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Chimeric antigen receptor T cells to target CD79b in B-cell lymphomas

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ABSTRACT

Background Chimeric antigen receptor (CAR) T cells targeting CD19 mediate potent and durable effects in B-cell malignancies. However, antigen loss or downregulation is a frequent cause of resistance. Here, we report development of a novel CAR T-cell therapy product to target CD79b, a pan B-cell antigen, widely expressed in most B-cell lymphomas.

Methods We generated a novel anti-CD79b monoclonal antibody by hybridoma method. The specificity of the antibody was determined by testing against isogenic cell lines with human CD79b knock-in or knock-out. A single-chain variable fragment derived from the monoclonal antibody was used to make a panel of CD79b-targeting CAR molecules containing various hinge, transmembrane, and co-stimulatory domains. These were lentivirally transduced into primary T cells and tested for antitumor activity in in vitro and in vivo B-cell lymphoma models.

Results We found that the novel anti-CD79b monoclonal antibody was highly specific and bound only to human CD79b and no other cell surface protein. In testing the various CD79b-targeting CAR molecules, superior antitumor efficacy in vitro and in vivo was found for a CAR consisting CD8α hinge and transmembrane domains, an OX40 co-stimulatory domain, and a CD3ζ signaling domain. This CD79b CAR specifically recognized human CD79b-expressing lymphoma cell lines but not CD79b knock-out cell lines. CD79b CAR T cells, generated from T cells from either healthy donors or patients with lymphoma, proliferated, produced cytokines, degranulated, and exhibited robust cytotoxic activity in vitro against $CD19^+$ and $CD19^-$ lymphoma cell lines and patient-derived lymphoma tumors relapsing after prior CD19 CAR T-cell therapy. Furthermore, CD79b CAR T cells were highly efficient at eradicating pre-established lymphoma tumors in vivo in three aggressive lymphoma xenograft models, including two cell line-derived xenografts and one patient-derived xenograft. Notably, these CAR T cells did not demonstrate any significant tonic signaling activity or markers of exhaustion.

Conclusion Our results indicated that this novel CD79b CAR T-cell therapy product has robust antitumor activity against B-cell lymphomas. These results supported initiation of a phase 1 clinical trial to evaluate this product in patients with relapsed or refractory B-cell lymphomas.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Chimeric antigen receptor (CAR) T cells targeting CD19 are highly effective but relapse is common due to antigen loss or downregulation. Targeting alternative B-cell antigens is needed to improve outcomes in these patients.

WHAT THIS STUDY ADDS

⇒ We developed a novel CAR T-cell therapy product targeting CD79b using a novel monoclonal antibody and a novel CAR design. The CD79b CAR T cells were highly effective against various preclinical models of B-cell lymphomas.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The results of these preclinical studies supported initiation of a clinical trial for evaluation of this novel CD79b-targeting CAR T-cell therapy product in patients with B-cell lymphoma.

INTRODUCTION

Four autologous chimeric antigen receptor (CAR) T-cell therapy products, all targeting CD19, have recently been approved for relapsed or refractory (r/r) B-cell lymphomas including large B-cell lymphoma (LBCL), mantle cell lymphoma (MCL), and follicular lymphoma (FL).[1–5] In pivotal trials, durable remissions lasting more than 2 years have been observed in ~40–50% of these patients. However, >50% of patients relapse or progress suggesting alternative therapies are needed for these patients. Indeed, recent studies showed that the median survival of patients with LBCL and patients with MCL relapsing after CD19 CAR T-cell therapy is 6 months or less highlighting a major unmet need.[6–8] We and others have observed that about 30–40% of LBCL tumors relapsing after CD19 CAR T-cell therapy have loss or downregulation of CD19, indicating antigen escape as a major mechanism of resistance.[6 9–11] Antigen loss has also been reported in patients with MCL after CD19 CAR T-cell therapy.[7] However,

expression of other pan-B-cell antigens such as CD20, CD22, CD79a, and CD79b was not altered in the relapsing tumors with or without CD19 loss[11–14] providing a rationale to target alternative antigens in these patients.

We chose to develop a novel CAR T-cell therapy product to target CD79b, a pan-B-cell antigen that is expressed on the cell surface as a heterodimer with CD79a and serves as a co-receptor for the B-cell receptor in normal B cells and leads to initiation of the signal transduction cascade activated by the B-cell receptor complex. Consistent with its expression in mature normal B cells, CD79b was previously reported to be highly expressed across various mature B-cell malignancies including LBCL, MCL, FL, marginal zone lymphoma, Burkitt lymphoma, lymphoplasmacytic lymphoma, and hairy cell leukemia whereas its expression is lower in chronic lymphocytic leukemia.[15 16] Here, we describe the development of a CAR T-cell therapy product to target CD79b using a novel monoclonal antibody and demonstrate its antitumor activity in vitro and in vivo in multiple preclinical models of B-cell lymphomas.

MATERIALS AND METHODS

Cell lines and culture

The human B-cell malignancy cell lines, Daudi (Burkitt's lymphoma), Jeko-1 (MCL), SUDHL-6 (LBCL), and NALM-6 (B-cell acute lymphoblastic leukemia) were obtained from American Type Culture Collection and cultured according to the supplier's recommendations. The above cell lines were transduced to express a firefly luciferase and green fluorescent protein (GFP). Isogenic cell lines of SUDHL6 with CD19 knock-out (KO), CD79b KO, and CD19 and CD79b double KO were generated by CRISPR/Cas9 approach as previously described.[11]

CAR constructs

A novel murine anti-CD79b monoclonal antibody (clone 28B) was generated by hybridoma approach (online supplemental methods). Second-generation anti-CD79b (clone 28B) or anti-CD19 (clone FMC63) CARs with single-chain variable fragment (scFv) were synthesized and cloned into a lentiviral backbone under the control of human EF-1α promoter. CARs included CD8α or CD28 hinge/transmembrane domains, CD28, 4–1 BB, or OX40 co-stimulatory domains, and CD3ζ signaling domain, a T2A skip element encoding a self-cleaving peptide, and enhanced GFP (eGFP) as a transduction marker.

Lentivirus generation

Lentivirus for CAR transduction was generated by transfection of 80% confluent Lenti-X-293T cells with the transfer, packaging, and envelope plasmids (ABM 3rd Generation Packing System Mix) using Lipofectamine 3000 (Invitrogen). Virus supernatant was harvested at 24 and 48 hours post transfection, centrifuged, and filtered through a 0.45 mm cellulose acetate filter, concentrated by Lenti-X-Concentrator (Takara) at 1,500 g 4°C for 45 min and re-suspended in Opti-MEM medium (Gibco).

CAR T-cell generation

Buffy coats were obtained from healthy blood donors from local blood bank and blood samples were obtained from patients with lymphoma by phlebotomy under studies approved by the University of Texas MD Anderson Cancer Center Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation and cryopreserved in aliquots. Frozen PBMC were thawed and cultured overnight in complete media with interleukin (IL)-2 (20 IU/mL, Prometheus Therapeutics and Diagnostics). Next day, T cells were isolated from PBMC by the Pan T cell Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Purified T cells were resuspended in complete media at a concentration of 10^6 cells/mL and activated with ImmunoCult Human CD3/CD28/CD2 T cell Activator (STEMCELL Technologies) for 48 hours. Activated T cells were then plated in a 12-well plate at 1×10^6 cells/well in the presence of 10 µg/mL of Vectofusin-1 (Miltenyi Biotec) and cells were spin-transduced with lentivirus at 1000 g at 32°C for 2.0 hours. Two days later, transduction efficiency was determined by flow cytometry as percentage of GFP^+ cells. Transduced T cells were expanded in complete media with 200 IU/mL of human IL-2. Cells were split every 2–3 days to maintain a concentration of $\leq1\times10^6$ cells/mL. CAR T cells were used in functional assays either fresh or cryopreserved at day 13 after transduction. Untransduced donor-matched T cells that were similarly activated with ImmunoCult Human CD3/CD28/CD2 T cell Activator and expanded in vitro were used as controls where appropriate.

Jurkat-Lucia NFAT reporter cell line activation

Jurkat-Lucia NFAT reporter T cells (InvivoGen) were transduced lentivirally with CAR constructs and co-cultured for 18–24 hours with target cells at 1:1 ratio or with anti-CD3/CD28 antibodies (BD Biosciences). NFAT activation was determined by measuring Lucia luciferase activity in cell culture supernatant using QUANTI-Luc and a luminometer.

Flow cytometry

Cells were stained with the indicated fluorescently labeled antibodies and resuspended in a staining buffer consisting of 1× phosphate-buffered saline (PBS) and 1% fetal bovine serum. Antibodies used for flow cytometric analysis include CD3, CD4, CD8, CD19, CD25, CD27, CD45RA, CD62L, CD79b, CD107a/b, CD127, PD-1, TIM3, LAG-3, 2B4, TIGIT, pCD3ζ, and pERK1/2. Surface expression of CD79b CAR was detected using fluorochrome labeled recombinant human CD79b-Fc protein (Creative BioMart) or goat anti-mouse IgG F(ab)2 fragment (Jackson ImmunoResearch). Surface expression of CD19 CAR was detected by fluorochrome labeled CD19 extracellular domain-Fc protein (ACROBiosystems).

Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo V.10 software (Tree Star).

Degranulation, proliferation, cytotoxicity, and cytokine assays

For degranulation assay, CAR T cells were co-cultured for 6 hours with target cells at 1:1 ratio. Brefeldin A, monensin, and fluorescently labeled CD107a/b antibodies were added after 2 hours of co-culture. Samples were acquired by flow cytometer for analysis at the end of co-culture. For proliferation and cytotoxicity assays, CAR T cells were labeled with CellTrace Far Red (Thermo Fisher Scientific) and co-cultured with target cells labeled with CellTrace Violet (Thermo Fisher Scientific) at the indicated effector:target (E:T) ratios in each experiment. CAR T-cell proliferation was assessed by gating on diluted CellTrace Far Red labeled T cells. For cytotoxicity assays, dead target cells were determined at the end of co-culture by flow cytometry using LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific). Absolute cell number of live tumor cells was calculated using CountBright absolute counting beads (Thermo Fisher Scientific) by flow cytometry. Cell culture supernatants from cytotoxicity assay were harvested and analyzed for cytokine levels using Meso Scale Discovery multiplex platform. For all functional studies, effector T-cell numbers were normalized based on CAR expression. All assays were performed in replicate wells of two or more in each experiment.

Phosflow analysis

CAR T cells were rested in complete media without IL-2 on ice for 1 hour and then stimulated by cross-linking with 5 µg/mL of mouse anti-human CD3 monoclonal antibody UCHT1 (BD Biosciences) and mouse anti-human CD28 monoclonal antibody CD28.2 on ice for 15 min followed by goat anti-mouse Ig antibody (BD Biosciences). For the test sample, CAR T cells were stimulated with 5 µg/mL recombinant human CD79b-Fc fusion protein on ice for 15 min followed by an anti-human IgG antibody (BD Biosciences). The cells were then incubated for 5 min at 37°C. Next, the cells were fixed by BD Phosflow Fix Buffer I for 15 min at 37°C, washed twice with 1× PBS, permeabilized with cold BD Phosflow Perm Buffer III on ice for 30 min and blocked with human AB serum. Next, cells were washed with a cold staining buffer twice and stained with anti-CD247 (CD3ζ pY142, BD Biosciences) and anti-ERK1/2 (pT202/pY204, BD Biosciences) for 30 min. Untreated CAR T cells served as negative control. After staining, cells were washed twice and resuspended in a 250 µL staining buffer containing 1% bovine serum albumin before analysis by flow cytometry. CAR^+ and CAR^- T cells were gated as GFP^+ and GFP^- cells.

In vivo studies

All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center (Study Number: 00000331-RN03). Tumor cells were injected intravenously into NSG mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1}Wjl/SzJl, Jackson Laboratories) of 6–10 weeks age. Cryopreserved or fresh CAR T cells were normalized for CAR expression by the addition of activated untransduced donor-matched T cells and given as a single intravenous injection after tumor engraftment was confirmed by bioluminescence imaging. Tumor burden was monitored using Xenogen IVIS Lumina (Caliper Life Science) spectra imaging apparatus after intraperitoneal injection of 3 mg D-Luciferin (PerkinElmer, Waltham, Massachusetts, USA). Luminescence images were normalized and analyzed using Living Image software (PerkinElmer, Waltham, Massachusetts, USA).

Statistical analysis

Barplots present estimated quantities as mean plus or minus SD, as indicated in the figure legends. Significance of in vitro assays was determined by a two-sided Student's unpaired t-test, Mann-Whitney U test, or ordinary one-way analysis of variance with Dunnett's multiple comparisons correction. The method of Kaplan and Meier was used to generate survival curves, and a two-sided log-rank test was applied to assess differences in survival functions. All statistical analyses were performed with Prism V.8 software V.8.0 (GraphPad). Statistical significance was indicated as ns (not significant) $p>0.05$; *$p<0.05$, **$p<0.01$; ***$p<0.001$; or ****$p<0.0001$.

RESULTS

CD79b expression pattern in normal and malignant cells is favorable for CAR T-cell targeting

To confirm that CD79b is a safe and appropriate target for CAR T-cell therapy, we determined its expression in normal and malignant cells. By quantitative PCR on 20 normal tissues, we found that CD79b transcripts were present only in lymphoid tissues such as spleen and lymph node but absent in non-lymphoid normal tissues (online supplemental figure 1A). This result was consistent with analysis of publicly available gene expression data sets from 79 human tissues (BioGPS),[17] which showed that CD79b expression was highly restricted to B cells among normal tissues and has an expression pattern like other pan B-cell antigens such as CD19, CD20, and CD79a (online supplemental figure 1B). Among malignant cell types, CD79b transcripts and protein were present in a broad range of B-cell lymphoma cell lines including Daudi, HBL-1, Jeko-1, SUDHL-6, SUDHL-4, and U2932 but had no expression in Jurkat and J76 T-cell lymphoma/leukemia cell lines (online supplemental figure 1C,D). Consistent with this, using publicly available gene expression data sets (Oncomine and The Cancer Genome Atlas Program), we found that CD79b transcript is highly expressed in multiple B-cell lymphoma subtypes such as Burkitt lymphoma, diffuse LBCL, FL, and MCL but absent in acute myeloid leukemia and various solid tumors (online supplemental figure 1E,F).

Importantly, CD79b expression was not altered in CD19 KO lymphoma cell lines (online supplemental figure 1G). Together, these results suggest that targeting CD79b with CAR T-cell therapy is unlikely to cause any on-target, off-tumor adverse effects except for B-cell aplasia and that it is likely to be a good therapeutic target for a variety of B-cell lymphomas including tumors with CD19 loss.

Monoclonal antibody targeting human CD79b was highly specific

Monoclonal antibodies targeting CD79b were generated in house by hybridoma technology (online supplemental methods). We screened 2496 hybridoma fusions by ELISA and flow cytometry assays against lymphoma cell lines and mouse fibroblast L cells transduced with human CD79b and identified the monoclonal antibody clone 28B, that specifically bound to human CD79b-transduced L cells but not parental L cells (online supplemental figure 2 and data not shown). The binding of the monoclonal antibody was not inhibited by polatuzumab, an anti-CD79b antibody previously approved for use in patients with r/r LBCL as an antibody drug conjugate[18] suggesting that the two antibodies likely bind to different epitopes (online supplemental figure 3A). In contrast, the binding of clone 28B was markedly diminished against Jeko-1 CD79b^{KO} cells as compared with the parental or Jeko-1 CD19^{KO} cells (online supplemental figure 3B). Furthermore, screening of the anti-CD79b monoclonal antibody clone 28B against an array of 5828 full-length human plasma membrane and cell surface-tethered human secreted proteins further 398 human heterodimers expressed in human cells[19] showed that the tested antibody bound to only CD79b when it was expressed either alone or as a heterodimer with CD79a. No other interactions were identified for the selected antibody, indicating high specificity for the primary target CD79b (data not shown).

Phenotypic profile of CAR T cells targeting human CD79b

To determine the optimal design of the CD79b-targeting CAR molecule, we generated six second generation CAR constructs with scFv derived from clone 28B with different hinge/transmembrane (H/TM; either CD8α or CD28) and co-stimulatory (CD28, 4-1BB, or OX40) domains. All constructs had CD3ζ signaling domain and eGFP as a transduction marker (figure 1A). Lentiviral transduction into primary human T cells showed good and sustained transduction efficiency of all six CAR constructs as assessed by eGFP expression. However, binding to recombinant CD79b-Fc protein was better when CD8α H/TM domain was used compared with CD28 H/TM domain (figure 1B,C; online supplemental figure 4A). The CAR T cells were successfully expanded in vitro and consisted of both CD4$^+$ and CD8$^+$ T cells, with predominantly naïve-like (CD45RA$^+$CD62L$^+$) and terminally differentiated (CD45RA$^+$CD62L$^-$) phenotypic subsets (online supplemental figure 4B-D).

CD79b-targeting CAR T cells proliferated, produced cytokines, degranulated, and exhibited cytotoxic effects in vitro

To compare the function of CD79b-targeting CAR T cells generated using the six different CAR constructs, we co-cultured them with Daudi Burkitt lymphoma tumor cells. In a 4-day proliferation assay, we observed that both CD4$^+$ and CD8$^+$ CD79b CAR T cells proliferated significantly in response to Daudi tumor cells, compared with untransduced T cells. The proliferation of CD79b CAR T cells with CD8α H/TM domain was numerically higher compared with CARs with CD28 H/TM domain. The proliferation of CD79b CAR T cells was comparable to CD19 CAR T cells (figure 2A,B).

Assessment of supernatants by multiplex cytokine assay 24 hours after co-culture showed that CD79b CAR T cells produced significant amounts of IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, and/or IL-17A in response to Daudi tumor cells compared with untransduced T cells. The cytokine production by CD79b CAR T cells was comparable to CD19 CAR T cells. There were no significant differences between the different CD79b CAR constructs (figure 2C and data not shown).

We also found that both CD4$^+$ and CD8$^+$ CD79b CAR T cells degranulated significantly in response to Daudi tumor cells compared with untransduced T cells and it was comparable to the same effect observed in CD19 CAR T cells. Degranulation was numerically superior with CD79b CAR T cells containing CD8α H/M domain compared with CD79b CAR T cells containing CD28 H/TM domain (figure 3A,B).

In cytotoxicity assays, we observed that CD79b CAR T cells induced significant lysis of Daudi tumor cells compared with untransduced T cells and the cytotoxic activity was comparable to the one induced by CD19 CAR T cells. The cytotoxic activity was superior with CD79b CAR T cells containing CD8α H/TM domain compared with CD79b CAR T cells containing CD28 H/TM domain (figure 3C–E).

CD79b CAR T cells eradicated tumors and prolonged survival in a lymphoma xenograft model

In a cell line-derived xenograft model of Daudi Burkitt lymphoma, we found that CD79b CAR T cells induced rapid elimination of established tumors and significantly improved survival like the effect observed with CD19 CAR T cells. In contrast, rapid tumor growth was noted in mice treated with untransduced T cells. Consistent with the cytotoxicity results in vitro, the tumor control and survival were superior with CD79b CAR T cells containing CD8α H/TM domain compared with CD79b CAR T cells containing CD28 H/TM domain with the best survival observed with CD79b CAR T cells containing CD8α H/TM domain and OX40 co-stimulatory domain (figure 4A–D; online supplemental figure 5A). In surviving mice, we did not observe any evidence of weight loss at any point during the duration of the experiment, suggesting that the CD79b CAR T cells were well tolerated like the CD19

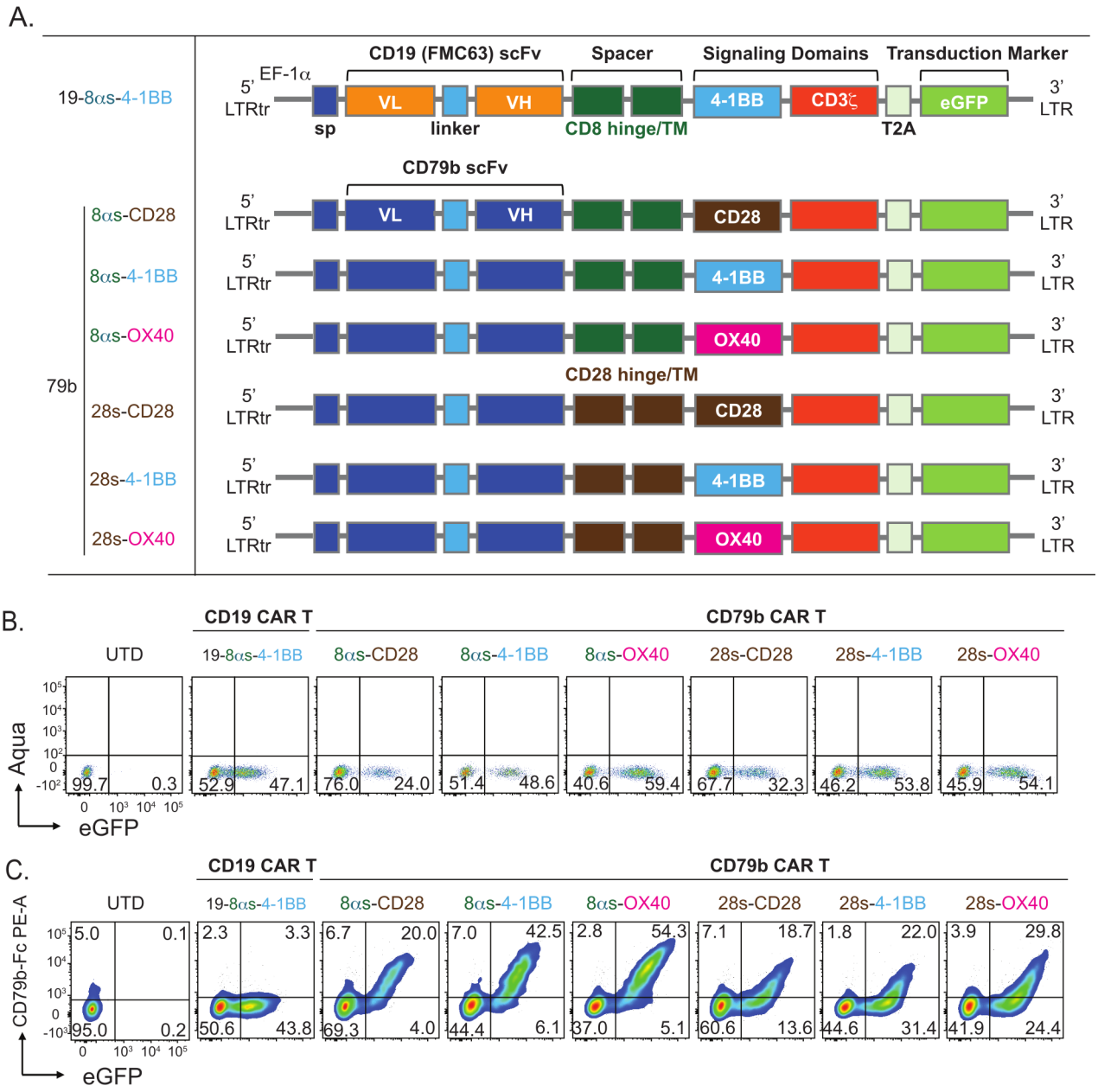

Figure 1 Schematic of different CD79b CAR constructs and transduction efficiency in primary T cells. (A) Schematic design of CAR constructs to target CD19 or CD79b. (B and C) CAR constructs shown in panel A were transduced lentivirally into primary human T cells and transduction efficiency was determined by flow cytometry by assessing eGFP expression and CAR expression is shown by demonstrating binding to fluorescently labeled recombinant human CD79b-Fc protein. Data is representative of CAR T cells generated from two different donors. CAR, chimeric antigen receptor; eGFP, enhanced green fluorescent protein; LTR, long terminal repeat; LTRtr, LTR truncated; scFv, single chain variable fragment; sp, signal peptide; VL, variable light chain; VH, variable heavy chain; TM, transmembrane domain.

CAR T cells (online supplemental figure 5B). Assessment of peripheral blood sample at day 94 from a mouse treated with CD79b-CD8α-OX40 CAR T cells that had no evidence of tumor by bioluminescence imaging, showed that the CAR T cells were still detectable ~3 months after infusion and were comprised of both $CD4^+$ and $CD8^+$ T cells. Importantly, the CAR T cells did not show significant expression of exhaustion markers such as programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin domain 3 (TIM-3) or T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), although lymphocyte-activation gene 3 (LAG-3) was increased in $CD8^+$ T cells (online supplemental figure 5C).

Patient-derived CD79b CAR T cells and efficacy against patient-derived lymphoma tumor cells

To demonstrate that functional CD79b CAR T cells could be generated from patients relapsing after prior CD19 CAR T-cell therapy, we generated CAR T cells from two patients with LBCL relapsing ~3 months after CD19 CAR T-cell therapy. Transduction efficiency of CD79b CAR in

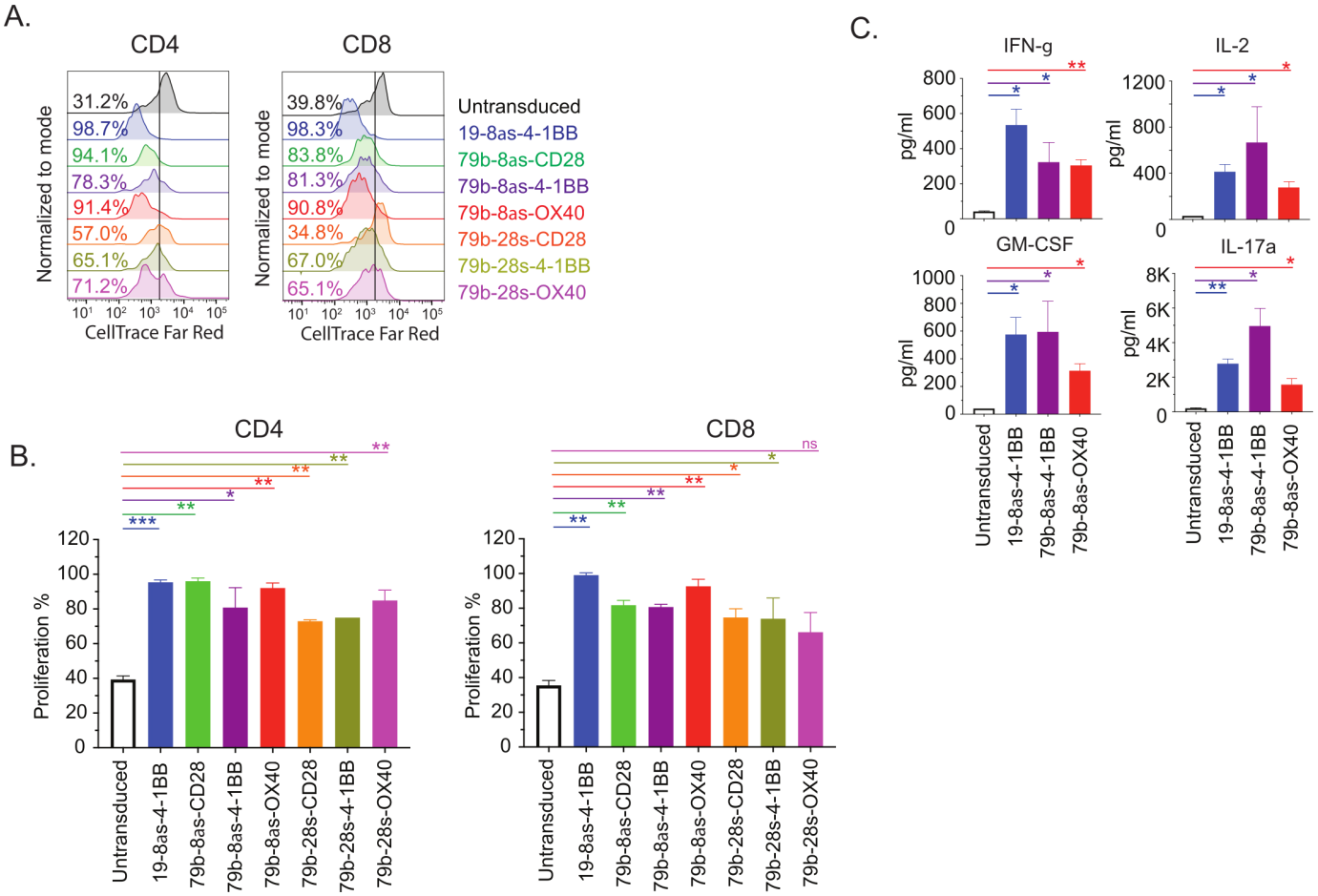

Figure 2 CD79b CAR T cells proliferated and produced cytokines in response to lymphoma cells in vitro. CAR T cells targeting CD19 or CD79b generated from healthy donor T cells were co-cultured with Daudi Burkitt lymphoma tumor cells at an effector:target ratio of 1:1. Untransduced T cells were used as controls. (A) Representative flow cytometric plots illustrating proliferation of $CD4^+$ and $CD8^+$ CAR T cells after 96 hours of co-culture. (B) Summary results showing proliferation from replicate wells (N=2). (C) Cytokine production by CAR T cells in response to Daudi lymphoma cells in replicate wells (N=2) after 24 hours of co-culture. Data represents mean±SD and is representative of at least three separate experiments from three different donors. ***p<0.001; **p<0.01; *p<0.05; (ns) p>0.05. CAR, chimeric antigen receptor; IFN, interferon; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor.

these patients-derived T cells was ~50% and their phenotypic profile was largely similar to CD79b CAR T cells generated from normal donors (online supplemental figure 6A-C). Importantly, in vitro they showed robust degranulation, proliferation, cytotoxic activity, and cytokine production against Daudi tumor cells (figure 5A–G).

To determine the activity of CD79b CAR T cells against lymphoma tumors cells derived from patient-derived xenografts (PDX), we tested them against two samples: one high-grade B-cell lymphoma, PDX300 sample that is $CD19^{Lo}CD79b^+$ and the other a double-hit lymphoma, PDX203, that is, $CD19^+CD79b^+$, both derived from patients relapsing within 3 months after prior CD19 CAR T-cell therapy (figure 6A). We found that CD79b CAR T cells proliferated significantly in response to both tumor samples, produced cytokines, and efficiently lysed (>80%) the tumor cells at low E:T ratio compared with untransduced T cells (figure 6B–D; online supplemental figure 7A,B). The significant lysis of target tumor cells and cytokine production were highly reproducible when CD79b CAR T cells derived from multiple donors were tested (figure 6E,F). However, some variation in background activity with untransduced T cells was observed likely due to variation in magnitude of human leukocyte antigen (HLA)-mismatch between the different donor CAR T cells and target tumor cells, which could result in variable allogeneic response across different experiments. More importantly, CD79b CAR T cells demonstrated significant antitumor activity against the PDX tumors in mice (figure 6G–J).

Specificity of CD79b CAR T cells

To confirm that CD79b CAR specifically reacted to CD79b protein, first, we demonstrated that they are bound to recombinant human CD79b protein but not recombinant human CD19 or CD79a protein (figure 7A). Next, we transduced the CARs into Jurkat-Lucia NFAT reporter cells and determined their signaling capability (figure 7B). Culture of these transduced Jurkat cells with anti-CD3/anti-CD28 antibodies or Daudi Burkitt lymphoma cells, induced

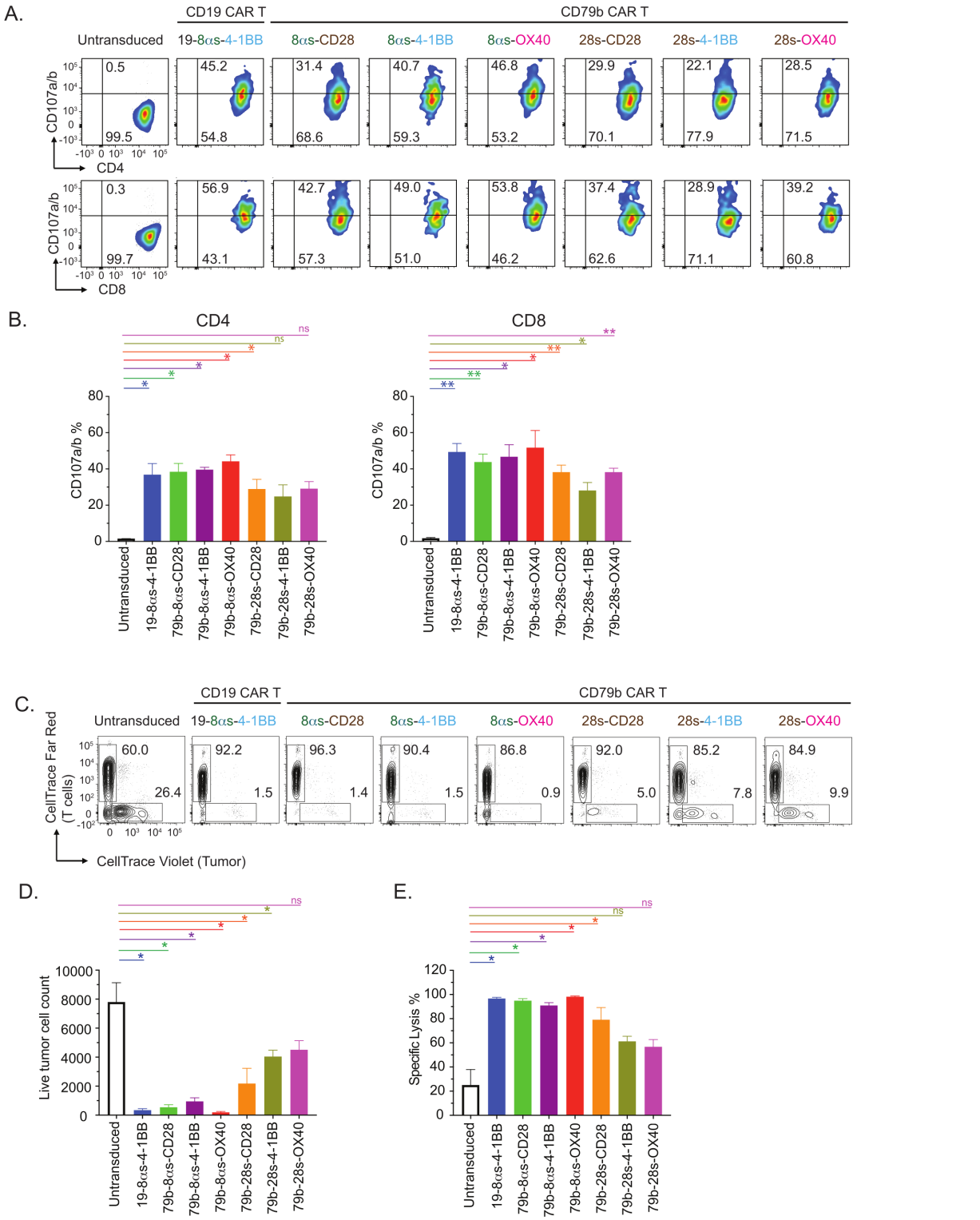

Figure 3 CD79b CAR T cells degranulated and exhibited cytotoxic activity against lymphoma cells in vitro. CAR T cells targeting CD19 or CD79b were generated from healthy donor T cells and co-cultured with Daudi Burkitt lymphoma tumor cells at an effector:target ratio of 1:1. Untransduced T cells were used as controls. (A) Representative flow cytometric plots illustrating CD107a/b degranulation on $CD4^+$ and $CD8^+$ CAR T cells after 6 hours of co-culture are shown. (B) Summary results showing degranulation from replicate wells (N=2). (C–E) CAR T cells labeled with CellTrace Far Red were co-cultured with Daudi lymphoma tumor cells labeled with CellTrace Violet at an effector:target ratio of 1:1. Cytotoxicity assay was assessed after 96 hours by flow cytometry by counting live tumor cells using TruCOUNT beads. Untransduced T cells were used as controls. (C) Representative flow cytometric plots showing percentage of live tumor and T cells after end of co-culture. Summary results of cytotoxicity assay from replicate wells (N=2) showing absolute live tumor cell count (D) or specific lysis (E) of Daudi tumor cells. Data represents mean±SD and is representative of at least three separate experiments from three different donors. ***p<0.001; **p<0.01; *p<0.05; (ns)p>0.05. CAR, chimeric antigen receptor.

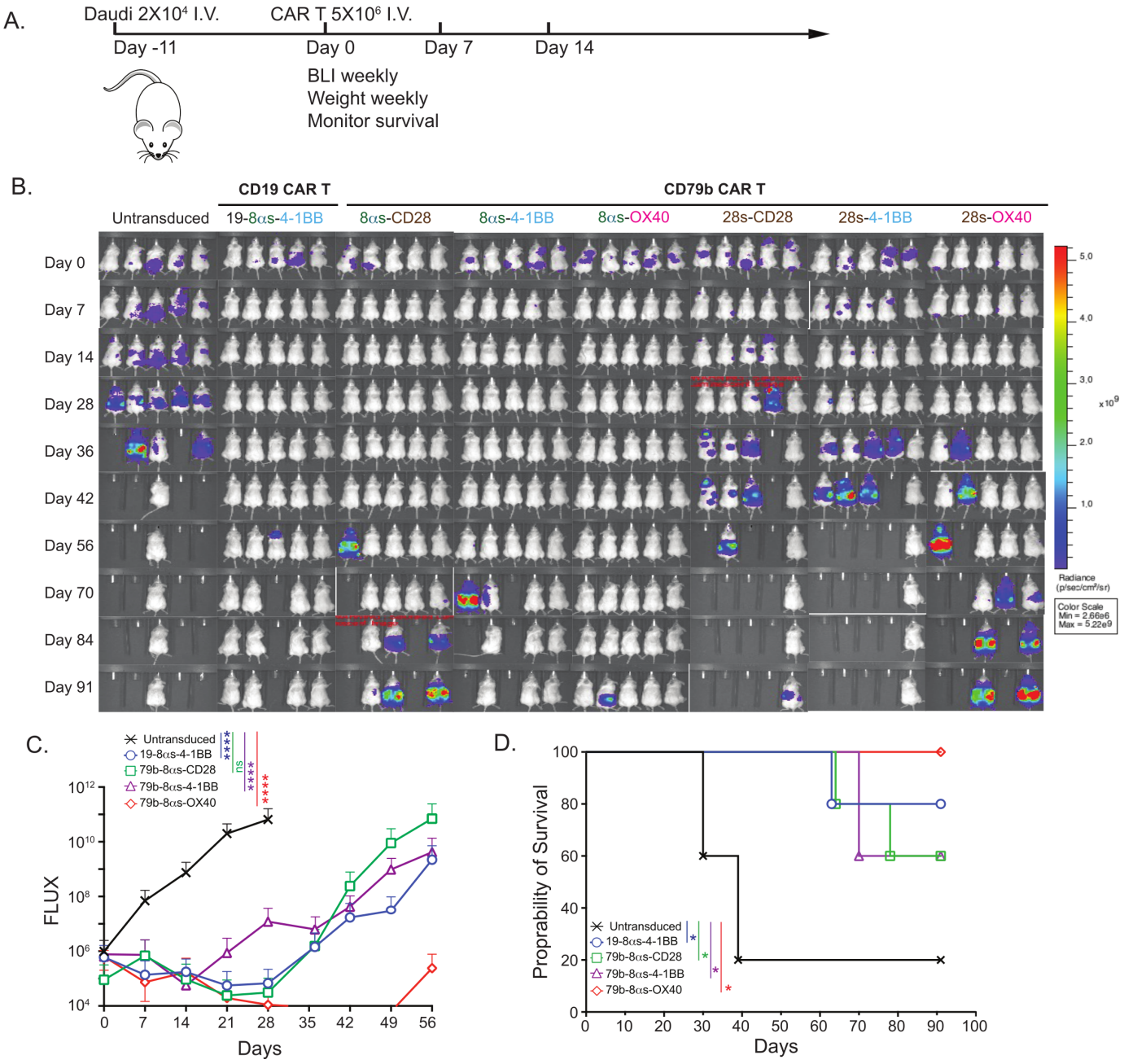

Figure 4 CD79b CAR T cells exert antitumor effects in vivo. (A) Schematic overview of xenograft mouse model. Luciferase-labeled Daudi Burkitt lymphoma cells were injected intravenously into NSG mice at 2×10^4 tumor cells/mouse. After 11 days, mice were treated with untransduced T cells, CD19 CAR T cells, or CD79b CAR T cells via tail vein injection at 5×10^6 CAR$^+$ T cells/mouse. (B) Tumor burden assessed by bioluminescence imaging at the indicated time points is shown. (C) Average radiance (photons/sec/cm^2/steradian) of groups of mice at different time points is shown. Significance among treatment groups was determined by comparing area under the curve for each CAR-T group to untransduced control T-cell group in an ordinary one-way ANOVA with Dunnett's multiple comparisons test (n=5 mice per group, mean±SD is shown). (D) Kaplan-Meier survival curves of groups of mice treated with different T cells. (****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; (ns) p>0.05, ANOVA). ANOVA, analysis of variance; CAR, chimeric antigen receptor; I.V., intravenous.

strong luciferase activity compared with untreated cells (figure 7C). Co-culture of CAR-transduced Jurkat-Lucia NFAT reporter cells with isogenic cell lines of SUDHL-6 LBCL cell line with CD19 and/or CD79b KO showed that CD79b CAR recognized both parental and CD19^{KO} cells but the luciferase signal was significantly reduced in response to CD79b^{KO} or CD19^{KO}CD79b^{KO} cells. In contrast, the CD19 CAR recognized both parental and CD79b^{KO} cells but not CD19^{KO} or CD19^{KO}CD79b^{KO} cells (figure 7D; online supplemental figure 8). We did not observe any significant spontaneous luciferase activity in CAR-expressing cells suggesting minimal or no antigen independent tonic signaling (figure 7C,D). Lack of tonic signaling and specificity was also confirmed by phosflow

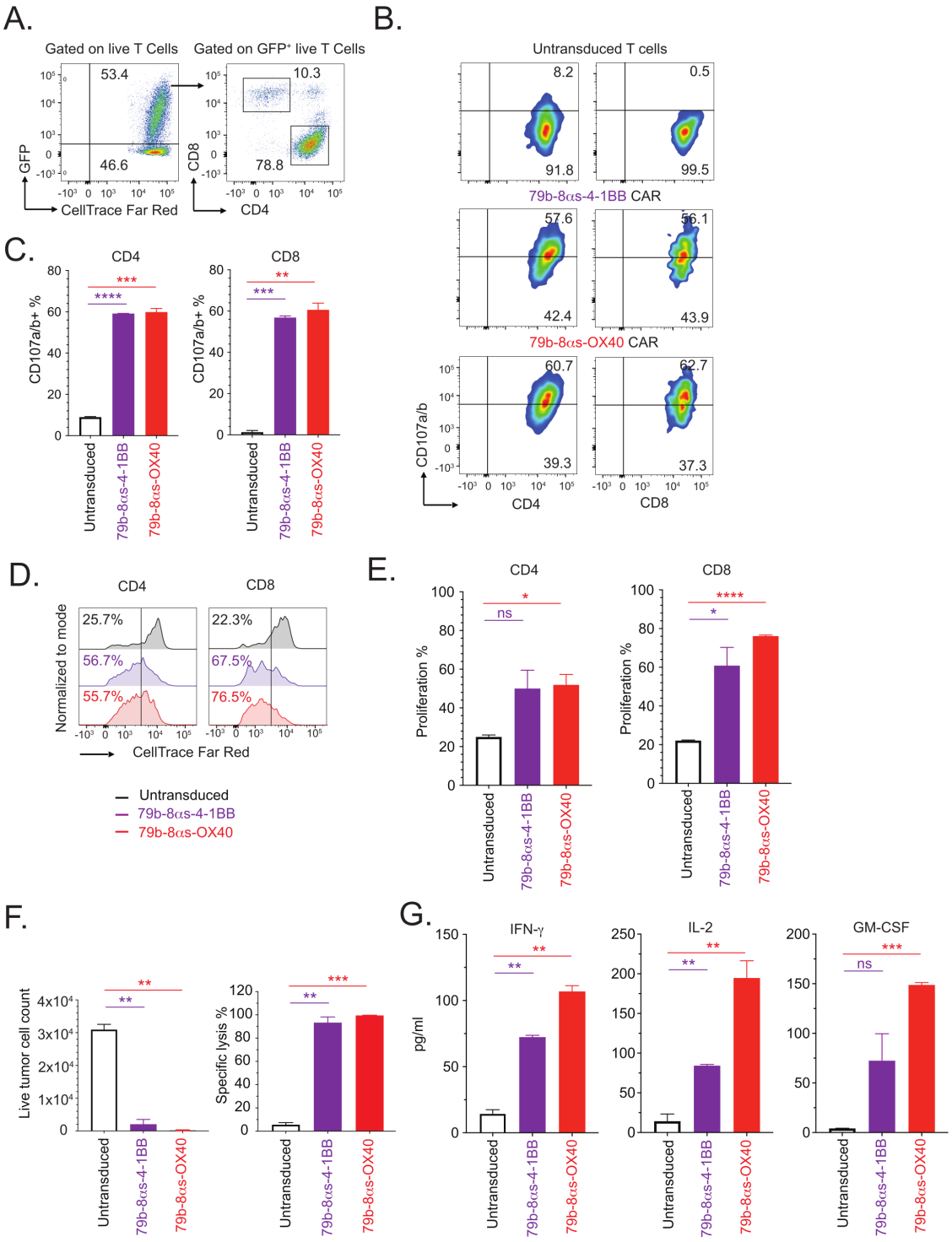

Figure 5 CD79b CAR T cells generated from patients with lymphoma relapsing after autologous CD19 CAR T-cell therapy are functional. CAR T cells targeting CD79b were generated from T cells isolated from two patients with large B-cell lymphoma relapsing after autologous CD19 CAR T-cell therapy and co-cultured with Daudi Burkitt lymphoma tumor cells at an effector:target ratio of 1:1. Untransduced T cells were used as controls. Representative data from one patient is shown. (A) Representative flow cytometric plots showing transduction efficiency of 79b CAR and proportion of $CD4^+$ and $CD8^+$ T cells. (B) Representative flow cytometric plots illustrating CD107a/b degranulation by $CD4^+$ and $CD8^+$ CAR T cells after 6 hours of co-culture. (C) Summary results showing degranulation from replicate wells (N=2). (D) Representative flow cytometric plots illustrating proliferation of $CD4^+$ and $CD8^+$ CAR T cells after 96 hours of co-culture. (E) Summary results showing proliferation from replicate wells (N=2). (F) Summary results of cytotoxicity assay from replicate wells (N=2) showing absolute live tumor cell count (*left panel*) or specific lysis (*right panel*). (G) Cytokines production by CAR T cells co-cultured with Daudi lymphoma cells in replicate wells (N=2) after 24 hours of co-culture. Data is representative of at least three separate experiments with T cells from two different patients. ***p<0.001; **p<0.01; *p<0.05; (ns) p>0.05. CAR, chimeric antigen receptor; IFN, interferon; IL, interleukin; GFP, green fluorescent protein; GM-CSF, granulocyte-macrophage colony-stimulating factor.

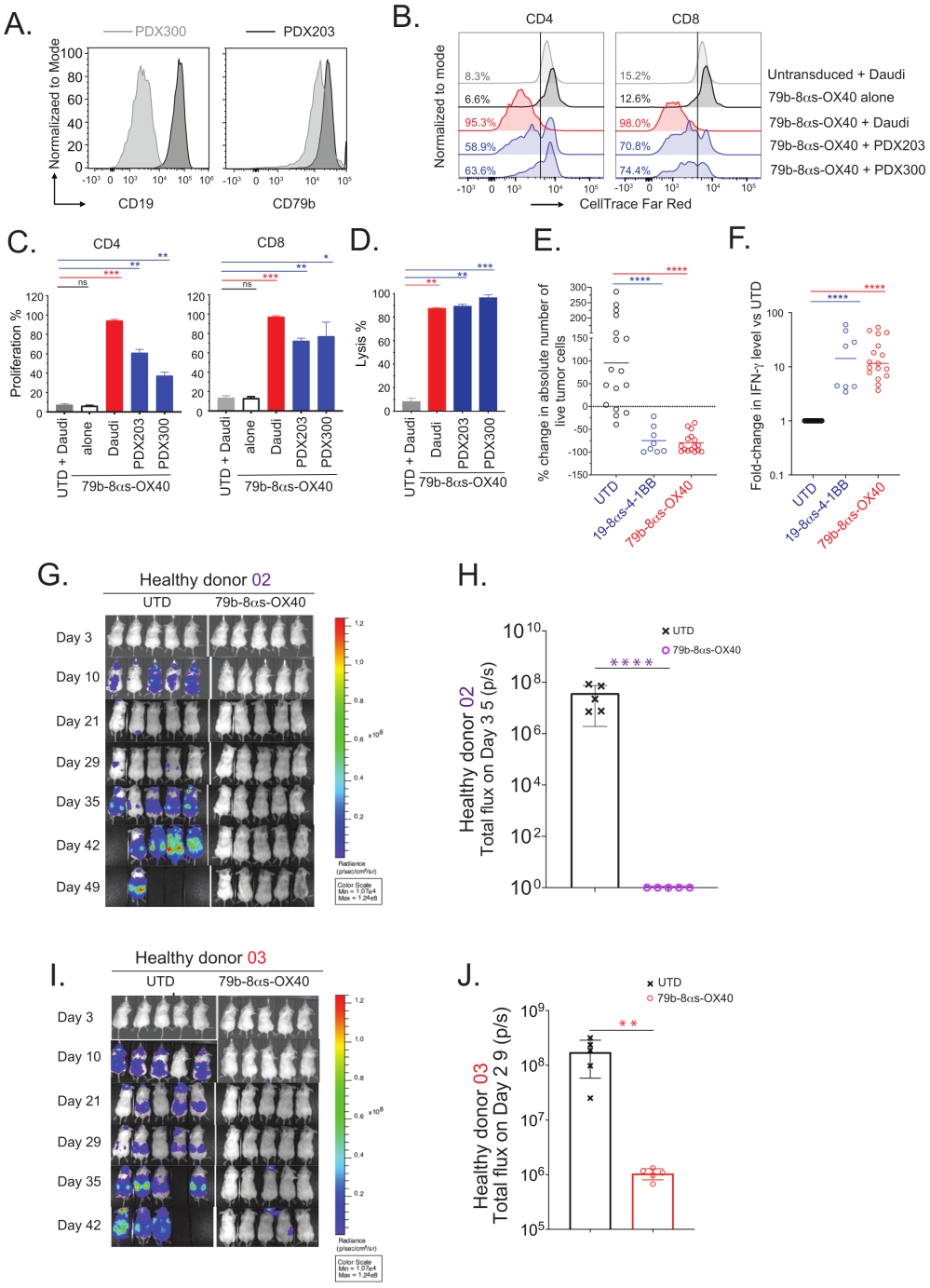

Figure 6 Efficacy of CD79b CAR T cells against patient-derived lymphoma tumor cells. CAR T cells targeting CD79b were generated from primary healthy donor T cells, labeled with CellTrace Far Red and co-cultured with Daudi Burkitt lymphoma tumor cells or lymphoma tumor cells derived from two PDXs (PDX203 and PDX300) for 96 hours. Untransduced T cells were used as controls. (A) Histograms showing expression of CD19 and CD79b in the two tumor samples. (B) Representative flow cytometric plots illustrating proliferation of $CD4^+$ and $CD8^+$ CARs T cells in response to tumor cells at an effector:target (E:T) ratio of 0.6:1. (C) Summary results showing proliferation from replicate wells (N=2). (D) Summary results of cytotoxicity assay from replicate wells (N=2) showing specific lysis at E:T ratio of 0.6:1. (E and F) Pooled data from 17 experiments testing CD79b CAR T cells generated from five donors is shown for per cent change in absolute number of live tumor cells on day 4 in an in vitro cytotoxicity assay compared with day 0 (E) and fold change in interferon-γ production compared with untransduced T cells after 24 hours of co-culture (F). Target tumor cells in these experiments included Daudi, Jeko-1, SUDHL6, or PDX203. Each dot represents data from one experiment and mean values of data from replicate wells from each experiment are shown. (G–J) Luciferase-labeled PDX203 5D4 tumor cells (0.2×10^6/mouse) were injected via tail vein into NSG mice on day −4 and CD79b CAR T cells or untransduced T cells (5×10^6/mouse) derived from two different donors were injected via tail vein on day 0. Tumor burden was monitored by bioluminescence imaging (G and I). Average radiance (photons/sec/cm^2/steradian) of groups of mice on days 35 and 29 are shown (H and J). (n=5 mice per group, mean±SD is shown). ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; (ns) p>0.05. CAR, chimeric antigen receptor; PDX, patient-derived xenografts.

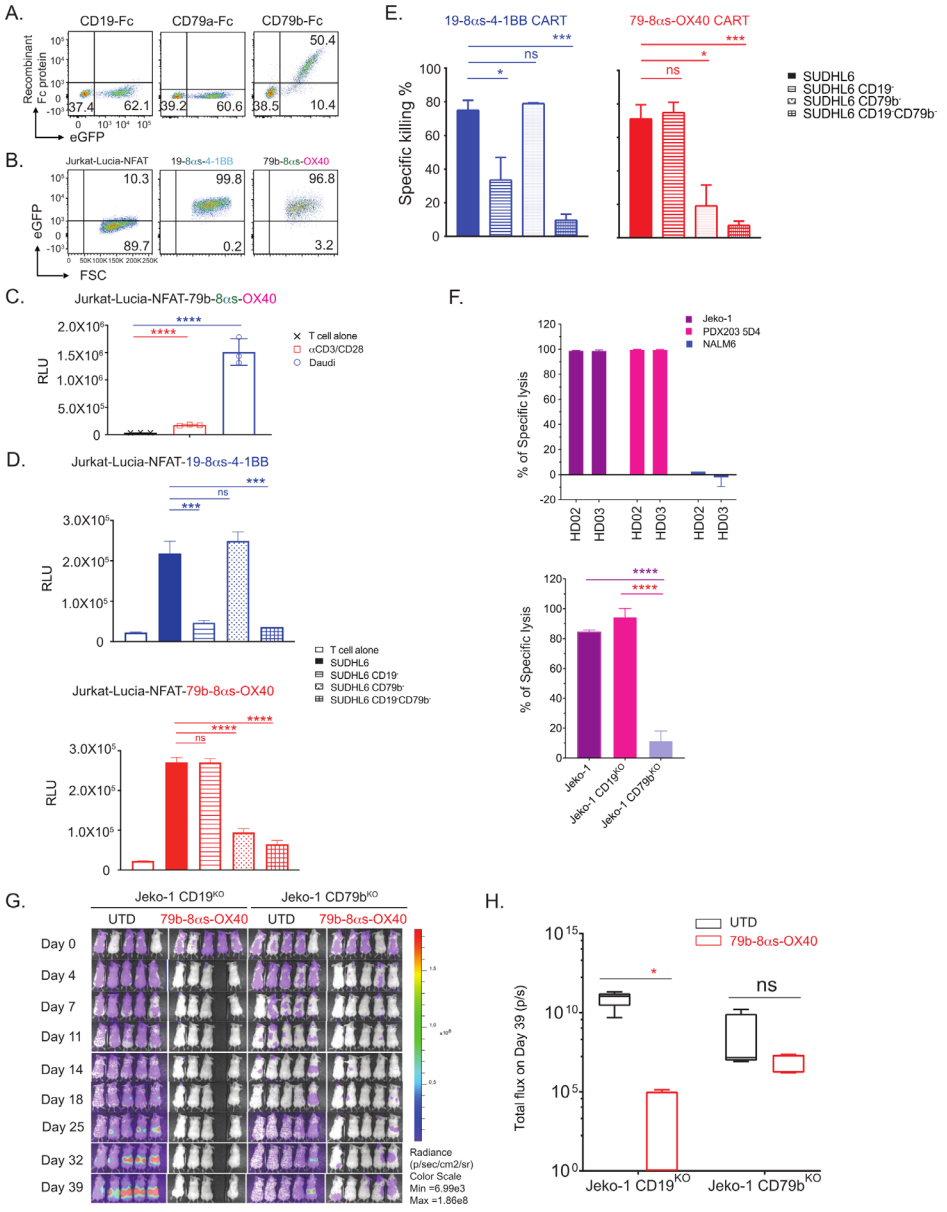

Figure 7 CD79b CARs specially recognized human CD79b protein. (A) CAR T cells targeting CD79b were generated from primary healthy donor T cells, stained with fluorochrome labeled recombinant human CD19-Fc, CD79a-Fc, or CD79b-Fc proteins, and assessed by flow cytometry. Transduction efficiency determined by eGFP expression and binding of recombinant proteins is shown. (B–D) CAR constructs targeting CD19 or CD79b were transduced lentivirally into Jurkat-Lucia NFAT reporter cell line and cultured alone or with the indicated lymphoma cell lines at an effector:target (E:T) ratio of 1:1 or with anti-CD3 and anti-CD28 monoclonal antibodies. NFAT activation was determined after 24 hours by measuring luciferase activity. Percentage of Jurkat-Lucia NFAT reporter cells expressing CD19 or CD79b CAR was determined by assessing eGFP transduction marker by flow cytometry (B). Luciferase activity shown in Jurkat-Lucia NFAT reporter cells transduced with 79b CAR construct and cultured alone or with anti-CD3/CD28 antibodies or Daudi Burkitt lymphoma cells (C). Luciferase activity shown in Jurkat-Lucia NFAT reporter cells transduced with the indicated CD19 or CD79b CAR constructs and cultured alone or with isogenic SUDHL6 lymphoma cell lines (parent, $CD19^{KO}$, $CD79b^{KO}$, or $CD19^{KO}CD79b^{KO}$). (E) Cytotoxic activity of CD19 or CD79b CAR T cells generated from healthy donor T cells against isogenic cell lines of SUDHL6 (parent, $CD19^{KO}$, $CD79b^{KO}$, or $CD19^{KO}CD79b^{KO}$). (F) Cytotoxic activity of CD79b CAR T cells against wild type Jeko-1 and PDX203 5D4 ($CD19^{+}CD79b^{+}$), Jeko-1 $CD19^{KO}$, Jeko-1 $CD79b^{KO}$, or NALM6 acute lymphoblastic leukemia ($CD19^{+}CD79b^{-}$) cell lines at an E:T ratio of 1:1. The absolute number of live tumor cells at the end of culture period from this experiment are shown in online supplemental figure 10. (G and H) Luciferase-labeled Jeko-1 $CD19^{KO}$ or Jeko-1 $CD79b^{KO}$ tumor cells (0.2×10^{6}/mouse) were injected via tail vein into NSG mice on day −4 and CD79b CAR T cells or untransduced T cells (5×10^{6}/mouse) were injected via tail vein on day 0. Tumor burden was monitored by bioluminescence imaging at the indicated time points (G) and average radiance (photons/sec/cm^2/steradian) of groups of mice on day 39 is shown (H). Data represent the mean±SD of replicate wells (N=2) where applicable and is representative of at least three separate experiments from two different donors (C–F). ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; (ns) p>0.05. CAR, chimeric antigen receptor; eGFR, enhanced green fluorescent protein; HD, healthy donor; KO, knock-out; RLU, relative luminescence units.

assay, which showed that baseline levels of phosho-CD3ζ and phospho-Erk1/2 were comparable between CD79b CAR^+ and CAR^- primary human T cells but their levels were enhanced in CAR^+ but not CAR^- T cells in response to recombinant human CD79b protein (online supplemental figure 9A,B).

Consistent with the above results, CD79b CAR T cells induced significant lysis of both parental and $CD19^{KO}$ SUDHL6 cells, but the lysis was significantly diminished against $CD79b^{KO}$ and $CD19^{KO}CD79b^{KO}$ cells. In contrast, CD19 CAR T cells induced significant lysis of both parental and $CD79b^{KO}$ SUDHL6 cells, but lysis was significantly reduced against $CD19^{KO}$ and $CD19^{KO}CD79b^{KO}$ cells (figure 7E). Furthermore, we observed that CD79b CAR T cells caused significant lysis of wild type Jeko-1 and PDX203 5D4 tumor cells and $CD19^{KO}$ Jeko-1 cells but not $CD19^+CD79b^-$ NALM6 or $CD79b^{KO}$ Jeko-1 cells (figure 7F; online supplemental figure 10). Lastly, CD79b CAR T cells eradicated established Jeko-1 $CD19^{KO}$ tumors in mice but the tumor eradication was not sustained in mice injected with Jeko-1 $CD79b^{KO}$ tumors (figure 7G and H). It is also noteworthy that the CD79b CAR T cells demonstrated antitumor activity in vitro and in vivo against Daudi tumor cells that have high levels of various co-stimulatory molecules as well as against other tumor models (Jeko-1, SUDHL6, PDX203, and PDX300) where these molecules were either absent or markedly lower (online supplemental figure 11). Taken together, these results indicated that CD79b CARs specifically recognized CD79b protein and provided activation signals to T cells in response to CD79b-expressing tumor cells.

DISCUSSION

Our results show that our novel CD79b-targeting CAR T-cell product, generated using a novel monoclonal antibody, is highly effective against B-cell lymphomas and specifically reacts to CD79b-expressing tumor cells both in vitro and in vivo. This product could potentially be used for the treatment of patients with most mature B-cell malignancies including diffuse LBCL, FL, MCL, marginal zone lymphoma, Burkitt lymphoma, lymphoplasmacytic lymphoma, and hairy cell leukemia.[15 16] While polatuzumab vedotin, an antibody drug conjugate targeting CD79b, has been approved for patients with LBCL, it has not been shown to be curative in r/r settings.[18] As compared with loncastuximab tesirine, an anti-CD19 antibody drug conjugate,[20] CAR T-cell therapies targeting CD19 have been much more effective in terms of complete response rates and improved durability of responses.[1–3 21] Therefore, development and evaluation of CD79b-targeting CAR T-cell therapy is warranted for patients with B-cell lymphoma despite the availability of polatuzumab vedotin. Loss of CD79b has only rarely been reported in LBCL after treatment with polatuzumab vedotin.[22] Importantly, since the binding of our antibody to its target is not inhibited in the presence of polatuzumab, this product could potentially be used even in patients after recent exposure to polatuzumab vedotin.

We demonstrated that our CD79b-targeting CAR T-cell product was effective against both CD19+ and CD19– lymphomas. This could offer significant clinical benefit to patients relapsing after prior CD19 CAR T-cell therapy since at least one-third of their tumors may have antigen loss.[6 10 11] While there have been prior preclinical studies of CAR T-cell products targeting CD79b,[13 16 23 24] there have been no clinical reports to date. Our CD79b-targeting CAR T-cell product uses a novel monoclonal antibody with CD8α hinge and transmembrane domain and OX40 co-stimulatory domain, which is distinct from prior products. Interestingly, we found that CD79b CAR T-cell products using CD28 hinge and transmembrane domain were less effective than those with CD8α hinge and transmembrane domain. While the exact mechanism for this variation is unknown, it is possible that it may be related to differences in the length of the hinge region between the two, which could alter the tertiary structure of the CAR molecules and binding to the cognate antigen.[25] In addition, the CD8α hinge is almost twice as long compared with CD28, which likely facilitates better engagement of the target and therefore better synapse formation between the CAR T cell and tumor cell.[24] A longer hinge is likely critical for targeting CD79b as its extracellular portion is relatively short with only one immunoglobulin-like domain. In a recent study comparing 12 different co-stimulatory molecules, OX40 was shown to provide the most effective co-stimulatory signal for CAR T cells by promoting proliferation, reducing apoptosis and exhaustion, and enhancing cytotoxicity through activation of NF-κB, MAPK, and PI3K-AKT pathways.[26] Consistent with this, we found that our construct had no significant tonic signaling activity, was not associated with exhaustion, was highly effective, and resulted in long-term persistence of CAR T cells in vivo.

Since CD79b is a pan-B-cell antigen and is expressed on normal B cells, B-cell aplasia is an expected on-target off-tumor effect with this product similar to CD19 CAR T-cell therapy.[27] Although B-cell aplasia can be associated with hypogammaglobulinemia and risk of recurrent sinopulmonary infections, it is expected to be manageable with immunoglobulin replacement therapy as needed.[21 27 28] In fact, despite the B-cell aplasia after CD19 CAR T-cell therapy, only about one-third of adult patients with B-cell lymphoma needed immunoglobulin replacement therapy to prevent recurrent infections.[1–5 28 29] The restricted expression of CD79b in normal tissues together with the absence of any off-target binding of our antibody is expected to minimize any other unexpected toxicity with this product. The use of OX40 co-stimulatory domain in our product is different from the approved CD19 CAR T products, which use either CD28 or 4-1BB co-stimulatory domains. However, OX40 co-stimulatory domain has been used with a CD19 and CD22 dual targeting CAR T cell product, in a clinical trial in patients with r/r acute lymphoblastic leukemia, and

was found to be safe without any incidence of severe cytokine release syndrome or neurological toxicity.[30] Therefore, taken together with the absence of any toxicities in our mouse models, this novel CAR design is expected to be safe in patients.

Besides CD19 and CD79b, CAR T-cell therapy products targeting other pan-B-cell antigens are also in various stages of development including those targeting CD20, CD22, and CD79a.[24 31–33] Such products are also likely to be effective in patients relapsing after CD19 CAR T-cell therapy with or without CD19 loss. Whether there is a potential advantage among targeting these other pan-B-cell antigens remains to be seen. Recently, CAR T-cell therapy targeting CD22 was shown to be highly effective in patients with LBCL relapsing after CD19 CAR T-cell therapy, but was also associated with increased incidence of immune effector cell-associated hemophagocytic lymphohistiocytosis-like syndrome for unclear reasons.[31] While CD20 is a well-established target in B-cell lymphomas, the plethora of CD20-targeting agents that are currently in use or in clinical development, including naked monoclonal antibodies, bispecific T-cell engagers, and CAR T-cell therapies, is expected to increase the risk of tumors relapsing with CD20 loss.[34–38] Bi-specific and tri-specific CAR T-cell therapy approaches targeting CD19 with CD20, CD22, CD79a, and CD79b are also in various stages of preclinical and clinical development.[10 13 24 39 40] Ultimately, such multispecific CAR T-cell therapy approaches may be needed to minimize immune escape due to antigen loss and improve outcomes in these patients.

In summary, we developed a CD79b-targeting CAR T-cell therapy product with a novel monoclonal antibody and a novel CAR design that is highly specific and has robust antitumor activity against aggressive B-cell lymphomas in preclinical models. These results supported the initiation of a phase 1 clinical trial to evaluate this product in patients with r/r B-cell lymphomas (NCT05773040).

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Contributors FC and SSN conceived the project, designed the experiments, analyzed the results, and wrote the manuscript. FC and JC designed and performed most of the experiments. FC, JC, JL, TJD, S-qK, XC, ZZ, SK and FV developed CAR constructs, generated lentivirus, generated CAR T cells, and/or performed experiments. HY and MG generated patient-derived xenografts. LTV and LB generated monoclonal antibody by hybridoma technology. RS provided statistical support. RED performed CRISPR/Cas9 knockout experiments. SSN supervised the overall project. All authors participated in data analysis, interpretation, manuscript writing, and approval of the final submitted version. SSN serves as guarantor for the overall content.

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Competing interests FC has intellectual property related to cell therapy. JC has intellectual property related to cell therapy. JL has intellectual property related to cell therapy. FV receives research support from Allogene and Geron corporation. SSN received research support from Kite/Gilead, BMS, Allogene, Precision Biosciences, Adicet Bio, and Sana Biotechnology; served as Advisory Board Member/Consultant for Kite/Gilead, Merck, Sellas Life Sciences, Athenex, Allogene, Incyte, Adicet Bio, BMS, Bluebird Bio, Fosun Kite, Sana Biotechnology, Caribou, Astellas Pharma, Morphosys, Janssen, Chimagen, ImmunoACT, Orna Therapeutics, Takeda, and Synthekine; has stock options from Longbow Immunotherapy; and has intellectual property related to cell therapy.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by MD Anderson Cancer Center Institutional Review Board (IRB). Study IDs approved by IRB: 2005-0656 and LAB04-0717. Participants gave informed consent to participate in the study before taking part.

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