Molecular Therapy
Original Article

Feasibility and preclinical efficacy of CD7-unedited CD7 CAR T cells for T cell malignancies

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Chimeric antigen receptor (CAR)-mediated targeting of T lineage antigens for the therapy of blood malignancies is frequently complicated by self-targeting of CAR T cells or their excessive differentiation driven by constant CAR signaling. Expression of CARs targeting CD7, a pan-T cell antigen highly expressed in T cell malignancies and some myeloid leukemias, produces robust fratricide and often requires additional mitigation strategies, such as CD7 gene editing. In this study, we show fratricide of CD7 CAR T cells can be fully prevented using ibrutinib and dasatinib, the pharmacologic inhibitors of key CAR/CD3ζ signaling kinases. Supplementation with ibrutinib and dasatinib rescued the *ex vivo* expansion of unedited CD7 CAR T cells and allowed regaining full CAR-mediated cytotoxicity *in vitro* and *in vivo* on withdrawal of the inhibitors. The unedited CD7 CAR T cells persisted long term and mediated sustained anti-leukemic activity in two mouse xenograft models of human T cell acute lymphoblastic leukemia (T-ALL) by self-selecting for CD7$^-$, fratricide-resistant CD7 CAR T cells that were transcriptionally similar to control CD7-edited CD7 CAR T cells. Finally, we showed feasibility of cGMP manufacturing of unedited autologous CD7 CAR T cells for patients with CD7$^+$ malignancies and initiated a phase I clinical trial (ClinicalTrials.gov: NCT03690011) using this approach. These results indicate pharmacologic inhibition of CAR signaling enables generating functional CD7 CAR T cells without additional engineering.

INTRODUCTION

Chimeric antigen receptor (CAR)-modified T cells are effective in patients with treatment-resistant B lineage malignancies, in part because of high expression by malignant cells of targetable antigens not expressed by vital tissues. Extending this approach to other antigens is often complicated by "on-target, off-tumor" toxicity, compromising the safety of these treatments. For example, few antigens specific for T cell leukemia and lymphoma can be targeted without producing substantial damage to normal T cells, including to CAR T cells themselves. Expression of CARs specific for most T lineage antigens induces CAR T cell fratricide, thus requiring additional engineering to mitigate self-targeting.[1] However, genome editing or inserting additional transgenes to alleviate fratricide increases the cost and complexity of these therapies.

CD7 is a pan-T cell antigen highly expressed in most T cell acute lymphoblastic leukemias (T-ALLs) and lymphomas, as well as in several subtypes of mature T cell lymphoma, making it an attractive target for cellular immunotherapy.[1] However, high CD7 expression on normal T cells causes substantial fratricide on transduction with a CD7 CAR. Strategies have been developed to remove surface CD7 antigen through genome editing or an intracellular protein expression blocker (PEBL).[2–4] Both methods yield fratricide-resistant CD7 CAR T cells that demonstrate high activity in preclinical models of CD7$^+$ lymphoid and myeloid malignancies. Early clinical results indicate that these CD7 CAR T cells can induce remissions in patients with recalcitrant T cell malignancies but do not fully eliminate endogenous T cells because of the presence of CD7$^-$ T cell subsets resistant to CAR T cell cytotoxicity.[5] The CD7$^-$ T cell compartment represents a minority of circulating T cells and contains both CD4$^+$ and CD8$^+$ T cells, mainly from effector and memory compartments.[6,7] The

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expression of a CD7 CAR on these CD7$^-$ T cells is not expected to cause fratricide, thus potentially allowing manufacturing functional CD7 CAR T cells without additional engineering. However, enriching the final T cell product for CD7$^-$ CAR T cells would either require additional cell sorting prior to CAR transduction or rely on CAR-mediated elimination of CD7$^+$ T cells that could accelerate differentiation and exhaustion of CD7$^-$ CAR T cells during *ex vivo* expansion and thus limit their therapeutic efficacy.

To overcome these limitations, we tested whether CD7 CAR-mediated fratricide in T cells can be temporarily minimized by blocking CAR signaling with pharmacologic inhibitors of key signaling kinases. Src family kinases Lck and Fyn play a central role in initiating and propagating signaling from the CD3ζ chain, leading to the activation of downstream cascades via Itk. In addition, the CD28 costimulatory endodomain elicits Lck-dependent signaling and can directly recruit and activate Itk.[8] In this study, we investigated whether supplementing ibrutinib and dasatinib—pharmacologic inhibitors of Itk and Lck/Fyn, respectively—to CD7 CAR T cells generated from whole peripheral blood T cells mitigated their fratricide and prevented terminal differentiation by reversibly blocking deleterious CAR signaling during *ex vivo* expansion. We assessed the anti-leukemic activity of these CD7 CAR T cells on removal of pharmacologic inhibitors and explored the mechanism by which the unedited CAR T cells produced sustained anti-tumor activity in mouse xenograft models of human T-ALL. We also demonstrated the feasibility of cGMP manufacturing autologous unedited CD7 CAR T cells for patients with CD7$^+$ T cell malignancies and initiated a phase I clinical trial.

RESULTS

Pharmacologic inhibition of CAR signaling prevents fratricide of CD7 CAR T cells

Because most T cells express high levels of CD7, transduction with a CD7 CAR induces strong fratricide.[2–4] We hypothesized that this fratricide can be minimized by pharmacologic inhibition of signaling emanating from the CAR-embedded CD3ζ and CD28 endodomains. Because both molecules activate cytotoxic signaling in T cells via Lck/Fyn and Itk kinases, we utilized dasatinib and ibrutinib to selectively inhibit these signaling mediators and suppress unwanted CAR-driven cytolysis (Figure 1A). Healthy donor peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3/anti-CD28 antibodies in the presence of 200 nM ibrutinib followed by gammaretroviral transduction with a clinically validated CD7 CAR vector containing a CD28 costimulatory domain (Figure 1B).[2] To prevent interference with initial T cell stimulation, dasatinib was added on the day of transduction at the final concentration of 200 nM. Transduced CD7 CAR T cells were expanded in the presence of both ibrutinib and dasatinib, interleukin (IL)-7, and IL-15. These CD7-unedited CD7 CAR T cells expanded with the pharmacologic inhibitors (hereafter PI CAR T cells), retained surface expression of the CAR, and had reduced intensity of CD7, possibly as a result of antigen masking by the CAR (Figure 1C). Control unedited CD7 CAR T cells cultured without ibrutinib and dasatinib (Figure 1B) had high CAR expression with a moderate reduction of surface levels of CD7 (Figure 1C) and showed extensive fratricide (Figure 1D), precluding downstream functional analyses. In contrast, both CD7-edited CD7 CAR T cells, where the expression of the CD7 gene was disrupted using CRISPR-Cas9 prior to CAR transduction (Figure 1B, hereafter CD7 knockout [KO] CAR T cells), and PI CAR T cells retained high viability and produced normal expansion *ex vivo* (Figure 1D). Pharmacologic inhibition of CAR signaling also preserved minimally differentiated T cell populations that were partially decreased in CD7 KO CAR T cells because of residual CAR signaling inducing differentiation (Figure S1). Similarly, PI treatment also suppressed fratricide and terminal differentiation of T cells expressing a CD7 CAR with the 4-1BB costimulatory endodomain, restoring their viability and expansion to a similar level with CD7 KO CAR T cells (Figure S2). However, PI CAR T cells expanded less than non-transduced (NT) T cells (Figure S2C), consistent with the toxicity of tonic 4-1BB signaling in T cells transduced with a gammaretroviral vector,[9] which relies on TRAF2 signaling and cannot be fully blocked by Lck/Itk inhibitors. In light of these findings, we performed the rest of the studies with the CD28-costimulated CD7 CAR. These results indicate that the expansion of unedited CD7 CAR T cells in the presence of ibrutinib and dasatinib minimizes deleterious CAR signaling and the resulting fratricide, as well as terminal differentiation of T cells.

PI CAR T cells regain cytotoxicity on removal of ibrutinib and dasatinib

Blockade of CAR signaling protects PI CAR T cells from fratricide but also inhibits tumor-directed cytotoxicity. To test whether PI CAR T cells regain their anti-tumor function on withdrawal of ibrutinib and dasatinib, we generated unedited CD7 CAR T cells from multiple healthy donors and expanded them *ex vivo* in the presence of ibrutinib and dasatinib for 7 days, following which the T cells were washed and cryopreserved (Figure 1B). After thawing, we cocultured PI CAR T cells with the T-ALL cell lines Jurkat or CCRF-CEM in the absence of ibrutinib, dasatinib, or exogenous cytokines. We observed cytokine production from PI CAR T cells as early as 4 h post-stimulation, indicating rapid acquisition of effector function on withdrawal of the pharmacologic inhibitors (Figure S3). In addition, they produced significant cytotoxicity against both cell lines, although CCRF-CEM cell killing and CAR T cell expansion were attenuated compared with CD7 KO CAR T cells at a 1:4 effector-to-target ratio (Figures 1E, S4A, and S4B), likely because withdrawal of the pharmacologic inhibitors also led to resumed fratricide of PI CAR T cells. Notably, PI CAR T cells showed higher cytotoxicity and prolonged T cell survival than CD7 KO CAR T cells in a stress test once the effector-to-target ratio was lowered to 1:10 (Figures 1F, S4C, and S4D), potentially because of their less differentiated status and reduced self-targeting in a tumor-saturated environment. These studies demonstrate that removal of ibrutinib and dasatinib restored CD7-directed cytotoxicity in PI CAR T cells.

PI CAR T cells produce robust anti-leukemic activity *in vivo*

Although most T cells are CD7$^+$, a subset naturally lacks CD7 expression. This population is highly variable in frequency among healthy donors, constituting a mean of 7.8% of CD4$^+$ and 2.3% of CD8$^+$

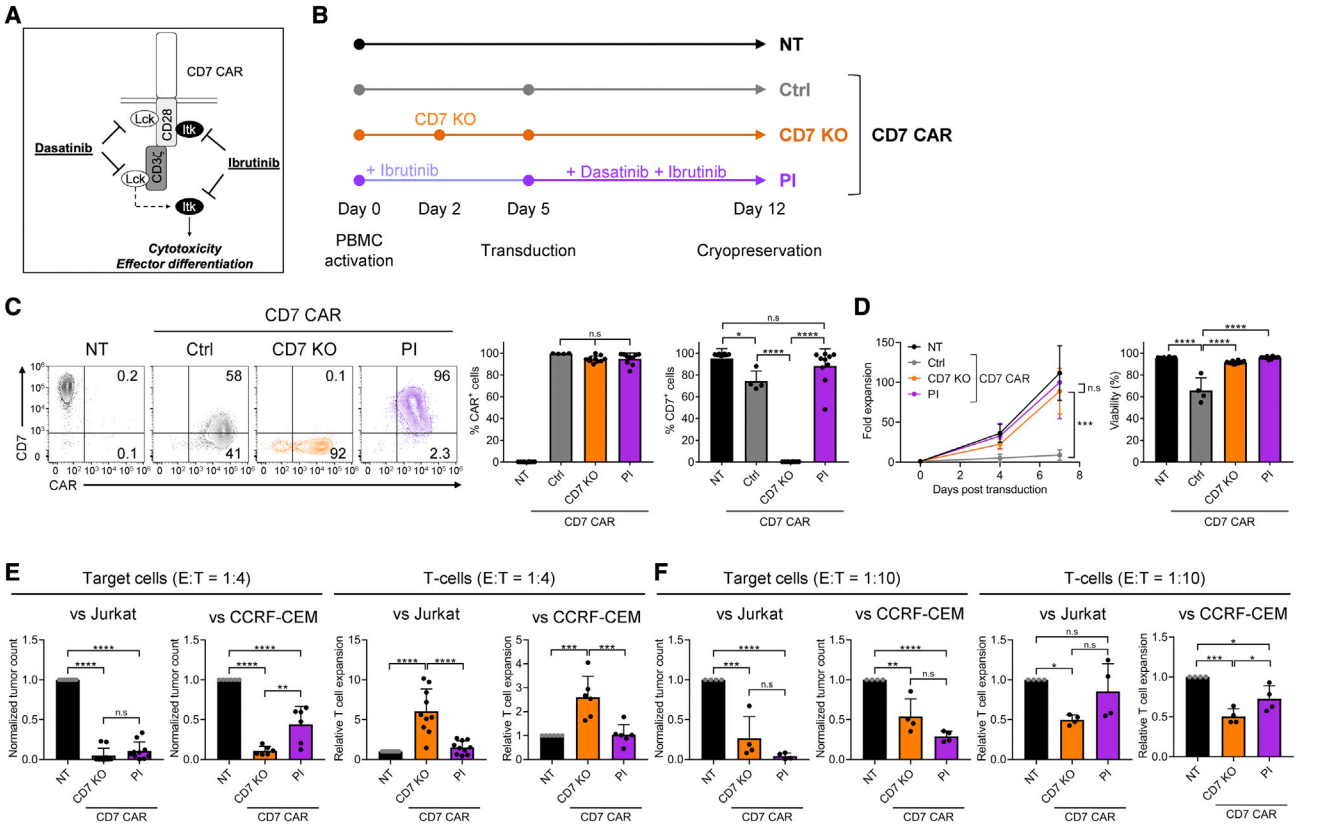

Figure 1. Dasatinib and ibrutinib prevent CD7 CAR T cell fratricide, and the inhibitory effect is reversible

(A) A schematic diagram showing the effect of dasatinib and ibrutinib on CAR signaling. (B) An outline of CAR T cell generation. (C) Representative flow plots (left) and summary of percent of CAR$^+$ and CD7$^+$ cells (right) on day 7 post-transduction for each specified T cell type (mean ± SD, Ctrl CD7 CAR T, n = 4; other conditions, n = 10). (D) Top: *ex vivo* fold expansion of specified T cell types over time (mean ± SD, n = 8). Bottom: cell viability on day 7 post-transduction determined by flow cytometry (mean ± SD, Ctrl CD7 CAR T, n = 4; other conditions, n = 10). (E) Left: cytotoxicity of specified effector T cells against Jurkat (mean ± SD, n = 10) or CCRF-CEM (mean ± SD, n = 6) target cells at 72 h after coculture setup at a 1:4 effector-to-target ratio. Right: expansion of specified effector T cells when cocultured with Jurkat (left, mean ± SD, n = 10) or CCRF-CEM (right, mean ± SD, n = 6) target cells for 72 h at a 1:4 effector-to-target ratio. (F) Similar coculture setup with (E) at a 1:10 effector-to-target ratio (mean ± SD, n = 4). Statistical differences are calculated by one-way ANOVA with Tukey's multiple comparisons (C–F). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. n.s, non-significant.

T cells (Figure S5). These cells are expected to resist CD7-directed fratricide and therefore can produce sustained anti-tumor activity. We hypothesized that PI CAR T cells will target cancerous T cells early post-infusion and eventually self-select for the fratricide-resistant CD7$^-$ population of CD7 CAR T cells. To test this hypothesis, we engrafted CD7$^+$ Jurkat T-ALL cells that were modified to express firefly luciferase (FFluc) in NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice and injected freshly thawed CD7 CAR T cells intravenously 3 days later (Figure 2A). PI CAR T cells mediated potent anti-tumor activity (Figures 2B and 2C), significantly extending animal survival (Figure 2D).

To better characterize the kinetics of expansion and persistence of PI CAR T cells in leukemia-bearing mice, we generated FFluc-labeled CD7 CAR T cells (Figure S6A) and administered them to mice engrafted with Jurkat T-ALL (Figure 2E). PI CAR T cells expanded and persisted in most animals, protecting them from leukemia progression (Figures 2F and 2G). CD7 KO CAR T cells had inferior persistence and anti-tumor activity compared with PI CAR T cells. Their decreased function *in vivo* could result from electroporation and/or genotoxicity from CRISPR-mediated genome editing or reflect enhanced terminal differentiation of CD7 KO CAR T cells (Figure S1), or both. Indeed, CD7 KO CAR T cells generated in the presence of PI (KO + PI) showed enhanced expansion and persistence compared with PI CAR T cells without CD7 editing (Figures S6B–S6D). The long-term persistence and anti-tumor activity of PI CAR T cells were not specific to a particular T cell donor or the result of xenogeneic graft-versus-host responses, because we observed similar outcomes with PI CAR T cells derived from multiple donors in NOD.Cg-Prkdc^{scid}H2-K1^{tm1Bpe}H2-Ab1^{em1Mvw} H2-D1^{tm1Bpe}Il2rg^{tm1Wjl}/SzJ (NSG-MHC class I/II DKO [double-knockout]) mice engrafted with Jurkat T-ALL (Figure S7), albeit with varying magnitudes of expansion.

We further evaluated the activity of PI CAR T cells in a second model of T-ALL in which we inoculated NSG mice with CCRF-CEM T cell

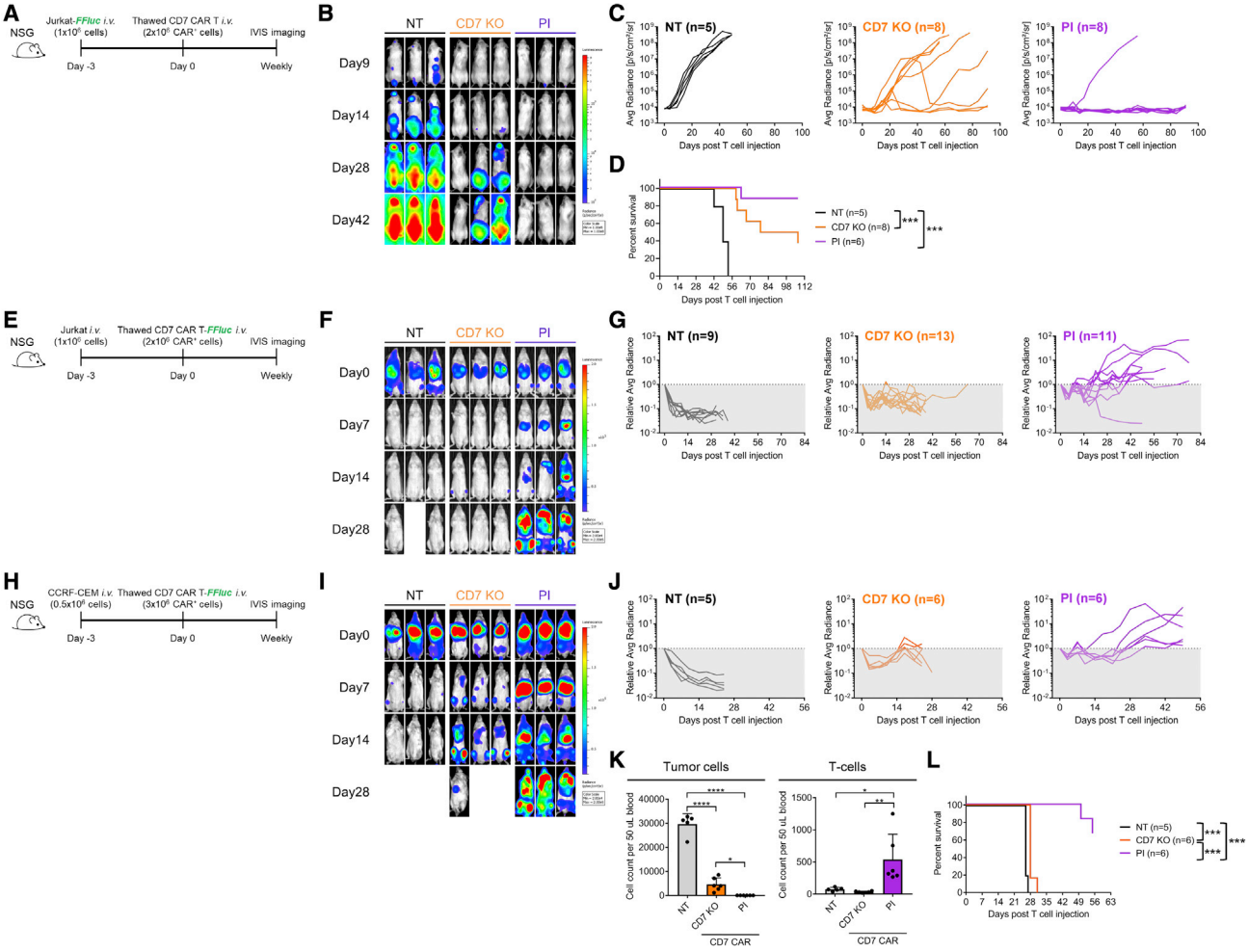

Figure 2. PI CAR T cells showed superior anti-tumor activity and long-term persistence *in vivo*

(A) Schematic of model setup for (B)–(D). (B) Representative IVIS images showing tumor bioluminescence. (C) Change of tumor bioluminescence over time in mice receiving the specified T cell treatment, as measured by IVIS imaging. Each line represents data from one individual animal. (D) Animal survival over time. (E) Schematic of model setup for (F) and (G). (F) Representative IVIS images showing bioluminescence from infused T cells. (G) Change of T cell bioluminescence over time in mice receiving the specified T cell treatment, as measured by IVIS imaging. Each line represents data from one individual animal. (H) Schematic of model setup for (I)–(L). (I) Representative IVIS images showing bioluminescence from infused T cells. (J) Change of T cell bioluminescence over time in mice receiving the specified T cell treatment, as measured by IVIS imaging. Each line represents data from one individual animal. (K) Absolute counts of infused CCRF-CEM tumor cells (left) and T cells (right) in 50 μL peripheral blood on day 15 after T cell infusion (mean ± SD, n = 5 for NT, n = 6 for CD7 KO, n = 6 for PI). (L) Animal survival over time. Statistical differences are calculated by log rank test (D and L) or one-way ANOVA with Tukey's multiple comparisons (K). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

leukemia followed by a single dose of FFluc-labeled CAR T cells 3 days later (Figure 2H). Compared with the Jurkat model, CCRF-CEM produces more aggressive tumors with typical leukemic distribution of malignant cells in peripheral blood and bone marrow.[10] While the infusion of CD7 KO CAR T cells resulted in a marginal survival benefit in this model (Figure 2L), PI CAR T cells produced long-term persistence and sustained anti-tumor activity, eradicating T-ALL blasts in peripheral blood in most animals and significantly extending animal survival (Figures 2I–2L). Overall, these results indicate persisting PI CAR T cells resist fratricide *in vivo* and produce sustained anti-leukemia activity in mouse xenograft models of human T-ALL.

Persisting PI CAR T cells lack CD7 gene expression and transcriptionally resemble CD7-edited CAR T cells

To determine the mechanism of fratricide resistance in persisting PI CAR T cells *in vivo*, we measured the expression of both the CD7 CAR and the CD7 antigen by flow cytometry on circulating CAR T cells 27 days post-infusion. In all animals, remaining PI CAR T cells had uniformly high expression of the CAR, whereas CD7 was undetectable, albeit being a minor population in the infusion product (Figure 3A). Loss of detectable CD7 was not a result of CAR-mediated antigen masking because PI CAR T cells lacked both protein and mRNA expression of the CD7 gene, measured by western blot and quantitative PCR (qPCR), respectively

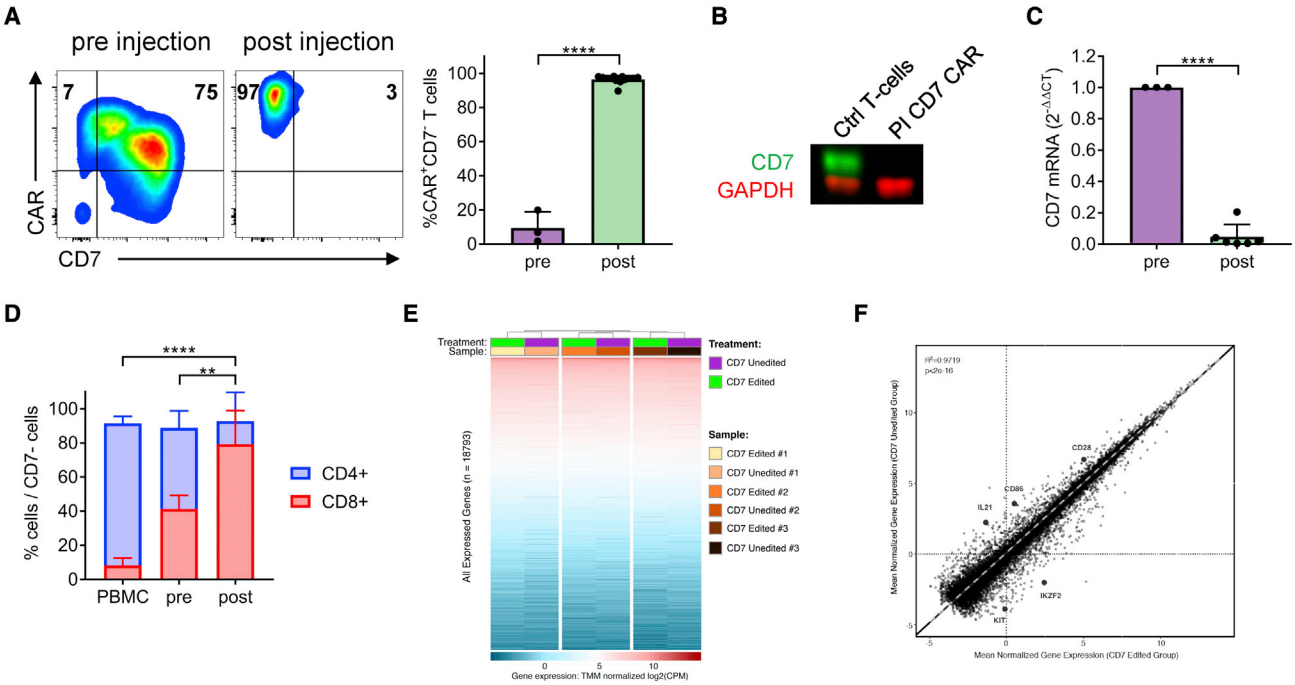

Figure 3. Persisting PI CAR T cells lack CD7 expression and transcriptionally resemble CD7-edited CAR T cells

(A) Representative flow plots (left) and summary of percent of CAR^+CD7^- cells (right) pre-infusion and at 27 days post-infusion (mean ± SD, n = 3 for pre-infusion, n = 13 for post-infusion). (B) CD7 protein expression in persisting control (Ctrl) non-transduced (NT) T cells and PI CAR T cells. (C) Quantitative PCR results showing relative CD7 mRNA level of post-infusion PI CAR T cells compared with pre-infusion (mean ± SD, n = 3 for pre-infusion, n = 6 for post-infusion). (D) Percent of $CD4^+$ and $CD8^+$ cells in the starting cell material (PBMCs), pre-infusion products (pre), and persisting $CD7^-$ PI CAR T cells post-infusion (post) (mean ± SD, n = 3 for PBMCs, n = 3 for pre-infusion, n = 13 for post-infusion). (E) Heatmap was plotted using normalized gene expression from each sample. Gene expression was normalized with Trimmed Means of M values (TMM) and log2 transformed counts per million (log2(CPM)). Results of unsupervised clustering are shown. (F) Scatterplot showing significantly high transcriptome profiling correlation between CD7-unedited and -edited CD7 CAR T cells. Mean normalized gene expression was calculated by averaging normalized gene expression (same method as described in E) from three biological replicates in each condition. p value and coefficient were calculated with linear regression. Highlighted are genes involved in regulating the immune function of T cells. Statistical differences are calculated by unpaired two-tailed *t* test (A and C). ****$p < 0.0001$. See Figures 2E, S6A, and S7A for model setup.

(Figures 3B and 3C). These data support the expansion of naturally $CD7^-$ CAR-transduced T cells, which are present in peripheral blood of healthy donors. Notably, while the endogenous circulating $CD7^-$ T cells in PBMCs are predominantly $CD4^+$, $CD8^+CD7^-$ T cells outgrew in culture and animal hosts (Figure 3D).

Next, we investigated whether the persisting $CD7^-$ PI CAR T cells were transcriptionally distinct from KO + PI CAR T cells isolated from mice (Figure S6D) using bulk RNA sequencing (RNA-seq). Unsupervised hierarchical clustering analysis revealed the CD7-unedited and CD7-edited CD7 CAR T cells were transcriptionally very similar (Figure 3E). The similarity of transcriptomes is also observed in Figure 3F, where the two transcriptomes of CD7-unedited and CD7-edited CD7 CAR T cells show a highly significant correlation ($R^2 = 0.97$; $p < 2e{-}16$). Out of the near 20,000 genes detected in the cells, only 102 showed a 2-fold differential expression (adjusted p value [p.adj] < 0.05) (Figures 3F and S8; Table S1). These results indicate the fratricide-resistant PI CAR T cells lack CD7 expression, persist long term, and are transcriptionally similar to CD7-edited CD7 CAR T cells.

cGMP manufacturing of functional autologous PI CAR T cells for patients with T-ALL

Pharmacologic inhibition of CAR-driven fratricide offers a straightforward method of cGMP-compliant manufacturing of functional CD7 CAR T cells without additional genetic engineering. However, because multiple lines of lymphotoxic chemotherapy in patients with treatment-refractory leukemia and lymphoma often change subset composition and the expansion potential of normal circulating T cells,[11] it is important to assess the feasibility of manufacturing functional unedited CD7 CAR T cells for these patients using a cGMP-compliant method. To evaluate the frequency of fratricide-resistant $CD7^-$ T cells in the starting cell material, we analyzed PBMCs of nine patients with $CD7^+$ T cell malignancies enrolled in clinical studies in our center. $CD7^-$ T cells constituted a mean of 9.52% of $CD4^+$ T cells and a mean of 3.38% of $CD8^+$ T cells in PBMCs of those patients (Figure 4A), similar to healthy donors (Figure S5). Based on these data and the preclinical results described above, we initiated a phase I clinical study of autologous unedited CD7 CAR T cells in patients with refractory or relapsed T cell malignancies (CRIMSON-NE,

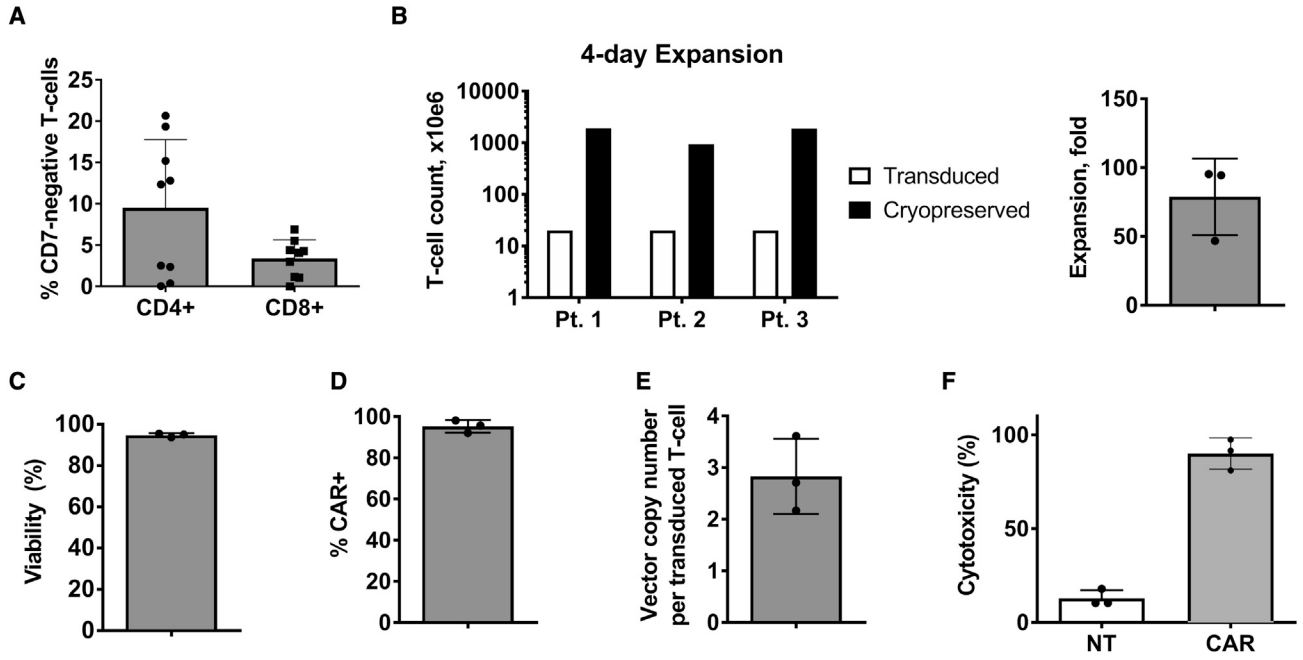

Figure 4. Characterization of cGMP-manufactured autologous PI CAR T cells for T-ALL patients

(A) Frequency of CD7$^-$ normal CD4$^+$ and CD8$^+$ T cells measured by flow cytometry in PBMCs collected from patients with T cell malignancies. (B) Left: absolute T cell counts for each patient at the time of transduction (transduced) and on the day of cryopreservation (cryopreserved, day 4 post-transduction). Right: fold expansion of PI CAR T cells between transduction and cryopreservation. (C) Viability of PI CAR T cells at the time of cryopreservation. (D) Percent of CAR$^+$ T cells at the time of cryopreservation. (E) Vector copy number per transduced T cell at the time of cryopreservation. (F) Cytotoxicity of PI CAR T cells and donor-matched NT T cells on coculture with FFluc-labeled Jurkat T-ALL cell line for 24 h. In all panels, each dot represents data from an individual patient. Means ± SD are shown.

ClinicalTrials.gov: NCT03690011). We developed a cGMP-compliant method of manufacturing PI CAR T cells and validated it by generating CAR T cell products from adult patients enrolled in the study protocol. Dasatinib concentration is increased to 500 nM during clinical manufacturing to ensure sufficient inhibition of CD7 CAR signaling across all patients without producing toxicity. PBMCs were obtained from three patients with recalcitrant T-ALL, processed in our cGMP facility, and stimulated with plate-bound CD3-and CD28-specific antibodies in the presence of ibrutinib. Three days later, T cells were transduced with a clinical-grade CD7 CAR gammaretroviral vector and expanded in the presence of ibrutinib, dasatinib, IL-7, and IL-15. We observed robust expansion of PI CAR T cells in all three patient products with a mean 78.8-fold expansion over 4 days following transduction (Figure 4B). At the end of the expansion, CAR T cells were counted and cryopreserved. Mean viability of cryopreserved CD7 CAR T cells was 94.7% as measured by flow cytometry (Figure 4C). CD7 CAR was highly expressed in all three products (mean transduction efficiency, 95.3%; Figure 4D) with mean vector copy number of 2.83 per transduced T cell (Figure 4E). Mean cytotoxicity of CD7 CAR T cells was 90.0% measured in a 24-h coculture assay with Jurkat T-ALL cells at a 1:2 effector-to-target ratio (Figure 4F). No residual CAR$^+$ T-ALL blasts were detected in final products by flow cytometry (data not shown), and all three lines met the release criteria.

DISCUSSION

We have shown that the *in vitro* fratricide of CD7 CAR T cells can be averted by reversible pharmacologic inhibition of CAR signaling with ibrutinib and dasatinib, thus obviating the need for CD7 gene editing. The resultant PI CAR T cells regain cytotoxicity on removal of inhibitors and eradicate human leukemia in xenograft models. Anti-tumor activity is sustained by persisting CD7$^-$ CD7 CAR T cells, which are selected *in vivo* and resist CD7-directed fratricide. We also demonstrated the feasibility of manufacturing functional autologous PI CAR T cells in cGMP conditions for patients with T cell malignancies.

Fratricide of CAR T cells targeting T lineage antigens is a common phenomenon. For example, expression of CARs specific to antigens expressed on T cells, like CD3ε, TCRβ, CD7, CD38, and NKG2D ligands, can produce strong internecine cytolysis that impairs T cell expansion.[2–4,12–15] Continuous ligand-driven CAR signaling also accelerates terminal T cell differentiation that limits the therapeutic potency of these cells.[16,17] Multiple approaches have been proposed to mitigate this unwanted activity. Genetic disruption of the target antigen on T cells prior to CAR transduction is one common method. We previously reported that knocking out the CD7 gene with CRISPR-Cas9 enabled the generation of functional CD7 CAR T cells with no detectable fratricide.[2,18] This approach can be combined with TCR gene editing to create non-alloreactive CD7 CAR T cells suitable for off-the-shelf use.[4] Similarly, genetic disruption

of the CD3ε gene reduces fratricide of CD3 CAR T cells.[12] An alternative approach is to disrupt intracellular trafficking of surface antigens by anchoring them in the endoplasmic reticulum using PEBL molecules. Preclinical studies have shown PEBL-mediated intracellular retention of CD7 or CD3ε proteins prevented their surface expression and minimized fratricide of CAR T cells targeting the respective antigens.[3,19] Further, inhibition of CAR-antigen binding with a blocking antibody helps reduce T cell cytolysis driven by an NKG2D-based CAR, which recognizes multiple ligands on T cells.[15] All these approaches can be utilized to attenuate fratricide and the associated terminal differentiation and functional exhaustion in T cells but require additional genetic manipulations and/or costly reagents.

Alternative methods of mitigating fratricide without additional modifications would streamline T cell manufacturing and reduce its complexity and costs. One notable example is a CAR recognizing CD5, a pan-T cell antigen expressed at high levels on normal T cells. The expression of a CD5 CAR on T cells induces only brief and limited fratricide followed by a rapid and complete loss of detectable CD5 on the cell surface, minimizing self-targeting in CAR T cells.[10,17] Other mechanisms of fratricide resistance include CAR-mediated epitope masking in *cis*, which reduces accessible antigen molecules on the cell surface and decreases fratricide intensity, as seen in T cells transduced with 4-1BB- or CD38-specific chimeric receptors.[14,20] Finally, targeting antigens with restricted expression to certain T cell subsets (such as TRBC1 or CD4) helps limit fratricide by sparing populations lacking those antigens.[13,21] Targeting CD7 falls under this scenario, as we have shown in this study that CD7 CAR ablates normal $CD7^+$ T cells and selects for fratricide-resistant $CD7^-$ lymphocytes.

Mechanisms of intrinsic fratricide resistance that rely on antigen neutralization often produce undesirable ligand-driven CAR signaling that enhances T cell differentiation to effector and effector memory populations. Specifically, CD3ζ chain signaling via Src kinases Lck and Fyn activates key signaling mediators, such as Itk, LAT, and PLCγ, and triggers downstream signaling cascades. Signaling from the CD28 endodomain augments CD3ζ signaling by recruiting and activating Grb2, Lck, and Itk.[8] Because this signaling network contributes to terminal T cell differentiation, blocking these pathways during CAR T cell manufacture would lead to cell products with a less differentiated phenotype, which is often desired in the adoptive cell therapy setting. Here we used pharmacologic blockade of Itk and Src kinases Lck/Fyn with ibrutinib and dasatinib to fully prevent T cell activation and degranulation during *ex vivo* expansion.

Recent studies have demonstrated that dasatinib can reversibly block tonic CAR signaling and halt T cell activation, exhaustion, and fratricide.[22–26] Further, addition of ibrutinib has been shown to help limit differentiation of *ex vivo* expanded T cells.[27,28] We found that combining both inhibitors in non-toxic concentrations effectively minimized fratricidal CAR signaling and inhibited terminal T cell differentiation. This approach enabled us to generate a CD7 CAR T cell product with a minimally differentiated phenotype that mediates potent and sustained anti-tumor activity *in vivo* without the need for additional genetic engineering. Of note, the CD28 costimulatory endodomain also directly recruits the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and activates downstream Akt-mTOR and nuclear factor κB (NF-κB) pathways, further contributing to T cell proliferation and effector differentiation. Neither ibrutinib nor dasatinib is known to directly inhibit the PI3K-Akt pathway, and therefore it may still remain active in PI CAR T cells. If so, this signaling axis did not accelerate T cell differentiation, because the subset composition of PI CAR T cells closely resembled that of control donor-matched NT T cells. In our system, addition of dasatinib was sufficient to protect CAR T cells from fratricide and preserve most of the undifferentiated T cells because we observed minimal differences in CAR T cell expansion and phenotype between dasatinib alone and the combination with ibrutinib (Figure S9). Of note, the expansion of T cells expressing a 4-1BB-costimulatied CD7 CAR could not be fully rescued with ibrutinib and dasatinib because the toxicity was driven by gammaretroviral vectors harboring CARs with a 4-1BB endodomain,[9] at least in part, through the TRAF2-NFkB signaling axis insensitive to those tyrosine kinase inhibitors.

Consistent with prior reports,[22–24,27] pharmacologic inhibition of CAR signaling was fully reversible as PI CAR T cells regained their cytotoxicity shortly after inhibitor removal. Reactivation of CAR signaling led to fratricide, limiting the expansion of PI CAR T cells in tumor coculture assays and delaying their initial expansion *in vivo*. Although these events could have impacted early anti-leukemic activity of CAR T cells, fratricide-resistant $CD7^-$ PI CAR T cells outgrew over time and persisted long term. In most clinical scenarios, the infused CAR T cells are initially surrounded by malignant cells, meaning a CD7-unedited CD7 CAR T cell will likely encounter a leukemic cell before targeting another CAR T cell. Therefore, it is plausible that $CD7^+$ PI CAR T cells will contribute to short-term anti-leukemic activity before succumbing to fratricide. In the long term, $CD7^-$ PI CAR T cells will establish more sustained persistence and cytotoxicity.

Our study also supports the potential of adopting $CD7^-$ T cells as a platform for engineered cell therapy. CD7 is one of the earliest T lineage markers expressed in early thymic immigrants, most thymocytes, and peripheral T cells, as well as NK cells. Functionally, CD7 is a transmembrane protein that provides costimulation and modulates adhesion in T cells.[29] However, the functional importance of CD7 in peripheral T cells is not clearly defined, and mice lacking CD7 have a largely unperturbed and competent T cell compartment. In humans, loss of CD7 has been documented in a small subset of circulating T cells, which are predominantly $CD4^+$ and have the $CD45RA^-$ $CD45RO^+$ memory phenotype.[6,7,30] The frequency of $CD7^-$ circulating T cells increases with age.[30] Expansion of $CD7^-$ $CD4^+$ and $CD8^+$ T cells has also been documented in the settings of viral infections (HIV, Epstein-Barr virus [EBV]), rheumatoid arthritis, and other inflammatory conditions.[30–36] These and other studies implied that the lack of CD7 is associated with the terminal

differentiation of chronically stimulated T cells but also suggested that T cells lacking CD7 are more resistant to activation-induced apoptosis.[37] Our results showed $CD7^-$ CD7 CAR T cells persisted long term in immunodeficient mice and suppressed leukemia relapse, suggesting these cells might be capable of producing sustained anti-tumor activity in patients with T cell malignancies. It is unclear whether the persisting $CD7^-$ CD7 CAR T cells originate from $CD7^-$ peripheral blood T cells or downregulate CD7 expression during T cell manufacturing and *in vivo* expansion. Notably, in most mice, the majority of persisting $CD7^-$ CAR T cells were $CD8^+$, in stark contrast with human endogenous PBMCs where $CD4^+$ T cells dominated the $CD7^-$ subset. This may be caused by the proliferative advantage of $CD8^+$ over $CD4^+$ T cells during both *ex vivo* and *in vivo* expansion and/or that CAR signaling favored the expansion of $CD8^+$ T cells in this model. Further, gene expression analysis showed that the outgrowing CD7 CAR T cells lacking CD7 had a similar transcriptional profile to that of CD7 CAR T cells with CD7 gene KO. However, we were unable to compare the transcriptome of $CD7^-$ and $CD7^+$ CD7 CAR T cells expanded without pharmacologic inhibitors because of the fulminant fratricide of the latter. Therefore, additional studies are needed to elucidate the functional capacity and composition of $CD7^-$ T cells and assess their potency as a broader platform for engineered cell therapy.

Taken together, these results support efficacy and feasibility of manufacturing unedited CD7 CAR T cells with potent anti-tumor activity by using pharmacologic inhibition of CAR-mediated fratricide in lieu of genome editing or incorporating additional transgenes. Based on this work, a phase I clinical trial of unedited CD7 CAR T cells manufactured with this method for patients with T cell malignancies (CRIMSON-NE, ClinicalTrials.gov: NCT03690011) has been initiated at Baylor College of Medicine.

MATERIALS AND METHODS

Donors and cell lines

PBMCs were obtained from healthy volunteers and patients with T cell hematologic malignancies after informed consent on protocols approved by the Baylor College of Medicine Institutional Review Board (H-45017 and H-43761). Jurkat, clone E6-1 (acute T cell leukemia cell line), and CCRF-CEM (acute T cell lymphoblastic leukemia cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA). Jurkat and CCRF-CEM cells were maintained in RPMI-1640 medium (Gibco BRL Life Technologies, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL Life Technologies) with 2 mM L-GlutaMAX (Gibco BRL Life Technologies). Cells were maintained in a humidified atmosphere containing 5% carbon dioxide (CO_2) at 37°C. All cell lines have been routinely tested for Mycoplasma.

Generation of retroviral constructs and retrovirus production

A second-generation 28z.CAR construct targeting CD7 was previously developed in our laboratory.[2,18] In brief, the CAR construct is composed of an scFv domain (clone 3A1e) followed by IgG-derived hinge and C_H3 spacer with CD28 transmembrane/costimulatory and CD3ζ signaling domains. In some experiments, we utilized a second-generation CD7.CAR with the costimulatory endodomain replaced with 4-1BB. Gammaretroviral vectors were generated as previously described.[38]

Generation of CAR-modified T cells and gene-modified cell lines

To obtain activated T cells, we plated 1×10^6 PBMCs in each well of a non-tissue culture-treated 24-well plate pre-coated with 500 μL of OKT3 (1 mg/mL; Ortho Biotech, Bridgewater, NJ, USA) and anti-CD28 (1 mg/mL; BD Biosciences, San Jose, CA, USA) antibodies. Cells were cultured in complete CTL medium containing 45% RPMI-1640 medium, 45% Click's medium (Irvine Scientific), 10% FBS, and 2 mM L-GlutaMAX. IL-7 (10 ng/mL) and IL-15 (10 ng/mL) were added on the following day. To generate CD7-edited CD7 CAR T cells, we stimulated PBMCs as described above, then CD7 gene was genomically disrupted using the CRISPR-Cas9 system on day 2 as previously described.[2] CD7 CAR transduction was performed on day 5 where retroviral supernatant was plated in a non-tissue culture-treated 24-well plate pre-coated with recombinant fibronectin fragment (FN CH-296; Retronectin; TAKARA BIO, Otsu, Japan), and centrifuged at $2000 \times g$ for 90 min. After removal of the supernatant, OKT3/CD28-activated PBMCs were resuspended in complete CTL medium supplemented with IL-7/IL-15 at a final concentration of 0.1×10^6/mL, and 2 mL of cell suspension was added to each virus-loaded well, which was subsequently spun at $1{,}000 \times g$ for 10 min and then transferred to a 37°C, 5% CO_2 incubator. In the condition where we generated unedited CD7 CAR T cells in the presence of pharmacologic inhibitors, ibrutinib (200 nM; Catalog# S2680; Selleckchem) was added on day 0 and a combination of dasatinib (200 nM; Catalog# S1021; Selleckchem) and ibrutinib (200 nM) was added on the day of transduction. Concentrations of ibrutinib and dasatinib were selected based on titration experiments designed to determine minimal concentrations of inhibitors that consistently produced full inhibition of CAR signaling. For the generation of CD7 CAR and GFP/FFluc co-transduced T cells, GFP/FFluc transduction, CD7 KO, and CD7 CAR transduction were performed on days 2, 3, and 6 after initial stimulation, respectively. Transduced cells were transferred to and maintained in tissue-culture-treated plates, and half of the medium was replaced every 2–3 days with CTL medium supplemented with fresh cytokines, dasatinib, and ibrutinib where needed.

Flow cytometry

Cells were stained with fluorochrome-conjugated antibodies for 20 min at 4°C. All samples were acquired on a Gallios Flow Cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA) or FACSCanto (BD Biosciences), and data were analyzed using Kaluza 2.1 Flow Analysis Software (Beckman Coulter Life Sciences) or FlowJo (BD Biosciences). Antibodies used in this study are listed below: Alexa Fluor 647 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific (Cat# 109-605-098; Jackson ImmunoResearch, West Grove, PA, USA), CCR7-fluorescein isothiocyanate (FITC) (clone 150,503, Cat# 561271; BD Biosciences), CD3-PerCP (clone SK7, Cat# 347344; BD Biosciences), CD45-phycoerythrin (PE) (clone

HI30, Cat# 555483; BD Biosciences), CD8-PerCP (clone SK1, Cat# 347314; BD Biosciences), CD28-PE (clone CD28.2, Cat# 561793; BD Biosciences), tumor necrosis factor alpha (TNF-α)-PC7 (clone MAb11, Cat# 557647; BD Biosciences), interferon γ (IFNγ)-V450 (clone B27, Cat# 560371; BD Biosciences), CD3-allophycocyanin (APC)-A750 (clone UCHT1, Cat# A66329; Beckman Coulter Life Sciences), CD45RA-APC-A750 (clone 2H4LDH11LDB9 [2H4], Cat# A86050; Beckman Coulter Life Sciences), CD4-KrO (clone 13B8.2, Cat# A96417; Beckman Coulter Life Sciences), CD8-PB (clone B9.11, Cat# A82791; Beckman Coulter Life Sciences), CD62L-ECD (clone DREG56, Cat# IM2713U; Beckman Coulter Life Sciences), CD27-PC7 (clone 1A4CD27, Cat# A54823; Beckman Coulter Life Sciences), CD7-PC7 (clone CD7-6B7, Cat# 343114; BioLegend, San Diego, CA), CD7-PE (clone CD7-6B7, Cat# 343106; BioLegend), human leukocyte antigen (HLA)-A2-PB (clone BB7.2, Cat# 343312; BioLegend), and IL-2-APC/Fire750 (clone MQ1-17H12, Cat# 500352; BioLegend).

Coculture experiments

In the coculture experiments, freshly thawed 10,000 CAR^+ cells were cocultured with 40,000 GFP^+ target cell lines in 200 μL in one well of 96-well flat-bottom plates. Cells were harvested and analyzed by flow cytometry on days 0, 1, and 3. To quantify cell counts by flow cytometry, 10 μL/sample of CountBright Absolute Counting Beads (Thermo Fisher Scientific, Invitrogen, Grand Island, NY, USA) was added, and 7-AAD (BD Biosciences) was added to exclude dead cells. Acquisition was halted at 2,000 beads. Results were reported as normalized cell counts based on cell counts in control conditions (NT cells + target cells) at each time point.

Intracellular cytokine staining

To measure cytokine production, we cocultured 0.2×10^6 CAR^+ cells with 0.2×10^6 target cells in 200 μL of medium in the presence of monensin (BD GolgiStop; BD Biosciences) for 4 h. After the incubation, cells were stained for cell surface markers and Fixable Viability Stain 700 (BD Biosciences). Stained cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's protocol. Cytokines IFNγ, TNF-α, and IL-2 were stained and analyzed by flow cytometry.

In vivo models

Breeder pairs of NSG mice (stock no. 005557) and NSG-MHC class I/II DKO mice (stock no. 025216) were purchased from the Jackson Laboratory and bred in the Baylor College of Medicine animal facility. Both female and male littermates (aged 8–12 weeks) were used for experiments. All animal experiments were conducted in compliance with the Baylor College of Medicine IACUC (protocol no. AN-4758). To evaluate the *in vivo* anti-tumor effect of CD7 CAR T cells, 1 million Jurkat-GFP/FFluc cells were engrafted into each NSG mouse by intravenous injection. Three days later, freshly thawed 2×10^6 CD7 CAR T cells were injected intravenously. To track T cell expansion and persistence, we injected Jurkat (1×10^6 cells/animal) or CCRF-CEM (0.5×10^6 cells/animal) cells intravenously into either NSG or NSG-MHC class I/II DKO mice, and freshly thawed CD7 CAR T cells labeled with GFP/FFluc were injected 3 days later (2×10^6 CAR^+ cells for the Jurkat model and 3×10^6 CAR^+ cells for the CCRF-CEM model). Tumor cell growth or T cell expansion/persistence was evaluated by injecting mice intraperitoneally with 100 μL D-luciferin (30 mg/mL; PerkinElmer, Waltham, MA, USA) followed by bioluminescence imaging using an IVIS Lumina II imaging system (Caliper Life Sciences, Hopkinton, MA, USA) and analyzed by Living Image software (Caliper Life Sciences). To quantify tumor cells and T cells in mouse peripheral blood, we stained 50 μL of blood obtained by tail-vein bleeding with CD3, CD4, CD7, CD8, CD45, and HLA-A2, and then treated with RBC Lysis Buffer (BioLegend) to lyse red blood cells. $CD45^+CD3^+HLA\text{-}A2^+$ cells (infused T cells) and $CD45^+CD3^+HLA\text{-}A2^-$ cells (tumor cells) were counted by a flow cytometer using CountBright Absolute Counting Beads. Mouse peripheral blood was first treated with RBC Lysis Buffer, then stained with anti-Fc antibody, washed, and stained with CD3, CD4, CD7, CD8, CD45, and HLA-A2, to evaluate CAR expression on T cells.

Western blot and qPCR

To evaluate CD7 protein and mRNA level in CD7 CAR T cells *in vivo*, we extracted human T cells from mouse spleen by treating mashed spleen samples with RBC Lysis Buffer. The collected cells were cultured *in vitro* with IL-7 and IL-15 for 2–4 weeks, and then total protein or total RNA was extracted. At the time of protein/total RNA extraction, more than 95% of cells were positive for CD45, CD3, and HLA-A2 (infused T cells) in all samples. For western blot, cell lysates were run on the Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA) and wet transferred onto nitrocellulose. Blot was probed with anti-CD7 antibody (Clone: EPR4242, Cat# ab109296; Abcam, Waltham, MA, USA) and anti-GAPDH antibody (Clone: 6C5, Cat# sc-32233; Santa Cruz Biotechnology, Dallas, TX, USA) followed by Goat anti-Mouse IRDye 680RD (Cat# 925-68070; LI-COR Biosciences, Lincoln, NE, USA) and Goat anti-Rabbit IRDye 800CW (Cat# 925-32211; LI-COR Biosciences). Blots were developed using the LI-COR Odyssey CLx (LI-COR Biosciences). For qPCR for CD7 mRNA, total RNA was extracted by RNeasy kits (QIAGEN, Germantown, MD, USA), then cDNA was generated by Superscript III (Thermo Fisher Scientific, Invitrogen). qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) in the CFX85 Real-Time system (Bio-Rad). Primer sequences used are listed below: ACTB forward, 5′-AGAGCTACGAGCTGCCTGAC-3′, ACTB reverse, 5′-GGATGCCACAGGACTCCA-3′; CD7 forward, 5′-CCAGGACAACCTGACTATCACC-3′, CD7 reverse; 5′-AGCA TCTGTGCCATCCTTG-3′.

RNA-seq and data analysis

Total RNA samples for qPCR as described above were further treated with RNase-Free DNase (QIAGEN, Germantown, MD) to remove contaminating genomic DNA. mRNA library preparation and next generation sequencing were performed by Baylor College of Medicine Genomic and RNA Profiling Core using Illumina NovaSeq 6000 (read length: 100 bp paired ends; number of reads per sample: 20 million).

RNA-seq reads were aligned to the human genome (GRCh38, primary assembly) and transcriptome (Gencode version 38 primary assembly gene annotation) using STAR version 2.7.9a. The following non-standard parameters were used for STAR alignment: –outFilterMultimapNmax 1 –outSAMstrandField intronMotif– outFilterType BySJout –alignSJoverhangMin 8 –alignSJDBove 3 –alignEndsType EndToEnd. Only uniquely aligned reads were retained for differential gene expression analysis. Individual gene expression was obtained by counting reads over genes from the same annotation as alignment using featureCounts version 1.5.0-p. Differential gene expression analysis was conducted using DESeq2. Significantly differentially expressed genes (DEGs) were defined as genes with $|\log2FC| \geq 1$ and false discovery rate (FDR) ≤ 0.05. Raw counts were converted to the log2 counts per million (lcpms) scale, 102 DEGs were extracted, and Z score normalization was applied on lcpm scaled data across samples for each individual DEG. Both unsupervised clustering heatmaps were produced using Euclidean clustering.

cGMP manufacturing of unedited CD7 CAR T cells for patients with T cell malignancies

Autologous CD7 CAR T cells were produced in the cGMP facility from patients enrolled on the CRIMSON-NE study using a manufacturing method that closely resembles the research-grade process outlined above. In brief, freshly thawed PBMCs from patients with $CD7^+$ T cell malignancies were plated in T75 flasks coated with anti-CD3/anti-CD28 antibodies in the presence of 200 nM ibrutinib. No T-ALL blast depletion or T cell selection was performed prior to plating. Three days later, T cells were collected, counted, and transduced with a clinical-grade gammaretroviral vector encoding CD7 CAR using retronectin-coated flasks. Immediately following transduction, dasatinib was added to the final concentration of 500 nM along with recombinant IL-7 (5 ng/mL) and IL-15 (5 ng/mL). Final concentration of dasatinib was selected after validation runs to ensure full suppression of CAR signaling in all patient-derived products during cell expansion. Cells were moved to G-Rex culture devices on the next day and allowed to expand in the fresh medium supplemented with ibrutinib, dasatinib, and IL-7/IL-15 cytokines for 3 additional days. On day 4 post-transduction, T cells were collected, counted, and cryopreserved according to US Food and Drug Administration (FDA)-approved cGMP SOPs. CAR expression and the presence of malignant T cells were measured by flow cytometry for each product. Potency of CD7 CAR T cell products has been assessed by coculturing with $CD7^+$ Jurkat T-ALL cells modified to express FFluc and quantifying residual tumor cells by measuring luminescence on addition of D-luciferin. Average gammaretroviral vector copy number per T cell has been quantified by qPCR using TaqMAN primers specific to CAR sequence.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). Statistical tests used in each experiment are described in the figure legends.

DATA AVAILABILITY

RNA-seq data will be shared publicly in an online depository prior to publication. All other data generated for this study will be made available on reasonable request to the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2022.09.003.

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AUTHOR CONTRIBUTIONS

N.W. and F.M. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. R.Z. and C.C. performed the analysis of the RNA-seq data. R.M. and D.G.v.L. performed experiments and/or analyzed data. M.S. and L.D.S. assisted with initial pilot experiments. V.C.B., J.S.-R., H.H., S.W., B.M., H.Z., and S.G.T. manufactured cGMP CD7 CAR T cell lines or performed release testing. L.C.H. enrolled patients on the CRIMSON-NE protocol and is a lead clinical principal investigator of the study. H.E.H. and M.K.B. advised on the study. M.M. conceptualized, directed, and funded the study; designed experiments; analyzed and interpreted data; and wrote the manuscript. All authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

H.E.H. is a cofounder with equity in Allovir and Marker Therapeutics; serves on advisory boards for Gilead Sciences, GSK, Tessa Therapeutics, Fresh Wind Biotherapeutics, Novartis, and Kiadis; and has received research funding from Tessa Therapeutics and Kuur Therapeutics. M.K.B. is a cofounder with equity in Allovir, Marker Therapeutics, and Tessa Therapeutics and serves on advisory boards for Tessa Therapeutics, Allogene Therapeutics, Memgen, Kuur Therapeutics, Walking Fish Therapeutics, Tscan, Abintus, and Turnstone Biologics.

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