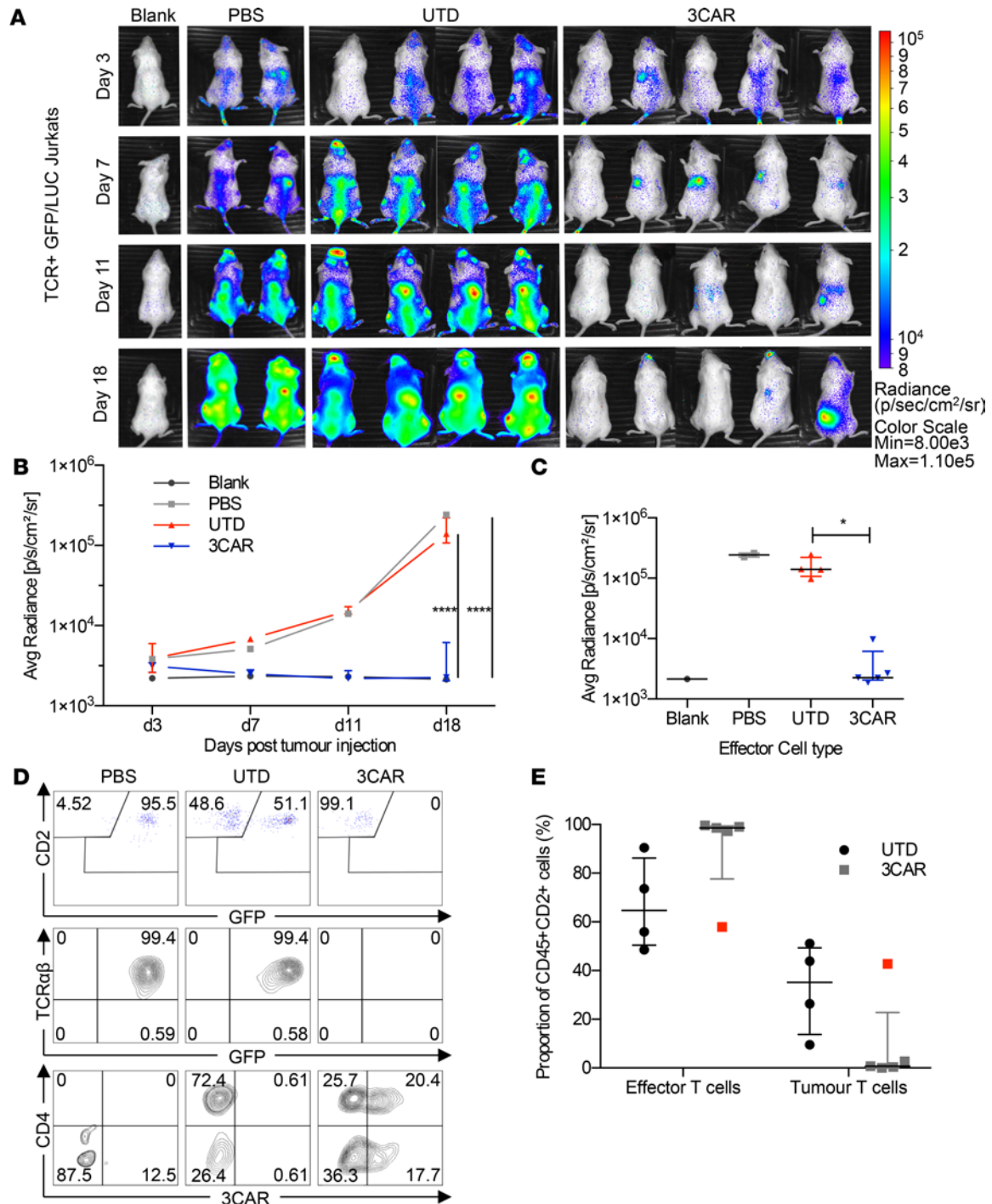
T cells from a non-HLA-matched allogeneic donor could be used in an off-the-shelf manner for refractory T cell malignancy. However, elimination of effector cells will be critical because, whereas suppression of the normal B cell compartment by persisting CAR19<sup>+</sup> T cells can be addressed by administration of replacement immunoglobulin therapy, long-term T cell lymphopenia mediated by 3CAR would be prohibitive. Inclusion of a suicide gene mechanism could be used for elimination of 3CAR cells, and we have confirmed the feasibility of incorporating such elements in our lentiviral system. Alternatively, additional conditioning and/or serotherapy could be used to eradicate 3CAR, ahead of donor-derived T cell reconstitution in the allogeneic stem cell transplantation setting, and this has been achieved using combinations of serotherapy and chemotherapy. Highly effective, high-depth 3CAR cell-based eradication of host T cell immunity may also be of broader interest as a transplant conditioning strategy as an alternative to intensive chemotherapy-based lymphodepletion.

## Methods

**Cell lines.** TCR/CD3<sup>+</sup> and TCR/CD3<sup>-</sup> Jurkat cells (acute T cell leukemic cell line from ATCC) were maintained in culture in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (MilliporeSigma).

**Generation of anti-CD3 CAR.** 3CAR was derived by codon optimization (GeneArt) of variable heavy-chain and variable light-chain antigen binding elements of the mouse anti-human CD3 monoclonal antibody, OKT3 sequence (16). The scFv was linked to activation domains derived from 41BB and CD3 $\zeta$ . The resultant 3CAR construct was cloned into a lentiviral vector (pCCL) under the control of a PGK promoter (Figure 1). Vector stocks pseudotyped with a vesicular stomatitis virus glycoprotein (VSV-G) envelope were generated in 293T cells (ATCC) and yielded titres  $>1 \times 10^9$  transducing units/ml after ultracentrifugation (17) and included control LV-CAR19 vectors (18).

**Generation of CAR-modified T cells.** PBMC were obtained from healthy donors and were cultured in 48-well plates at a density of  $1 \times 10^6$ /ml in TexMACS (Miltenyi Biotec), 3% human serum (Seralab) +



**Figure 6. Antileukemic responses by 3CAR effector T cells against CD3<sup>+</sup>TCR<sup>+</sup> GFP/luciferase human leukemia in immunodeficient mice.** (A) Serial bioluminescence imaging (BLI) of NSG mice (representative images from each cohort) following i.p. administration of D-luciferin substrate showing elimination of CD3<sup>+</sup> leukemia by 3CAR T cells but not by untransduced T cells. (B) Kinetics of systemic leukemia progression in mice following administration PBS ( $n = 2$ ), untransduced T cells ( $n = 4$ ), or 3CAR ( $n = 5$ ) effectors. Error bars represent median with interquartile range. Linear regression analysis showed significance between 3CAR vs. untransduced ( $****P < 0.0001$ ) and 3CAR vs. PBS ( $****P < 0.0001$ ) groups. (C) Average radiance values at termination (day 18) indicated significant difference in disease burden between untransduced and 3CAR effector-injected groups ( $*P = 0.0159$  by Mann-Whitney  $U$  test). Error bars represent median with interquartile range. (D) Upper panel, representative flow cytometry plots of T cells (gated on hCD45<sup>+</sup> cells) in BM. Middle panel, plots of leukemic T cell (CD3<sup>+</sup>TCR<sup>+</sup>GFP<sup>+</sup> Jurkat targets) after effector challenge (gated on hCD45<sup>+</sup>CD2<sup>+</sup> cells). Lower panel, plots of CAR T cells population (gated on hCD45<sup>+</sup>CD2<sup>+</sup>GFP<sup>-</sup> expression). (E) Proportion of GFP<sup>+</sup> Jurkat leukemia or GFP<sup>-</sup> effector T cells out of CD45<sup>+</sup>CD2<sup>+</sup> population in BM of untransduced or 3CAR-injected mice. Red marking highlights an animal that exhibited selective outgrowth of GFP<sup>+</sup>CD3<sup>-</sup> Jurkat cells after 3CAR therapy.

20 ng/ml human recombinant IL-2 (Miltenyi Biotec), and activated with TransAct reagent (Miltenyi Biotec). Activated T cells were electroporated with TALEN\_TCR\_2a Right and Left mRNA (TRAC TALEN mRNA) (15) (TriLink Biotechnologies). On day 2, cells were divided into untransduced controls or transduced with CD3-CAR vector at a MOI of 5.

*Flow cytometry.* Cells were stained with the following primary anti-human antibodies: mouse anti-human CD2 (clone LT2), mouse anti-human CD3 (clone BW264/56), mouse anti-human TCR $\alpha\beta$  (clone BW242/412), mouse anti-human CD4 (clone VIT4), mouse anti-human CD8 (clone BW135/80), mouse anti-human CD19 (clone LT19), mouse anti-human CD20 (clone LT20), mouse anti-human CD56 (clone AF12-7H3), mouse anti-human CD279 (PD-1; clone PD1.3.1.3), mouse anti-human CD62L (clone 145/15), and mouse anti-human CD45RA (clone T6D11) (all from Miltenyi Biotec); mouse anti-human TIM-3 (clone 7D3) and mouse anti-human LAG-3 (clone T47-530) (both from BD Pharmingen); mouse anti-human CD95 (clone DX2; BioLegend); and rat anti-mouse CD11b (clone M1/70; eBioscience).

To assess the efficiency of CD3-CAR transduction, cells were stained using a Biotin-SP (long spacer) AffiniPure Fab Fragment Goat Anti-Mouse IgG fragment-specific antibody (catalog 115-066-072; Jackson ImmunoResearch, Stratech Scientific Limited) followed by Streptavidin-APC (catalog 405207, BioLegend) or Streptavidin-FITC (catalog 405202, BioLegend). Cells were acquired on a Cyan or on a BD LSRII (BD Biosciences), and analysis was performed using FlowJo v10 (TreeStar Inc.).

*Chromium release assay of cytotoxicity.* Cytotoxic activity of 3CAR cells was assessed by  $^{51}\text{Cr}$  release assay. For this,  $5 \times 10^3$   $^{51}\text{Cr}$ -labeled TCR/CD3 $^+$  Jurkat cells, TCR/CD3 $^-$  Jurkat cells, or primary PBMC target cells were incubated with 3CAR or untransduced control effector cells at increasing effector/target ratios (E:T ratios) in 96-well microplates for 4 hours at 37°C.  $^{51}\text{Cr}$  release was then measured in a microplate scintillation counter (Wallac 1450 MicroBeta TriLux).

*Flow-based cytotoxicity assay.* CD3-specific CAR T cells or control nontransduced T cells were cocultured with TCR/CD3 $^+$  Jurkat cells or TCR/CD3 $^-$  Jurkat target cells that were loaded with CFSE dye (Cell Trace, Invitrogen) at the E:T ratio 1:1 (100,000 of both effector and target cells) for 24 hours. Dot plots show representative frequency of gated CFSE $^+$  tumor cells at the end of coculture (Figure 3C). CD3 CAR T cells or control nontransduced T cells also were cocultured with healthy donor PBMC, CD3 $^+$ , or CD3 $^-$  cells (MicroBeads, Miltenyi, Bergisch-Gladbach).

*Cytokine production.* 3CAR-transduced cells were thawed, washed, and resuspended in RPMI 10% FCS at a concentration of  $1 \times 10^6$ /ml. TCR/CD3 $^-$  Jurkat cells, TCR/CD3 $^+$  Jurkat cells, and healthy donor PBMC were all counted and also adjusted to a concentration of  $1 \times 10^6$ /ml. Cells were mixed at a 1:1 ratio in 24-well tissue culture plates and incubated at 37°C. After 24 hours, supernatants were harvested and stored at  $-80^\circ\text{C}$ . Levels of cytokine in the stored supernatants were quantified using the Th1/Th2/Th17 cytometric bead array kit (CBA; BD Biosciences) as per the manufacturer's protocol.

*Mixed leukocyte reaction and  $^3\text{H}$ -thymidine incorporation.* To assess allogeneic T cell proliferation in a mixed leukocyte reaction (MLR), 3CAR T cells were mixed with target PBMC or PBMC depleted of CD3 using CD3 MicroBeads, (Miltenyi, Bergisch-Gladbach), in RPMI 10% FCS at a 1:1 ratio in 96-well plates. To assess a 1-way MLR, targets cells were irradiated (30 Gy) prior to coculture. Targets alone were used as negative controls, and all cultures were run in triplicate. After coincubation for 5 days,  $^3\text{H}$ -thymidine (1  $\mu\text{Ci}$  per well) was added for 18 hours before harvesting onto filter mats for radioactivity measurement in a scintillation counter (Wallac 1450 MicroBeta TriLux).

*In vivo antitumor activity.* Ten-week-old female NSG mice (Charles River, strain: NSG [005557] from The Jackson Laboratory), were inoculated i.v. with  $10 \times 10^6$  TCR $^+$  or TCR $^-$  Jurkat T cell tumor targets by tail vein injection on day 0. The Jurkat T cell targets had been stably transduced to express both EGFP and luciferase and, following TALEN-mediated TCR $\alpha\beta$  disruption, had been sorted for TCR $^+$  and TCR $^-$  expressers. Tumor engraftment was confirmed by in vivo imaging of bioluminescence using an IVIS Lumina III In Vivo Imaging System (PerkinElmer, live image version 4.5.18147) on day 3 (19). TCR $^-$  or TCR $^-$ -injected mice were further injected on day 4 with either PBS ( $n = 2$ ),  $10 \times 10^6$  untransduced T cells ( $n = 4$ ), or  $10 \times 10^6$  3CAR T cells ( $n = 5$ ) per tumor group. Analysis of tumor clearance was performed by serial bioluminescent imaging on days 3, 7, 11, and 18, and processing of BM for the monitoring of tumor progression vs. clearance was carried out on day 18. BM samples were processed by a RBC lysis, followed by staining for flow cytometry.

*Primary T-ALL blasts.* Primary T-ALL patient samples are all from the ALL2003 trial, where peripheral blood was often sent at diagnosis instead of bone marrow, with information on CD3 $^+$  status and other surface antigens (Table 1) (a gift from Bloodwise Childhood Leukaemia Cell Bank, Stockport, United



Kingdom). To phenotype patient cells, cells were stained with the following primary antibodies: mouse anti-human CD3 (clone BW264/56; Miltenyi Biotec), mouse anti-human CD7 (clone M-T701), mouse anti-human CD19 (clone SJ25C1), and mouse anti-human CD34 (clone 8G12) (all from BD Biosciences). For FACS-Based Killing assay, CD3 CAR T cells, control CAR19 T cells, or control nontransduced T cells were cocultured with T-ALL patient sample target cells that were loaded with CFSE dye (Cell Trace, Invitrogen), at the E:T ratio 1:1(100,000 of both effector and target cells) for 24 hours.

**Statistics.** For in vitro studies, data points from individual donors are shown in all figures. Statistical significance in pairwise comparisons was determined by unpaired 2-tailed Student *t* test. For in vivo studies, a 2-tailed Man-Whitney *U* test was used for nonparametric comparison of grouped data, and values are presented as mean percentages of 3 or more samples with SEM or SD, or as a median with the 25th and 75th percentiles stated. Linear regression was used for the comparison of serial measurements, taking each *y* value as an individual point. In all experiments,  $P < 0.05$  was considered statistically significant. All statistical analysis was performed using GraphPad Prism software version 5.01.

**Study approval.** Human sample collections were approved by University College London (UCL) ethics committee and collected with written consent. Leukemia samples were provided by the Childhood Leukemia Cell Bank. All animal studies were approved by the UCL Biological Services Ethical Review Committee and licensed under the Animals (Scientific Procedures) Act 1986 (Home Office, London, United Kingdom).

## Author contributions

JR performed and collected data for in vitro experiments. CG and RP performed and collected data for in vivo experiments. UM and WQ designed the reagents. JR and WQ prepared the manuscript.

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