The Art and Science of Selecting a CD123-Specific Chimeric Antigen Receptor for Clinical Testing

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Chimeric antigen receptor (CAR) T cells targeting CD123, an acute myeloid leukemia (AML) antigen, hold the promise of improving outcomes for patients with refractory/recurrent disease. We generated five lentiviral vectors encoding CD20, which may serve as a target for CAR T cell depletion, and 2nd or 3rd generation CD123-CARs since the benefit of two costimulatory domains is model dependent. Four CARs were based on the CD123-specific single-chain variable fragment (scFv) 26292 (292) and one CAR on the CD123-specific scFv 26716 (716), respectively. We designed CARs with different hinge/transmembrane (H/TM) domains and costimulatory domains, in combination with the zeta (z) signaling domain: 292.CD8aH/TM.CD28z (28.28z), 292.CD28H/TM.CD28z (716.CD8aH/TM.CD28z), 292.CD8aH/TM.CD28z (8.28z), and 292.CD28H/TM.CD28z (28.28z).

Transduction efficiency, expansion, phenotype, and target cell recognition of the generated CD123-CAR T cells did not significantly differ. CAR constructs were eliminated for the following reasons: (1) 8.41BBz CARs induced significant baseline signaling, (2) 716.8.28z CAR T cells had decreased anti-AML activity, and (3) 28.41BBz CAR T cells had no improved effector function in comparison to 28z CAR T cells. We selected the 28.28z CAR since CAR expression on the cell surface of transduced T cells was higher in comparison to 28z CARs. The clinical study (NCT04318678) evaluating 28.28z CAR T cells is now open for patient accrual.

INTRODUCTION

Acute myeloid leukemia (AML) remains a clinically challenging disease because of its high morbidity, mortality, and relapse rates.3 Changes in supportive care have contributed to decreased treatment-related mortality in recent years. However, increased toxicities have dampened the benefit of intensive chemotherapy regimens on overall survival, and novel therapies are needed.

Although clinical studies are needed to assess the role of CAR design on the efficacy and safety of CD123-CAR T cell therapy, careful selection of CAR sequences is necessary to improve CAR T cell activity and minimize toxicity.

Several investigators have generated CD123-CARs with different endodomains, with a specific focus on CARs with a CD28 or 4-1BB endodomain for early phase clinical testing.12,14,15 Both CD28z- and 41BBz-CAR T cells targeting CD19 demonstrated potent antileukemia and lymphoma activity in humans, leading to their FDA approval.16,17 However, CD28z-CAR T cells persist for a shorter period of time in humans than do 41BBz-CAR T cells. Therefore, CD28z-CAR T cells are preferred for targeting CD1233 AML to limit HPC toxicity. CD28-CAR T cells, however, can recognize lower levels of antigens expressed on the cell surface of target cells than can 41BBz-CAR T cells, suggesting that CARs with a 41BBz endodomain are preferred.18 In addition, the nonsignaling components of CARs, including the H/TM domain, influence CAR expression and CAR T cell function.19–21

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A

B

C

D

E

F

NS

NS

NS

8.41BBz 8.28z 716.8.28z 28.28z 28.28.41BBz

CD20

CD123-CAR (%, %)

CD20 (%, %)

CD20-CAR+ (%)

CD20-CAR+ (%)

CD20-CAR+ (%)

CD20-CAR+ (%)

CD20-CAR+ (%)

CD20-CAR+ (%)

CD20-CAR+ (%)

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comparison of CAR T cells in preclinical studies is also needed to select a CAR construct for clinical testing. Here, we generated and compared five lentiviral vectors (LVs) encoding CD20, which may serve as a target for T cell product depletion, and CARs that differed in H/TM and signaling domains, with the goal of selecting a construct for future clinical testing. Although we only observed minor differences between constructs, we selected a CAR with a CD28 H/TM and CD28z signaling domain. This CAR will be evaluated in an FDA-approved bridge-to-transplant phase 1 clinical study designed to test CD123-CAR T cell safety and efficacy.

RESULTS

Generation of CD123-CARCD20 T Cells

We designed five bicistronic LVs encoding CD20, a 2A peptide sequence, and different CD123-CARs (Figure 1A). For four CARs, we used the CD123-specific scFv 26292 (292), and for one CAR the CD123-specific scFv 26716 (716), respectively. We generated CARs with different H/TM domains and costimulatory domains, in combination with the zeta (z) signaling domain. We used either CD28 or 41BB as costimulatory domains. In addition, we explored a 3rd generation CAR design with the zeta (z) signaling domain. We used either CD28 or 41BB as costimulatory domains. In addition, we explored a 3rd generation CAR design since at present the benefit of adding 41BB to CD28 costimulation is model dependent. Based on this, we constructed the following CARs: (1) CD20-2A-292.CD8aH/TM.41BBz (8.41BBz), (2) CD20-2A-292.CD8a H/TM.CD28z (8.28z), (3) CD20-2A-716.CD8aH/TM.CD28z (716.8.28z), (4) CD20-2A-292.CD28H/TM.CD28z (28.28z), and (5) 292.CD8aH/TM.CD28z.28.41BBz (28.28.41BBz). The amino acid sequence of the five CAR constructs is provided in the Supplemental Information (Figure S1). We generated T cells expressing CD123-CARs and CD20 (CD123-CARCD20) after lentiviral transduction, which mirrors our clinical-grade CAR T cell production process (Figure 1B). Transduction efficiency was determined by vector copy number (VCN) per cell and flow cytometry analysis. The mean VCN ranged from 1.31 to 2.25 (716.CD8aH/TM.CD28z, 716.8.28z, 28.28z, 28.41BBz, 28.28.41BBz). The mean fluorescence intensity (MFI) levels of CD123-CAR and CD20 expression were not different (Figure S2).

When we compared all five CAR constructs, we found no significant differences in VCN and transgene expression (p > 0.05). Comparison of CD8a and CD28 H/TM domain-containing CARs with the same signaling domain revealed a mean 2.3-fold (range, 1.9–2.6) higher MFI of CD28 H/TM CARs than that of CD8a H/TM CARs on the cell surface of T cells (Figure S3). In contrast, the mean fold changes for all other transduction parameters (i.e., percent CAR+, VCN, percent CD20+, and CD20 MFI) between T cells transduced with the CD20-2A-CD8a or CD28 H/TM CAR LVs were between 0.8 and 1.2 (Figure S3).

CAR Design Does Not Influence Expansion, Viability, or Phenotype of CD123-CARCD20 T Cell Products

CD123-CARCD20 T cells expanded more than 50-fold after 7 days in culture and exhibited viability of greater than 80% (Figures 2A and 2B; n = 5, p > 0.05). Determination of immunophenotype subsets (naïve, CCR7+CD45RO+; central memory [CM], CCR7+CD45RO+; terminally differentiated [TD], CCR7+CD45RO–; and effector memory [EM], CCR7+CD45RO–) on day 8 revealed an approximate CD4:CD8 ratio of 1:1 for all constructs (Figure S4A, n = 5, p > 0.05). Most T cells demonstrated an EM or CM phenotype (Figure 2C). Only non-transduced (NT) T cells contained a significant percentage of naïve T cells when compared with CD123-CARCD20 T cells (percentage of CD4+ cells in NT versus CD123-CARCD20 T cells: n = 5, p > 0.01; percentage of CD8+ cells in NT versus CD123-CARCD20 T cells: p < 0.0001). Flow cytometry analysis of the activation markers CD27, Tim3, and PD1 revealed no significant differences of single-positive (Figure 2B–2D; Figures S4B–S4D) or double-positive (Tim3+/PD1+) populations between CD123-CARCD20 T cells for either the CD4+ or CD8+ subsets (Figure 2D; n = 5, p > 0.05).

T Cells Expressing CD123-CARCD20 Recognize and Kill CD123+ Targets

To test the functional activity of CD123-CARCD20 T cells, we maintained effector cells in media alone or in co-cultures with either CD123+ (K562) or CD123+ (Molm13) cells and assayed for interferon-γ (IFN-γ) production. All CD123-CARCD20 T cell populations exhibited potent cytokine secretion in response to CD123+ target cells, as compared with NT T cells (Figure 3A; n = 5, IFN-γ secreted by CD123-CARCD20 T cells: range = 6,176–39,000 pg/mL; p < 0.0001). At baseline (media or K562 conditions), T cells expressing the 8.41BBz-CAR produced significantly higher IFN-γ levels (Figure 3A; p < 0.0001) than did the other CAR constructs. CD123-CARCD20 T cells exhibited significant in vitro antitumor activity against CD123+ target cells (Figure 3B; n = 5; p < 0.0001) but not against CD123− cells (K562). In contrast, NT T cells did not secrete IFN-γ or kill CD123+ target cells (Figure 3C). Thus, all CD123-CARCD20 T cell products had the desired specificity, and only the 8.41BBz-CAR induced significant IFN-γ production and thereby baseline T cell activation. In addition, all CD123-CARCD20 T cell populations were efficiently eliminated (Figure S4E; n = 15, p = 0.0007) in the presence of rituximab and complement, with no differences between constructs.

CD34+ HPCs Are Recognized to a Greater Extent by 716 Than by 292 scFv-Based CARs

Because we observed no difference in AML target recognition among the constructs, we next compared the potential on target/
off cancer toxicity of CD123-CARCD20 T cells against CD34+ HPCs in a standard colony-forming unit (CFU) assay at two effector to target ratios (E:T; 1:1 and 5:1). At an E:T ratio of 1:1, three (716.8.28z, 28.28z, and 28.28.41BBz) of the five evaluated CD123-CARCD20 T cell populations were cytotoxic to CD34+ target cells (Figure 4; n = 6 biological replicates). At an E:T ratio of 5:1, all CD123-CARCD20 T cells significantly reduced the number of CFUs formed (p < 0.05). At this higher E:T ratio, 716.8.28z CAR T cells induced a greater reduction in CFUs (Figure 4) than did the other CD123-CARCD20 T cells.

**DISCUSSION**

In this study, we generated five different LV constructs encoding CD20 and CD123-CARs that differed in their antigen binding, H/TM, and/or signaling domains. The CD123 CARCD20 T cell populations shared similar immunophenotypes and effector functions in vitro and in vivo, except for differences in baseline signaling and recognition of HPCs. On the basis
of our comprehensive analysis, we selected one construct for clinical testing.

The effector function of CAR T cells directly results from the interplay between the antigen recognition domain (e.g., scFv affinity and antigen density),18 length and flexibility of the H/TM domain,19–21 and the costimulatory domain selected.22,24,25 More recently, small amino acid variations in and around the H/TM domain were found to affect the activity of CD19-CAR T cells in patients.26 However, at present, no general rules of CAR design have emerged, and CAR design for a particular antigen remains largely empiric. We therefore tested two different scFvs (26292 and 32716) with CD8a or CD28 H/TM domains and CD28 and/or 4-1BB as costimulatory domains in our study.

Because CAR T cell function is influenced by the manufacturing process27–30 and our intent was to select a construct for clinical testing, we used a CAR T cell generation method that is established in our Current Good Manufacturing Practice facility. This method relies on CD4/CD8 selection, followed by activation, transduction, and expansion of CAR T cells in the presence of interleukin-7 (IL-7) and IL-15 in G-Rex culture devices. The resulting CAR T cell products exhibited a CD4 to CD8 ratio that approached 1:1, which is deemed favorable by other investigators.29,30 Of note, this was achieved without manufacturing CD4+ and CD8+ CAR T cells separately, simplifying the manufacturing process. The constructs expressing CD28 H/TM domains that also contained the same signaling domain resulted in higher levels of CAR expression than did their counterparts expressing CD8a H/TM. Although the influence of the H/TM domain on CAR cell surface expression has been reported by others,19–21,31 our study demonstrated that this is not due to differences in the transduction efficiencies of the CAR-encoding vectors.

The resulting CD123-CARCD20 T cells exhibited similar immunophenotypes, with a predominance of central and effector memory T cell subsets. The role that different T cell subsets play in determining CAR T cell efficacy is now emerging. For example, a recent study showed that CD19.28z-CAR T cell products containing a decreased amount of naive T cells correlate with increased progression-free survival (PFS) for poor risk and relapsed and refractory B cell non-Hodgkin’s lymphoma.32 In the same study, the presence of CD8+ CAR central memory T cells marginally improved PFS.32 Others have also reported that CD19-CAR T cell populations that are CD27+/PD-1−/CD8+ are a predictor for sustained remission.33

Of the CD123-CARs we generated, only the construct encoding a 4-1BB costimulatory domain induced baseline (tonic) signaling, which was evidenced by marked IFN-γ production without antigen-specific stimulation. However, this did not translate to increased expression of the markers associated with exhaustion, such as Tim3 and PD1, or decreased effector function. Nevertheless, we excluded this CAR as a potential candidate from clinical testing because of reports by others that tonic signaling can negatively affect CAR T cell function.22 While studies have indicated expression of exhaustion markers such as Tim3, PD1, and LAG3 correlate with CAR C T cell function,22 we have observed no or only transient differences in several models.34–36 In addition, a recent publication suggests that silencing of PD-1 has the potential to impair CAR T cell function.37 Since at present there is no clear definition of T cell exhaustion,38 investigators have focused on defining epigenetic programs that define T cell plasticity.39 Indeed our recent studies indicate that deletion of the de novo DNA methyltransferase (DNMT3A) in multiple human CAR T cell

![Figure 3. CD123-CARCD20 T Cells Recognize and Kill CD123+ Targets in an Antigen-Specific Manner](image-url)
systems resulted in a preservation of the CAR T cell’s ability to proliferate and mount an effector response during chronic antigen exposure.40 Thus, future studies are needed to define the epigenetic programs of CD123-CAR T cell populations.

We found that T cells expressing the 716 scFv-based CAR recognized HPCs to a greater extent than did 292 scFv-based CARs. Both scFvs bind to different epitopes; however, they have similar affinities for CD123 that are in the nanomolar range.41 Despite these similar affinities, only the 292 scFv-based immunotoxin imparted notable cytotoxic activity, indicating that the different binding sites within the extracellular domain of the antigen can affect the activity of immune-based approaches.42 This was also reported for CAR T cells.43,44 One study compared both scFvs in the context of CD28z-CARs and found no HPC toxicity for either CAR;14 however, these CARs contained a longer H domain (immunoglobulin G4 [IgG4]-Fc) than did ours. Recognition of HPCs by T cells expressing a 716 scFv-based 41BBz-CAR has been reported, and several safety switches are being actively explored to mitigate on target/off cancer toxicity. 292 and 716 scFv-based CAR T cells have the potential to recognize and kill HPCs in humans. Thus, having a suitable hematopoietic stem cell donor is one of the eligibility criteria of most current clinical studies evaluating the safety and efficacy of CD123-CAR T cells. Lastly, while CD123 splice variants have been described,45 a recent study suggest that there is evidence that normal HPCs express different splice variants than AML blasts.46 We and others have previously demonstrated that transgenic expression of CD20 in T cells is a promising strategy to eliminate T cells with the clinical grade CD20 antibody rituximab, and we therefore included CD20 in our CD123-CAR encoding LVs as a safety switch.12,47,48

We evaluated the antitumor activity of CD123-CARCD20 T cells at two dose levels. Although we did not observe significant differences between the CAR T cell groups at higher doses, the lower dose 716 scFv-based CD123-CARCD20 T cells significantly decreased antitumor activity. No difference in in vivo antitumor activity of 292 scFv- and 716 scFv-based CD28z-CD123-CAR T cells was reported, but only a single T cell dose was evaluated.15 The addition of a 4-1BB signaling domain to CD28.z-CAR T cells did not improve antitumor activity in our study, which is consistent with reports by others that the benefit of incorporating a 4-1BB signaling domain into CD28.z-CAR T cells is model dependent.22,23 One study compared the expansion of CD28.z- and CD28.41BBz-CAR T cells targeting CD19 in individual patients with lymphoma and observed increased CD28.41BBz-CAR T cell expansion with low disease burden.49 We selected the CD28.CD28z CAR for clinical development since it was expressed at higher levels on T cells in comparison to CD8a.CD28z CARs. Higher cell surface expression of CD28 H/TMs in comparison to CD8a H/TMs was not observed in a recent study.50 However, investigators found that CD28 H/TM CARs formed more stable immunological synapses than CD8a H/TMs,50 supporting the selection of our CD28.CD28z CAR for clinical testing.

Our study has several limitations; first, we did not determine the in vivo expansion and persistence of infused CD123-CAR T cells. These studies should ideally be performed in syngeneic mouse models to exclude xenogeneic CAR T cell stimulation as a confounding factor. In this regard, we are currently developing an immune competent model to evaluate CD123-CAR T cells. In addition, we did not compare 716-based (28.28z) CARs to 292-based CARs (292.28.z) directly in our initial studies. In subsequent studies we have now shown that there are no significant differences in transduction efficiency, phenotype, expansion, and effector function between both CAR constructs in vitro studies (Figures S6–S8).

In conclusion, no clear winner emerged from our evaluation of the five CAR constructs. However, subtle differences emerged, leading us to make an informed decision: one CAR demonstrated tonic signaling; one CAR had limited antitumor activity; and one CAR did not endow T cells with improved effector function despite having a more complex design (i.e., two costimulatory endodomains). Of the remaining two CARs, we selected the CAR with a CD28 H/TM domain for clinical testing because it resulted in higher levels of CAR expression. The safety and efficacy of CD123-CARCD20 T cells generated with the selected CAR will be evaluated in an approved clinical study (NCT04318678) that is now open for patient accrual.

MATERIALS AND METHODS

Cells and Culture Conditions

Deidentified apheresis products from healthy donors were purchased from Key Biologics (Memphis, TN, USA; 16761) and were used in accordance with the Helsinki Declaration. Authenticated K562 and...
Figure 5. CD123-CAR<sup>CD20</sup> T Cells Have Potent Antitumor Activity <i>In Vivo</i>

(A) Schematic of experimental design. (B–E) Animals were intravenously injected with Molm13-expressing luciferase, followed by infusion of either $3 \times 10^6$ or $1 \times 10^7$ effector T cells and in vivo imaging for evaluation of tumor burden. (B) Bioluminescence signal over time (total flux in photons/s) of group receiving $1 \times 10^7$ T cells. (C) Kaplan-Meier survival curves for animal groups receiving $1 \times 10^7$ T cells. Statistical significance was determined with a Wilcoxon rank-sum test ($p < 0.05$; blue shading in table). (D) Bioluminescence signal over time (total flux in photons/s) of group receiving $3 \times 10^6$ T cells. (E) Kaplan-Meier survival curves for animal groups receiving $3 \times 10^6$ T cells. Statistical significance was determined by Wilcoxon rank-sum test ($p < 0.05$; blue shading in table).
Molm13 cell lines were obtained from ATCC. Molm13 cells expressing a GFP firefly luciferase (Molm13.fluc) fusion molecule were previously described. All cell lines were maintained in RPMI culture media (GE Healthcare Life Sciences, Logan, UT, USA; SH30096.01) supplemented with 10% fetal bovine serum (FBS; GIBCO/Thermo Fisher Scientific, Waltham, MA, USA; 10082-147) and L-glutamine (GlutaMAX; GIBCO/Thermo Fisher Scientific; 35050-061).

LVs
The LV backbone used for this study has been previously described, except that the insulators were removed from the self-inactivating 3′ partially deleted viral long terminal repeats, according to the safety records of LVs in clinical trials. The expression cassette of the LV is under the control of the MND promoter (myeloproliferative sarcoma virus enhancer, negative control region deleted, d587rev primer-binding site substituted). Mini genes encoding CD20, 2A, and the CD123-specific CARs were synthesized by GeneArt (Thermo Fisher, Waltham, MA, USA) and subcloned by standard techniques. All cloned CD20-2A-CD123-CAR constructs were verified by sequencing at the Hartwell Center at St. Jude Children’s Research Hospital (St. Jude). Purified lentiviral particles were produced by the St. Jude Vector Core Laboratory by using transient transfection, followed by fast protein liquid chromatography purification.

CD4 and CD8 T Cell Isolation
CD4+/CD8+ T cells were magnetically isolated on a CliniMACS Plus instrument (Miltenyi Biotec; Bergisch Gladbach, Germany) with CD4 (Miltenyi Biotec; 130-030-401) and CD8 (Miltenyi Biotec; 130-030-801) microbeads and the enrichment program 1.1, per the manufacturer instructions. Aliquots of enriched CD4+/CD8+ T cells were cryopreserved and thawed before use for CAR T cell generation.

CD123-CAR T Cell Generation
Enriched CD4+CD8- T cells were resuspended at 1 × 10^6 per mL in X-VIVO 15 (Lonza, Walkersville, MD, USA; 04-744Q) supplemented with 5% human AB serum (Corning, Corning, NY, USA; 35-060-CI) and 10 ng/mL each IL-7 and IL-15 (Miltenyi Biotec; 170-076-111 and 170-076-114, respectively). Cells were activated by plating overnight with T cell TransAct (Miltenyi Biotec; 130-019-011). On day 1, 2 × 10^6 T cells were plated with LVs and transduced overnight at a multiplicity of infection of 10. After transduction, the cells were transferred to 6-well G-Rex plates (Wilson Wolf, New Brighton, MN, USA; 180102-1) and expanded for 7–10 days. On day 6, half of the media was removed and replaced with complete media with cytokines.

VCN
Transduced T cells were harvested, and total genomic DNA was isolated with the Zymo Research Quick-DNA 96-well kit (Zymo Research, Irvine, CA, USA; D3012). To determine the VCN per cell, we digested the genomic DNA with MspI and used as a template in PCR by using a digital droplet PCR instrument (QX200 Bio-Rad, Carlsbad, CA, USA). The following primer-probe sets were used to amplify the HIV psi sequence located on the vector genome and the endogenous control gene RPP30: 5′-ACTGAAAAAGGAAGGGAAC-3′, 5′-ACCCCATCTCTCTCTTCCTACGCC-3′, and probe 5′-FAM-AGCTCTCTGACGCAGAAGCTAAGGC-3′ and 5′-GGGCTGTCTCCCAAGATG-3′, 5′-GATTGGAACCTGCGAGGG-3′, and probe 5′-HEX-CTGACCTGAGGCTCT-3′, respectively. The reaction mixture contained ddPCR Supermix for probes without UTP (BioRad; 64180520). The cycled droplets were read with a QX200 droplet reader (Bio-Rad). The ratio of the numbers of molecules of these two genes was determined by the sample’s gene of interest relative copy number analyzed with QuantaSoft droplet reader software, version 1.7.4.0917 (Bio-Rad).

Flow Cytometry
Cells were stained with fluorochrome-conjugated primary antibodies for 30 min at 4°C and washed with fluorescence-activated cell sorting (FACS) buffer (2% FBS in 1× PBS) before analysis. For CAR staining, cells were washed with 1× PBS twice and then incubated with a recombinant CD123-Fc fusion protein (Abcam; ab88358) in PBS for 30 min at 4°C. The cells were then washed, incubated with the secondary antibody in FACS buffer for 30 min at 4°C, and washed with FACS buffer before analysis. Stained cells were analyzed with a CytoFLEX instrument (Beckman Coulter, Indianapolis, IN, USA) and FlowJo software. The following antibodies were used: CD4 (Clone OKT4, BV785, BioLegend; 317442), CD8 (Clone SK1, APC-Cy7, BD PharMingen; 557834), CCR7 (Clone REA546, PE, Miltenyi Biotech; 130-108-285), CD45RO (Clone UCHL1, APC, Tonbo; 20-00457-T100), Tim3 (Clone F38-2E2, PE-Cy7, Biolegend; 345014), PD1 (Clone EH12.2H7, BV421, Biolegend; 329920), CD20 (Clone 2H7, FITC, Tonbo; 35-0209-T100), and goat anti-human Fc-IgG (pooled goat antiserum, PE, Southern Biotech; 2048-09).

Cytotoxicity Assay
Cytotoxic activity was evaluated with a flow-cytometry-based assay. Target cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Cayman Chemical, Ann Arbor, MI, USA; 600120) for 20 min at 37°C. We incubated 50,000 CFSE-labeled target cells overnight either alone or with effector T cells in round bottom 96-well plates (Corning, Corning, NY, USA; 353077) at an E:T ratio of 3:1. The cells were washed and resuspended in PBS containing Count Bright Absolute Counting Beads (Life Technologies, Eugene, OR, USA; C36950). CFSE was measured by flow cytometry with a BD FACSLyric instrument (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (Becton Dickinson). Lysis was calculated with the following formula: % lysis = 100 – (average CFSE+ events per 100 beads/average of CFSE- events per 100 beads in wells with target alone) × 100.

Cytokine Production
Effector cells were grown in culture at a 2:1 ratio with target cells or in the presence of media alone for 24 h in a 24-well plate (Corning; 353047). Supernatants were collected, and IFN-γ was determined with a QuantiKinE ELISA kit (R&D, Minneapolis, MN, USA; SF50), according to the manufacturer’s instructions.
CFU Assay
An apheresis product of mobilized peripheral blood was purchased from Key Biologics (Memphis, TN, USA), and CD34⁺ cells were isolated by the Human Applications Laboratory at St. Jude with a Clin-iMACS device, per the manufacturer instructions (Miltenyi Biotec). A modified CFU assay was performed. In brief, CD34⁺ cells (5 × 10⁴) were incubated with CD123-CAR<sup>CD20</sup> T cells at ratios of 5:1 and 1:1 (T:CD34) for 4 h in 96-well round bottom plates. For each co-culture, three replicates (input equivalent of 2,000 CD34⁺ cells) were plated into 1 mL MethoCult H4434 media (Stem Cell Technologies, Vancouver, BC, Canada; 04434), following the manufacturer’s instructions. Colonies were counted 12–14 days later.

Safety Switch Activation Assay
T cells were resuspended in RPMI/1% human serum (Corning; 35-060-Cl; heat inactivated) and incubated for 1 h at 37°C with 10 μg of rituximab (Biogen, Cambridge, MA; 502-051021) and 10% baby rabbit complement (Cedarlane, Burlington, NC; CL3441) in a 96-well round bottom plate, as previously published. The cells were washed and analyzed by flow cytometry for CD20 expression. Percent transgene expression was determined by the following formula: (% CD20<sup>+</sup>_before − %CD20<sup>+</sup>_after)/%CD20<sup>+</sup>_before × (1−%CD20<sup>+</sup>_after).

Xenograft Model
In vivo experiments were performed under a protocol approved by the St. Jude Institutional Animal Care and Use Committee. Animals were housed in specific pathogen-free rooms for the duration of the experiments. Female NSG mice (NOD-SCID IL-2Rγnull, NSG, NOD-SCID gamma) were obtained from the St. Jude breeding colony at 8–10 weeks of age. The mice received 5 × 10⁶ Molm13 tumor cells modified to express a GFP::luc plasmid. Mice were subsequently performed in the St. Jude Center for Imaging and Therapeutics with a Xenogen IVIS-200 imaging system (IVIS, Xenogen, Alameda, CA, USA), as previously described. The mice were euthanized at predefined endpoints or when they met euthanasia criteria in accordance with St. Jude Animal Resource Center.

Statistics
Data were summarized using descriptive statistics. A Friedman or permutation test was used to examine overall differences in continuous variables between lentiviral CD123-CAR<sup>CD20</sup> constructs. The overall test was followed by pairwise comparisons with Wilcoxon signed-rank or paired-permutation tests, when appropriate. The constructs were then compared to the control sample with Wilcoxon signed-rank tests. The CD4:CD8 ratio for each construct was compared to a value of 1 with Wilcoxon signed-rank tests (i.e., H₀: ratio = 1; H₁: ratio ≠ 1). The bias-corrected and accelerated bootstrap confidence intervals are reported for the median ratio for each construct. The Kruskal-Wallis test was used to examine overall differences in bioluminescence on day 12 between constructs. The overall test was followed by pairwise comparisons with Wilcoxon rank-sum tests. A two-way repeated-measures ANOVA with a rank transformation was used to examine overall differences in bioluminescence over time and between constructs. Time, construct, and their interaction were considered in the model. Survival was compared among constructs with Wilcoxon rank-sum tests. Statistical analyses were conducted with R software, version 3.6.0 (Lucent Technologies, Murray Hill, New Providence, NJ, USA).

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.06.024.

AUTHOR CONTRIBUTIONS
Conceptualization, J.M.R., C.L.B., S.G., and M.P.V.; Data Analysis, A.S., N.S., J.M.R., S.G., and M.P.V.; Investigation, J.M.R., S.Z., F.Z., Y.-K., J.M., and A.V.; Resources, R.E.T. and B.R.; Writing – Original Draft, J.M.R., J.M., R.E.T., S.G., and M.P.V.; Writing – Review & Editing, J.M.R., S.Z., F.Z., Y.-K., J.M., A.V., R.E.T., A.S., N.S., C.L.B., B.R., S.G., and M.P.V.; Funding Acquisition, S.G., and M.P.V.; Supervision, S.G. and M.P.V.

CONFLICTS OF INTEREST
J.M.R., S.Z., F.Z., Y.-K., A.V., R.E.T., C.L.B., B.R., S.G., and M.P.V. have patent applications in the field of Cell and Gene Therapy.

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