HHS Public Access

Author manuscript

Clin Cancer Res. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

Clin Cancer Res. 2019 December 01; 25(23): 7046–7057. doi:10.1158/1078-0432.CCR-19-1337.

Chimeric antigen receptor T cells targeting CD79b show efficacy in lymphoma with or without co-targeting CD19

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Abstract

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Conflicts of interest Disclosures

M.O., T.B., and M.V.M. are listed as inventors on patents related to this work and held by the University of Southern Denmark, Massachusetts General Hospital, and Partners Health Care.

Purpose: T cells engineered to express a chimeric antigen receptor (CAR) against CD19 have recently been FDA-approved for the treatment of relapsed or refractory large B cell lymphoma. Despite the success and curative potential of CD19 CAR T cells, several reports describing disease relapse due to antigen loss are now emerging.

Experimental design: We developed a novel CAR construct directed against CD79b, a critical receptor for successful B cell development that remains highly expressed in several subtypes of B cell lymphoma, including mantle cell lymphoma (MCL). We tested CAR T cells directed against CD79b alone or in combination with CD19 targeting in a single construct, against cell line- and patient-derived xenograft models.

Results: We demonstrate CAR79b antigen-specific recognition and cytotoxicity against a panel of cell lines and patient-derived xenograft models of MCL. Importantly, we show that downregulation of CD19 does not influence surface expression of CD79b and that anti-CD79b CAR T cells alone or arranged in a dual-targeting format with a CD19 single-chain variable fragment (scFv) are able to recognize and eliminate CD19-positive, CD19-negative, and mixed CD19+/CD19- B cell lymphoma.

Conclusions: Our findings demonstrate that CAR T cells targeting CD79b alone or in combination have promise for treating and preventing CD19 antigen escape in B cell lymphomas.

Introduction

Non-Hodgkin lymphoma (NHL) is a large group of B cell malignancies accounting for about 4% of all tumors (1). Standard treatment for most subtypes of NHL involves a combination of therapies including rituximab and chemotherapy. Despite improvements in available therapies, NHLs carry a uniformly poor prognosis in the relapsed/refractory (r/r) setting. Adoptive immunotherapy utilizing T cells genetically modified to express a chimeric antigen receptor (CAR) has shown great efficacy as treatment of CD19-positive B cell malignancies. Approximately, 80% of NHL subtypes are derived from the B cell linage and retain expression of B cell markers, including CD19 and CD20 after malignant transformation. These surface antigens represent key targets for antibody-based therapeutics and CAR T cell therapy. In lymphoma, CAR19 therapy has a reported overall response rate in the 60–80% range, with approximately 40% of patients achieving long-term complete remission (2-6). Recently, these response rates led to the approval of two CAR19 products, axicabtagene ciloleucel, which bears a CD28 co-stimulatory domain, and tisagenlecleucel, which bears a 4–1BB co-stimulatory domain, for the treatment of r/r diffuse large B cell lymphoma (DLBCL). In addition, the tisagenlecleucel product has also been approved as treatment for children and young adults with r/r B cell acute lymphoblastic leukemia (ALL). However, clinical data reporting disease relapse due to CD19 antigen loss in both ALL and lymphoma patients are now emerging (2,7-9), highlighting an unmet clinical need for targeting novel surface antigens.

CD79b is part of the B cell receptor (BCR) signaling complex, and a critical receptor for the successful development and maintenance of mature B cells (10). CD79b expression is restricted to the B cell linage, and high expression is maintained on most subtypes of NHL, including mantle cell lymphoma (MCL), DLBCL, Burkitt’s lymphoma (BL), and follicular

lymphoma (FL) (10-12). Indeed, targeting CD79b with antibody-drug conjugates or bi-specific T-cell engagers (BiTEs) has been shown to be safe, well tolerated, and demonstrated early signs of efficacy (13-15).

Herein, we report on the development of a novel CAR product targeting CD79b. We initially confirmed expression of CD79b on patient-derived xenografts (PDX) and malignant cells in blood from MCL patients. We show that loss of CD19 at the DNA and RNA level does not interfere with CD79b surface expression, further supporting the use of CD79b as an alternative CAR T cell target in CD19-negative lymphomas. Importantly, we demonstrate potent antitumor effects of anti-CD79b CAR T cells, comparable to anti-CD19 CAR T cells, *in vitro* and *in vivo,* with prolonged remission in both cell line-based and patient-derived xenograft lymphoma models. Finally, anti-CD79b CAR T cells alone or arranged in a bi-specific format with an anti-CD19 CAR is able to eliminate CD19-positive, CD19-negative, and mixed CD19-expressing lymphomas *in vivo*.

Materials and methods

Cell lines and culture

The human cell lines Jeko-1, JVM-2 and Granta-519 (MCL), SuDHL-4 and SuDHL-6 (DLBCL), Raji and Daudi (Burkitt's lymphoma), and MM.1s (Multiple Myeloma) were obtained from American Type Culture Collection (ATCC) and cultured in accordance with the supplier's recommendations. Parental K562 were purchased from ATCC and modified to express CD19 (K562-CD19+), CD79b (K562-CD79b+), or CD19 and CD79b (K562-CD19+CD79b+). For some assays, cell lines were transduced to express a click-beetle luciferase and green fluorescent protein (CBG-GFP+). CBG-GFP+ cells were sorted on a FACSAria (BD) and new cultures established.

Mantle cell lymphoma patient samples

Blood from six patients diagnosed with MCL was collected after written informed consent was obtained at the Haematology-Pathology Research Laboratory, Odense University Hospital, approved by The Regional Committees on Health Research Ethics for Southern Denmark in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll gradient centrifugation at diagnosis and cryopreserved until further analysis.

Flow cytometry

Cells were stained with antibodies against phenotypic markers, run on a Fortessa X-20 (Becton Dickinson) or FACSCanto (Becton Dickinson) and data analyzed using FlowJo software (Tree Star) or Flow logic (Inivai technologies). The following antibodies, purchased from BD Bioscience and BioLegend, were used: BV421-CD19 (Clone HIB19), APC-CD19 (Clone 4G7), BB515-CD79b (Clone 3A2–2E7), PE-Cy7-CD79b (Clone CB3–1), PercPCy5.5-CD3 (Clone UCHT1), BUV395-CD3 (Clone UCTH1), PE-Cy7-kappa (G20–193), Alexa647-lambda (Clone JDC-12), PE-CD5 (UCHT2), APC-H7-CD20 (Clone 2H7), BV605-CD45 (Clone 2D1), and CD107a-AF700 (Clone H4A3). Cells were stained

for 15 minutes in Hank's Balanced Salt Solution (HBSS) supplemented with 2% FBS. 7AAD or DAPI was used to exclude dead cells prior to acquisition.

CRISPR/Cas9 Knockout

Jeko-1 CBG-GFP+ cells were transduced with Cas9 lentivirus and selected with blasticidin. Three CD19 guides from the Brunello library (16) were purchased from the Broad Institute as lentiviruses and used to transduce Jeko-1 CBG-GFP+ Cas9 cells. Following puromycin selection, CD19-negative cells were single-cell sorted and further expanded for use in *in vitro* and *in vivo* assays.

CAR constructs

Two second-generation anti-CD79b CARs, with either a light-heavy (CAR79b (L/H)) or a heavy-light (CAR79b (H/L)) single-chain variable fragment (scFv) configuration, were synthesized and cloned into a third-generation lentiviral backbone under control of the human EF1α promoter. All CARs included a CD8 hinge/transmembrane domain, 4–1BB and CD3ζ intracellular domains, a T2A skip element, and an mCherry fluorescent protein as a reporter gene for transduction efficiency.

Transduction and expansion of human T cells

Purified human T cells (STEMCELL Technologies, catalog #15061) from Leuko Paks of healthy donors were obtained from the Massachusetts General Hospital (MGH) blood bank under an IRB-exempt protocol in accordance with the U.S. Common Rule. For T cell expansion, T cells were activated (day 0) with anti-CD3/CD28-coated Dynabeads (Life Technologies, Catalog #111.32D) at a 3:1 bead-to-cell ratio. 24 hours after activation, T cells were lentivirally transduced to express the CAR construct. T cells were grown in RPMI media supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin (P/S), and 20 IU/mL of recombinant human IL-2. Cultures were maintained at $0.5e^6$ cells per mL and generally passaged every other day. For functional testing, cells were cryopreserved at day 9/10 of culture and directly used after thawing.

Cytotoxicity, activation, and cytokine assays

For all functional testing, different groups of CAR T cells were normalized for CAR expression by adding donor-matched and activated untransduced T cells. For cytotoxicity assays, CAR T cells were co-cultured with CBG-GFP+ target cells overnight at various ratios as indicated in each experiment. The percent specific killing was calculated based on luminescence measured with a Synergy Neo2 luminescence microplate reader (BioTek). For activation, Jurkat reporter (NFAT-luciferase) T cells transduced with CAR constructs were co-cultured overnight with target cells at a 1:1 ratio. Luciferase activity was used to measure percent specific activation using the Synergy Neo2 plate reader. For the analysis of cytokine production, CAR T cells were co-cultured overnight with target cells at a 1:1 ratio. Cell culture supernatants were collected and technical duplicates analyzed for level of cytokines, using a multiplex Luminex array (Luminex Corp., FLEXMAP 3D) in accordance with the manufacturer's instructions. All assays were performed in biological duplicates or more in

each experiment, as indicated by N, which corresponds to the number of healthy donor T cells tested.

In vivo studies

All animal experiments were performed in accordance with Federal and Institutional Animal Care and Use Committee (IACUC) requirements under an MGH-approved protocol. Animals were caged in groups with free access to food and water. NOD-SCID-γ chain−/− (NSG) mice (Jackson Laboratories) were engrafted with Jeko-1 CBG-GFP+ or patient-derived tumor cells at the cell number and route of administration specified in each experiment. Cryopreserved CAR T cells were normalized for CAR expression by the addition of activated untransduced donor-matched T cells and given as a single intravenous (IV) injection after tumor engraftment was established by bioluminescent imaging (BLI). To estimate the tumor burden, mice were imaged using an AMI spectra imaging apparatus after receiving an intraperitoneal (IP) injection of D-Luciferin (30 mg/mL). Images were analyzed with IDL software version 4.3.1. Animal welfare—including body weight, appearance, and tumor burden—was regularly monitored and animals were euthanized when they met pre-specified end points defined by the IACUC.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. A t test was performed when comparing two groups and a one-way analysis of variance (ANOVA) or a two-way ANOVA test, as appropriate, when comparing multiple groups. A p-value <0.05 was considered statistically significant.

Results

CD79b is expressed on human lymphoma subtypes and MCL patient cells

Expression of CD19 and CD79b on B cell tumor lines and MCL-patient-derived xenografts was evaluated by flow cytometry. CD79b and CD19 were expressed on human BL, DLBCL, and MCL, but not multiple myeloma as expected (Fig. 1 A and Supplementary Fig. 1A). In addition, CD79b expression has been reported in patients diagnosed with follicular lymphoma both at presentation and relapse (17). We noted a high frequency and homogeneity of CD79b-positive cells in the MCL PDX samples, to a greater degree than for CD19 (Fig. 1 B). Next, we evaluated the expression of CD79b and CD19 on malignant cells in blood from six patients diagnosed with MCL (Fig. 1 C). Malignant cells were gated as CD3-CD20+CD5+ B cells, and expression was analyzed by flow cytometry (Supplementary Fig. 1 B **and** C). All malignant cells expressed CD19, but unlike in the PDX models, the frequency CD79b-positive malignant cells appeared to be more variable.

Design of anti-CD79b CAR constructs

We designed two second-generation CAR constructs directed against CD79b. Both constructs included an anti-CD79b scFv connected via a CD8 hinge and transmembrane domain to an intracellular 4–1BB signaling domain and a CD3 zeta activation domain (Fig. 1 D). The scFv was synthesized in a light-heavy (L/H) or a heavy-light (H/L) orientation of the variable domains generating CAR79b (L/H) or CAR79b (H/L) CAR T cells. For

comparison, we generated a CAR19, and used the same hinge, transmembrane, and signaling domains as in the CD79b CARs. In order to determine transduction efficiency easily, we incorporated an mCherry fluorescent protein, separated from the CAR sequence by a T2A element. High transduction efficiencies of activated human T cells were routinely obtained using a third-generation, self-inactivating lentiviral vector system (Supplementary Fig. 2 A). We observed similar transduction efficiency across multiple donors for CAR19 compared to CAR79b (L/H) and CAR79b (H/L).

CD79b CAR T cells have potent effector functions *in vitro*

We designed a series of *in vitro* experiments to test the efficacy of the anti-CD79b CAR T cells. First, the ability of CAR T cells to be activated in response to antigen was tested. The different CAR constructs were transduced into the Jurkat-NFAT reporter T cell line. After transduction, Jurkat-NFAT cells were co-cultured with the MCL cell line Jeko-1, K562 transduced to express either CD19 or CD79b, anti-CD3/CD28 Dynabeads for positive control, or media for negative control. NFAT mediated luminescence demonstrated activation of Jurkat T cells in response to antigen-specific stimulation (Supplementary Fig. 2 B). We noted comparable levels of activation between CAR19 and CD79b CAR T cells in response to the CD19-positive MCL tumor cell line Jeko-1. Luminescent signal was only observed when CD79b CAR T cells were stimulated with K562-CD79b+ but not K562-CD19+, indicating that activation was antigen-specific. To evaluate the cytotoxic efficacy of the anti-CD79b CAR T cells, we performed tumor lysis assays against Jeko-1 transduced to express luciferase. CAR T cells were co-cultured overnight with tumor cells at various effector-target ratios (Fig. 1 E). Only anti-CD79b CAR T cells bearing the L/H scFv configuration showed increased cytotoxicity compared to untransduced T cells. Importantly, the ability of CAR79b (L/H) T cells to lyse tumor cells was comparable to CAR19. Next, we analyzed the production of cytokines from CAR T cells in response to antigen-specific stimulation. CAR T cells were co-cultured overnight with either Jeko-1 cells, anti-CD3/CD28 Dynabeads as a positive control, or media as a negative control. The pattern of cytokine production in response to Jeko-1 cells —especially with upregulation of Th1 cytokines IL-2, IFN-γ, GM-CSF and TNFα—was similar among different groups of CAR T cells (Fig. 1 F **and** Supplementary Fig. 2 C). In general, CAR19 produced slightly higher levels of cytokines compared to the CD79b CAR T cells. The levels of cytokine production correlating to optimal CAR-mediated killing is not known. Consistent with the cytotoxicity assays, CAR79b with the H/L configuration produced the lowest levels of cytokines in response to antigen. Together this set of *in vitro* experiments demonstrated superior antigen-specific effector functions of CAR79b (L/H) compared to CAR79b (H/L).

CAR79b eradicates MCL tumors and leads to prolonged survival *in vivo*

Next, we designed a series of xenograft models to test the efficacy of CAR79b (L/H) *in vivo*. In these experiments, we used donor-matched CAR19 and untransduced T (UTD) cells for comparison. NSG mice were intravenously injected with luciferase-positive Jeko-1 (CBG-GFP+) tumor cells, which incidentally, had a lower CD79b expression as determined by MFI compared to the PDX cells (Supplementary Fig. 1A). Seven days later, tumor burden was evaluated based on BLI, and mice received a single dose of CAR79b (L/H), CAR19, or UTD cells through the tail vein (Fig. 2 A). Fourteen days after CAR T cell injection,

complete tumor clearance was observed for groups of mice receiving either CAR79b or CAR19 (Fig. 2 B **and** C). We confirmed the persistence of CAR T cells both in peripheral blood at 14 days (Fig. 3 D) and in bone marrow at 28 days post-injection (Fig. 2 E). Overall, we observed no difference in tumor clearance or CAR T cell persistence between mice treated with either CAR79b or CAR19. Despite the advantage of using tumor cell lines to assess the efficacy of CAR T cells, they do not fully represent the tumor heterogeneity observed in patients. A more rigorous preclinical model to test CAR T cells is to use tumor cells directly derived from patients. Therefore, we tested the efficacy of CAR79b and CAR19 in an MCL PDX model. To do this, NSG mice were injected with luciferase-expressing MCL-PDX cells (DFBL.98848-V3). After we confirmed tumor engraftment, the mice were grouped according to BLI and injected with CAR79b, CAR19, or UTDs (Fig. 3 A). Fourteen days after the CAR79b injection, we observed tumor regression, with total tumor clearance at day 21 (Fig. 3 B **and** C). Mice treated with CAR19 or CAR79b survived and remained tumor-free until the end of the experiment (66 days post CAR T cell injection) (Fig. 3 D). We noted persistence of a small population of CAR79b in the collected bone marrow of mice at termination day, which was similar to the results for mice treated with CAR19 (Fig. 3 E). These results demonstrate the ability of CAR79b to mediate tumor clearance in different preclinical models of mantle cell lymphoma with similar efficacy as CAR19.

Loss of *CD19* at the DNA or RNA level does not reduce CD79b surface expression

Reported loss of CD19 leading to disease relapse is a major obstacle for the curative potential of CD19-directed CAR therapy (2). In ALL, an alternative splice product excluding the epitope targeted by CD19 CAR T cells has been reported as one mechanism of antigen escape and relapse (7), as have frameshift mutations in the *CD19* gene (18). CD19 and BCR cross-linking lowers the threshold for B cell activation (19,20), thereby augmenting B cell signaling. In lymphomas, CD79b and CD19 have been proposed to form a signaling complex that promotes survival (19). To this end, we tested whether the loss of *CD19* would interfere with the surface expression of CD79b. We used CRISPR/Cas9 technology to generate CD19-negative MCL cell lines. Jeko-1 cells were stably transduced to express Cas9 protein, and then transduced with lentiviruses encoding different CD19-targeted guides. CD19-negative Jeko-1 cells were single-cell clone sorted and expanded to establish cell lines. A complete knockout of *CD19* did not lead to reduction in surface CD79b expression in the generated cell lines (Supplementary Fig. 3 A **and** 3B). In addition, loss of *CD19* did not alter growth kinetics in negative cell lines compared to parental Jeko-1 (Supplementary Fig. 3 C). Importantly, the generated CD19-negative cell lines maintained surface CD79b expression and the loss of CD19 during a prolonged culture period (Supplementary Fig. 3 D). Similar findings were observed using a CD19 shRNA knockdown approach, whereby the downregulation of CD19 did not reduce CD79b expression in Jeko-1 cells (Supplementary Fig. 4 A **and** B). Together these data indicate no immediate relationship between CD19 and CD79b surface expression, leading us to hypothesize that the loss of CD19 in patients having received CAR19 therapy will not influence surface CD79b expression. Collectively, these results further strengthen CD79b as a potential target for CAR therapy in patients relapsed with CD19-negative lymphoma.

CAR79b eradicate CD19-negative MCL tumor *in vivo*

Next, we proceeded to test anti-lymphoma activity of CAR79b T cells against CD19-negative Jeko-1 cells *in vivo*. NSG mice were engrafted with CD19-negative Jeko-1 cells (F12 clone 5) and subsequently injected with a single dose of CAR19, CAR79b, or UTDs (Supplementary Fig. 5 A). A significant reduction in tumor burden was detectable only in the CAR79b group, starting at day 7 post-treatment, and was maintained for at least 14 days (Supplementary Fig. 5 B **and** C). At day 14 post CAR T cell injection, we detected allogeneic response of T cells from the UTD and CAR19 groups, which may be related to constitutive expression of Cas9, since allogeneic responses at these time points were not observed in non-Cas9-expressing Jeko-1 xenografts. Due to the strong allogenicity of the CD19-negative Jeko-1 cells, further experiments with the CD19-negative Jeko-1 lines required termination at day 14.

CAR79b shows efficacy *in vitro* in a tandem format with CAR19

One strategy to prevent relapse with CD19-negative disease is to target heterogeneous tumors with CARs that target multiple antigens simultaneously. Therefore, we designed two second-generation tandem CARs targeting both CD79b and CD19 (Fig. 4 A). No difference in transduction efficiency of human T cells from multiple donors was observed among the different tandem CAR constructs, but transduction efficiency of tandem bispecific CARs was slightly lower than the monospecific CARs, likely due to the size of the transgene (Supplementary Fig. 6A). Using the Jurkat-NFAT reporter assay, we observed antigen-specific activation of our tandem CAR T cells in response to K562-CD79b+, K562-CD19+, and K562-CD19-CD79b+ cells (Supplementary Fig. 6 B), thus demonstrating the ability of the tandem CARs to recognize both CD19 and CD79b. Degranulation of tandem CAR T cells in response to CD19-negative tumors was evaluated after five hours of co-culture with tumor cells (Supplementary Fig. 6 C). All CAR T cells expressed surface CD107a when incubated with parental Jeko-1 cells. While the tandem CAR T cells maintained the ability to degranulate in response to the CD19-negative tumor, Jeko-1 (F12 clone 5), a clear reduction was seen for CAR19 in response to these cells. The CAR79b-CD19 tandem CAR seemed to have reduced degranulation against the parental Jeko-1 cells in this assay. Next, we evaluated the ability of the tandem CAR T cells to produce cytokines in response to stimulation with Jeko-1 (Supplementary Fig. 6 D). In general, the level of Th1 cytokine production was higher for CAR19 and CAR79b than for the tandem CAR19–79b and CAR79b-19 compared to UTDs. When the tandem CAR T cells were stimulated with CD19-negative Jeko-1 cells, we detected higher levels of cytokines in the cell culture medium for CAR19–79b than CAR79b-19 (Supplementary Fig. 6 E). As expected, no cytokine production by CAR19 in response to the CD19-negative Jeko-1 cell line was detected. The ability of CAR79b to secrete effector cytokines in response to the CD19-negative Jeko-1 cell line was retained. In conclusion, these results demonstrate the ability of the tandem CAR T cells to maintain their effector functions independent of CD19 antigen expression, but there was not necessarily a clear advantage of one tandem CAR format over the other.

CAR79b-CD19 eradicates CD19-negative MCL tumors *in vivo* within mixed tumors

Next, we sought to test the ability of the tandem CAR T cells to lyse CD19-negative tumors in the context of a heterogeneous tumor in a xenograft model. We used a 1:1 mix of parental Jeko-1 and CD19-negative Jeko-1 cells as tumors, and tested CAR19, CAR79b, and the two tandem CARs, CAR19–79b and CAR79b-19, as treatment. Donor-matched untransduced T cells were used as controls for allogeneic effects. Briefly, NSG mice received an intravenous injection of a 1:1 mixture of tumor cells; at day 7, when tumors were established based on BLI, mice were re-grouped and received a single dose of UTD's, CAR19, CAR79b, CAR19–79b or CAR79b-19 (Fig. 4 B). Seven days post CAR T cell injection, we noted a reduction in tumor burden for CAR79b and CAR79b-19 compared to CAR19 and CAR19–79b, which was maintained through day 14 (Fig. 4 C **and** D). We detected the presence of CAR T cells in peripheral blood in all CAR treatment groups 14 days post CAR T cell injection (Fig. 4 E). Taken together, these data demonstrate that CAR79b and CAR79b-19, but not CAR19–79b, could lyse CD19-negative lymphoma within a heterogeneous tumor.

CAR79b-CD19 CAR T cells are effective against pure CD19-negative "relapsed" lymphoma and CD19^{+}CD79b^{+} "upfront" lymphoma

In order to test whether tandem bispecific CARs were still effective compared to monospecific CARs targeting CD79b in the CD19-negative relapse setting, we engrafted NSG mice intravenously with CD19-negative Jeko-1 cells one week before intravenous injection of CAR T cells, using donor-matched UTD and CAR19 serving as controls (Fig. 5 A). We observed that CAR79b and CAR79b-19 both cleared tumor by day 14, though monospecific targeting with CAR79b cleared tumor a few days earlier (Fig. 5 B and 5C). Next, to test whether monospecific or bispecific CARs would be equally effective in the "upfront" setting, we engrafted NSG mice with the parental Jeko-1 cells and compared treatment with CAR19, CAR79b, and CAR79b-19 to donor-matched UTD (Supplementary Fig. 7 A). CAR79b-19 and CAR-19 cleared the tumors by day 7, followed by CAR79b at day 11 of treatment (Supplementary Fig. 7 B **and** C). We confirmed the presence of CAR T cells in peripheral blood at day 14 (Fig. 6 **D**).

Finally, we used a stress model to better evaluate and compare the efficacy of monospecific CD79b and bispecific CAR T cells targeting CD19 and CD79b. We injected 1×10^{6} Jeko-1 cells into NSG mice followed by treatment with 1×10^{6} or 0.5×10^{6} UTD, CAR19 or CAR79b, CAR79b-19 bispecific T cells (Fig. 6 A). CAR19 and CAR79b-19 could still induce complete remission at both lower doses of T cells. CAR79b T cells delayed tumor progression but they were insufficient to achieve tumor eradication despite evident tumor remission at early time points (Fig. 6 B). We were able to detect CAR T cells in the blood at day 14 in some of the mice, which is perhaps explained by the lower dose of CAR T cells injected (Fig. 6 C). Interestingly, the presence of CAR T cells in the blood did not correlate with clearance of the tumor, as it does in human subjects.

Collectively, these results indicate that tandem CAR79b-19 T cells have the potential to treat both CD19-negative relapsed tumors that may have recurred after CD19-targeted therapies, as well as to treat tumors that still retain CD19 expression, as most B cell lymphomas would prior to treatment with CD19-targeted therapies.

Discussion

CD19 CAR T cell therapy has been highly efficacious as treatment of a variety of B cell malignancies, leading to recent approval by the FDA of two CD19-directed CAR T cell products, axicabtagene ciloleucel and tisagenlecleucel for r/r DLBCL. Despite the overall success rate, reports of patients relapsing with CD19-negative disease after CD19-directed CAR therapy are now emerging (2,7,8). This constitutes a significant obstacle for CD19-directed therapies, and efforts to target additional antigens are becoming increasingly important. Trials targeting the B cell antigen CD22 in B-cell ALL are already underway, but it has been noted that serial targeting of single antigens can result in serial antigen escape (21). To avoid antigen escape, CARs targeting both CD19 and CD22 simultaneously are also in clinical development (22,23). Unfortunately, CD22 expression is often lost in more mature B cell malignancies like lymphomas (24,25), making alternative antigen targets necessary.

The BCR consists of membrane-bound immunoglobulin and a transmembrane heterodimer consisting of CD79a and CD79b. The cytoplasmic tails of CD79a and CD79b contain an immunoreceptor tyrosine-based activation motif (ITAM), important for propagating signals within the B cell upon antigen ligation. Because of its signal-transducing properties, CD79b is an important mediator of development and maintenance of mature B cells. Its expression is restricted to the B cell compartment and lymphoid tissues, including bone marrow, tonsils, appendix, lymph nodes, and spleen, as well as most types of lymphoma (10-12,26). Many B cell lymphomas require continuous BCR signaling for their tumor growth, and several reports have established somatic mutations affecting the ITAM region of CD79b as a mechanism for enhanced tumorigenicity (19,27-29). Indeed, different drug modalities targeting CD79b have been shown to be effective and well tolerated (13-15,17,30) In an abstract presented at the American Association for Cancer Research in 2017, an anti-CD79b/CD3 BiTE showed inhibition of tumor growth in a humanized lymphoma xenograft mouse model. Encouragingly, an anti-cynoCD79b/CD3 BiTE was tested in a cynomolgus monkey model and was associated with an acceptable safety profile (15). The antibody-drug conjugate polatuzumab vedotin, directed against CD79b, was tested in a phase 1 dose escalation trial in cohorts of patients diagnosed with NHL or CLL (14). Objective responses were noted in 23 of 42 NHL patients, including responses in indolent NHL, DLBCL, and MCL. No objective responses were observed in the CLL cohort, potentially owing to the fact that CD79b surface expression is often downregulated or absent in this patient group (11,31). Collectively, these observations support CD79b as a potential target in lymphoma for CAR T cell therapy.

The loss of CD19 has been reported in up to 30% of patients treated with CD19-CAR T cells (2,7-9,32). Based on this, we hypothesized that potential antigen escape could be overcome or prevented by alternative or multiple antigen targeting. Therefore, we designed and validated the efficacy of a novel CAR construct directed against CD79b. First, we confirmed the expression of CD79b in a cohort of MCL patients. We noted some variability in the surface expression of CD79b. However, our Jeko-1 cells had lower CD79b expression as measured by MFI compared to our PDX, and yet was still responsive to the CAR79b cells. Nevertheless, selection of patients whose tumors have strong and uniform expression of

CD79b may be a useful approach for early clinical testing. In the present study, we demonstrated that T cells engineered to express a second-generation CAR directed against CD79b had the ability to eradicate CD79b-expressing tumors. The anti-CD79b CAR bearing the L/H configuration showed particularly superior antitumor efficacy compared to its H/L counterpart. Importantly, the CD79b CAR T cells showed compatible efficacy to the CD19 CAR T cells used as the current gold standard within the field.

CD19 has been shown to function as a co-receptor for the BCR by reducing the threshold for B cell signaling. In addition, CD79b and CD19 have been shown to form an alternative B cell signaling module that, via continuous ITAM/PI3K signaling, can promote the survival of lymphomas (19). Therefore, we sought to investigate if the loss of *CD19* would modulate the surface expression of CD79b. We used CRISPR/Cas9 to generate CD19-negative Jeko-1 cells. Knocking out *CD19* led to the loss of CD19 without altering the level of CD79b expression on the cell surface. Similar results were observed in a Burkitt's lymphoma cell line model, where CRISPR/Cas9-mediated knockout of *CD19* in Ramos cells did not reduce the expression of surface CD79b (19). Collectively, our results demonstrate no immediate correlation between the loss of *CD19* and CD79b surface expression. Although we realize that these data are based on tumor cell lines and therefore should be interpreted with caution, they do support the hypothesis that CD79b surface expression will be retained in patients who relapse with CD19-negative disease after CD19-targeted therapy. In ALL, resistance to CAR-19 can be mediated by the production of alternative CD19 transcripts lacking the epitope targeted by the CAR. This truncated version of CD19 failed to trigger CAR T cell killing but partly rescued defects in cell proliferation and pre-BCR signaling associated with CD19 loss (7). CD19 loss has also now been demonstrated at the genomic level, occurring via frameshift mutations (18). Whether CD19 expression loss in lymphoma is due to frameshift or splicing variants of CD19 remains to be established. In both genomic and RNA models of CD19 loss, we were able to demonstrate that CD79b expression was not affected. Furthermore, CD79b-directed CAR T cells, in both a monospecific and bi-specific confirmation with anti-CD19, were able to induce regression of CD19-negative tumor cells *in vivo*. Unfortunately, one weakness of our study is that our CD19-negative lymphoma tumors also expressed Cas9, and we observed that allogeneic effects against Cas9-expressing tumor cells reduced the usable timelines of our *in vivo* experiments. Immunogenicity in the general population against Cas9 was recently described (33).

Because it is likely that loss of CD19 reflects the selective pressure on the antigen exerted by CAR T cells, one strategy to prevent this problem is the development of novel CARs directed against multiple surface antigens. Therefore, we designed and validated the efficacy of a tandem CAR bearing scFvs directed against CD79b and CD19. We showed that one specific configuration of tandem CAR T cells showed *in vivo* antitumor activity against CD19-negative lymphoma, whereas CAR19 or a different configuration of the tandem CAR did not. Unfortunately, xenograft models of lymphoma are not adequate models in which to rigorously test the hypothesis that tandem CARs can prevent antigen escape. Although some xenograft models of leukemia can be used to model CD19 escape, such pre-B cell acute lymphoblastic leukemia models would also be expected to lack CD79b expression (34). Models of lymphoma that spontaneously result in antigen escape would be beneficial to the field.

In conclusion, CD79b represents a new target for the treatment of B-cell lymphoma. We report potent efficacy of monospecific CD79b and bispecific CD79b-CD19 CAR T cells *in vitro* and in xenograft models of lymphoma. CAR T cells targeting CD79b, particularly in combination with simultaneous targeting of CD19, could offer a new therapeutic option for patients who have CD19-negative recurrence of their lymphoma, or who have not had prior treatment with CD19-directed therapy, in the hopes of preventing recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Odense Patient data Explorative Network (OPEN) for establishing the REDCap database, the MGH Blood Bank for leuko paks, and the MGH Flow Cytometry Core facility for cell sorting. This work was supported in part by the Gabrielle's Angel Foundation (M.V.M), Velux foundation (M.O and T.B), and American Italian Cancer Foundation and Lymphoma Research Foundation (I.S).

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Statement of translational relevance: Complete remission has been observed in about 40% of lymphoma patients treated with anti-CD19 CAR T cells, but disease relapse can occur due to antigen escape. We have designed a CAR against CD79b, an antigen widely expressed in B cell lymphomas. We showed that targeting CD79b as a single antigen or in combination with CD19 using CAR T cells is effective *in vitro* and *in vivo* in xenograft and patient derived xenograft (PDX) lymphoma models. We further demonstrate that CAR T cells targeting CAR79b in a monospecific or bispecific configuration with CD19-targeting are able to clear CD19+ “upfront” lymphomas, heterogeneous tumors containing CD19+ and CD19- cells, and “relapsed” CD19-negative tumors. These results provide a rationale for clinical evaluation of CAR79b and CAR79b-19 T cells in patients.

Key points

- CD79b can be targeted with CAR T cells, and this antigen is retained on B cell lymphoma cell lines independent of loss of CD19.
- Primary human T cells transduced with CARs targeting CD79b alone or in a tandem CAR format with anti-CD19 eradicate CD19-positive, CD19-negative, and mixed CD19-expressing lymphoma cells in a xenograft model.

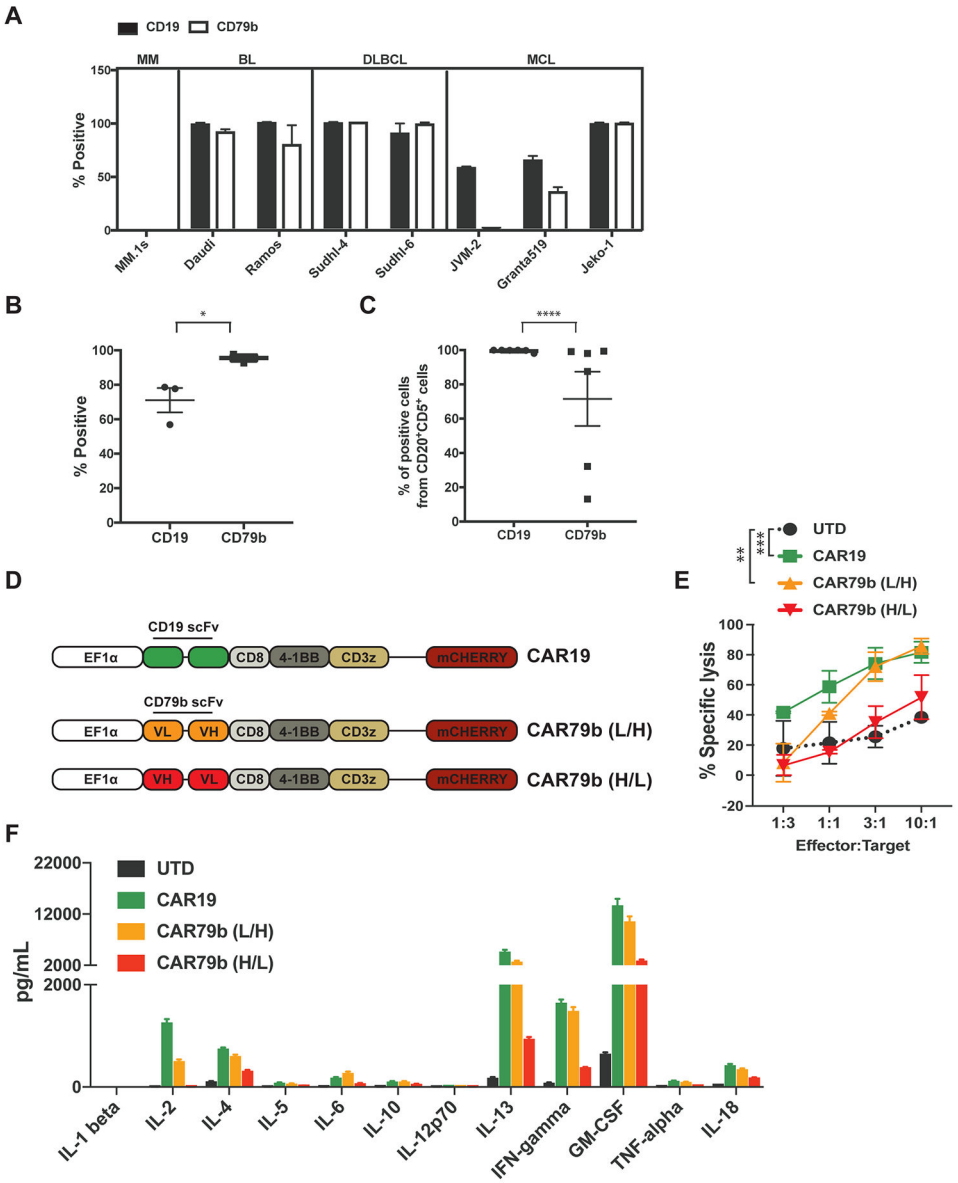

Figure 1: CD79b CAR T cells demonstrate potent effector functions *in vitro* against CD79b-positive lymphoma cell lines

(A) Bar graph showing the percent CD79b and CD19 expression on human B cell tumor cell lines determined by flow cytometry (B) Percent CD79b and CD19 expression on MCL-patient-derived xenografts determined by flow cytometry (mean±SEM shown, *<0.05, t test). (C) Percent CD79b and CD19 expression on circulating tumor cells obtained from MCL patients, gated as CD3-CD20+CD5+ (n=6 patients, mean±SEM shown, ****<0.0001, t test). MM: multiple myeloma, BL: Burkitt's lymphoma, DLBCL: diffuse large B cell lymphoma, MCL: mantle cell lymphoma. (D) Two second-generation chimeric antigen receptors against CD79b were constructed, utilizing a humanized antibody-derived single-chain variable fragment with a light-heavy (CAR79b (L/H), middle) or heavy-light (CAR79b (H/L), bottom) chain configuration. A second-generation CAR against CD19 was included as a control (CAR19, top). (E) Cytotoxicity of CAR T cells after overnight co-

culture with target cells at different effector-target ratios (n=2 healthy donors, mean±SEM shown, **<0.01, ***<0.001, ANOVA). (F) Effector cytokine production measured in collected cell culture supernatants after overnight co-culture of CAR T cells and target cells at a 1:1 ratio. Cytokine analysis was performed using a Luminex array (n=2 healthy donors, mean+SEM shown).

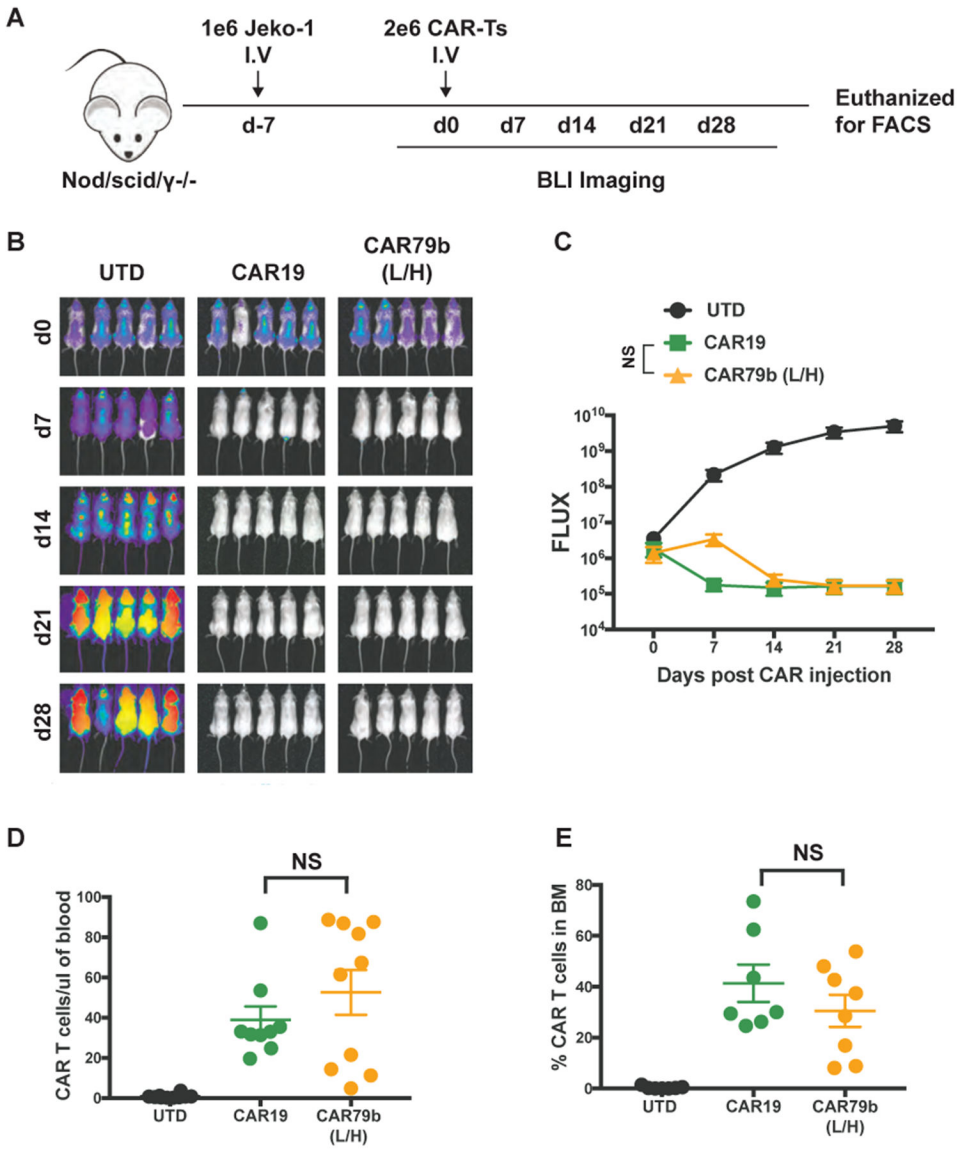

Figure 2: CAR79b (L/H) induced tumor clearance and persistence in an MCL xenograft model

(A) Schematic overview of xenograft experiment: NSG mice were engrafted with 1e^6 Jeko-1 (CBG-GFP+) cells and tumor development was monitored with bioluminescent imaging. At day 0, mice were grouped according to tumor burden and injected with 2e^6 CAR79b (L/H), CAR19 or untransduced control T cells (UTD). (B) Representative bioluminescent images of tumor burden over time for one experiment. (C) Average radiance [p/s/cm^2/sr] FLUX, of groups of mice at different time points (n=9–10 mice per group, mean±SEM shown, p=NS, two-way ANOVA). (D) Absolute numbers of CAR T cells in blood, quantified by flow cytometry and Trucount beads (mean±SEM shown, p=NS, ANOVA). (E) Persistence of CAR T cells in bone marrow, 14 days after infusion of UTDs, CAR19, or CAR79b (L/H) determined by flow cytometry (n=9–10 mice per group, mean±SEM shown, p=NS, ANOVA). Graphs are representative of two experiments with two different healthy donor T cells. NS; Non-significant.

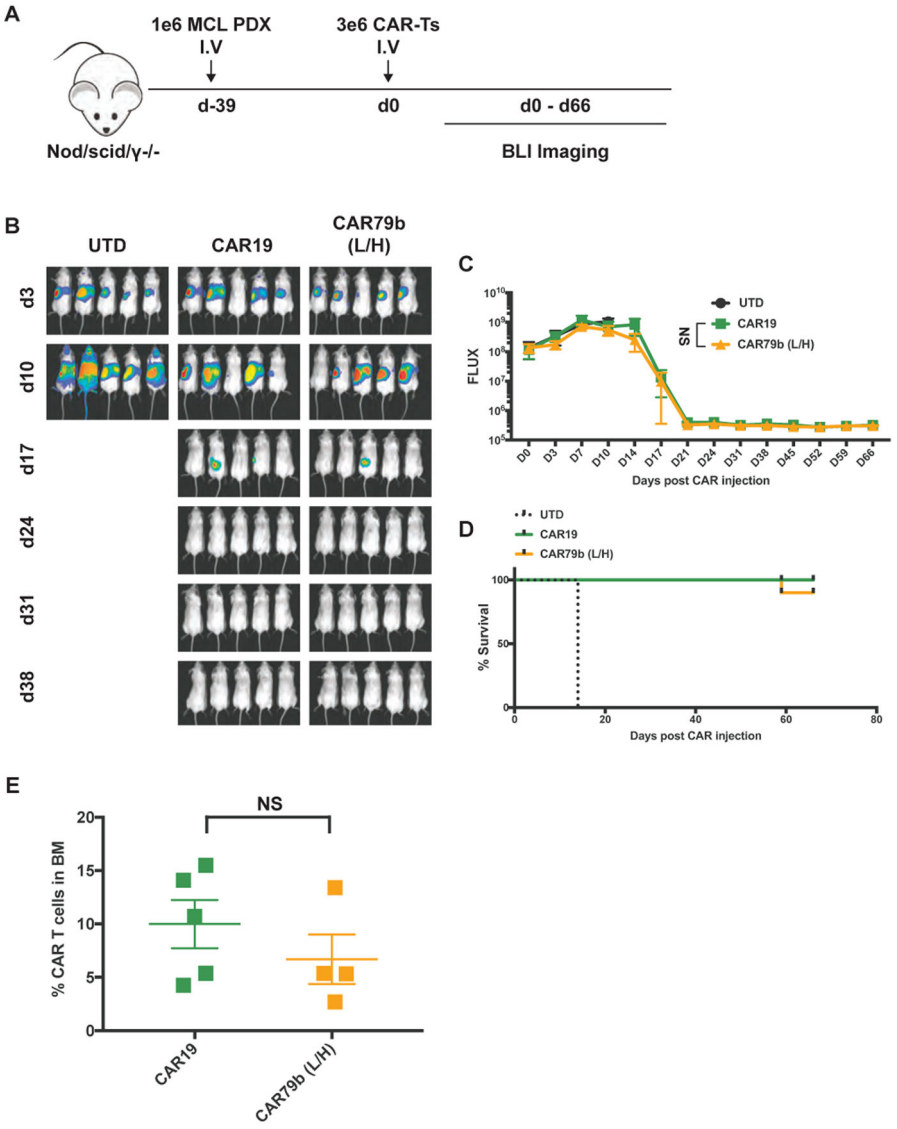

Figure 3: CAR79b (L/H) mediates tumor clearance and prolongs survival in an MCL-patient-derived xenograft model

(A) Schematic overview of patient derived xenograft experiment: NSG mice received an IV injection of $1e^6$ MCL-patient-derived cells (DFBL-98848-V3) in the tail vein. Tumor development was monitored by bioluminescent imaging. At day 0, mice were grouped according to tumor burden and received a single IV dose of $3e^6$ CAR79b, CAR19, or UTD control cells. (B) Representative bioluminescent images of tumor growth over time. (C) Average radiance [p/s/cm^2/sr] FLUX, of whole mice in the different treatment groups over time (n=4–5 mice per group, mean±SEM shown, p=NS, two-way ANOVA). (D) Kaplan-Meier survival curve of groups of mice receiving UTDs, CAR79b (L/H), or CAR19. (E) Persistence of CAR T cells in the bone marrow of mice 66 days after CAR T cell injection (n=4–5 mice per group, mean±SEM shown, p=NS, ANOVA). Graphs are representative of T cells from one healthy donor. NS; non-significant.

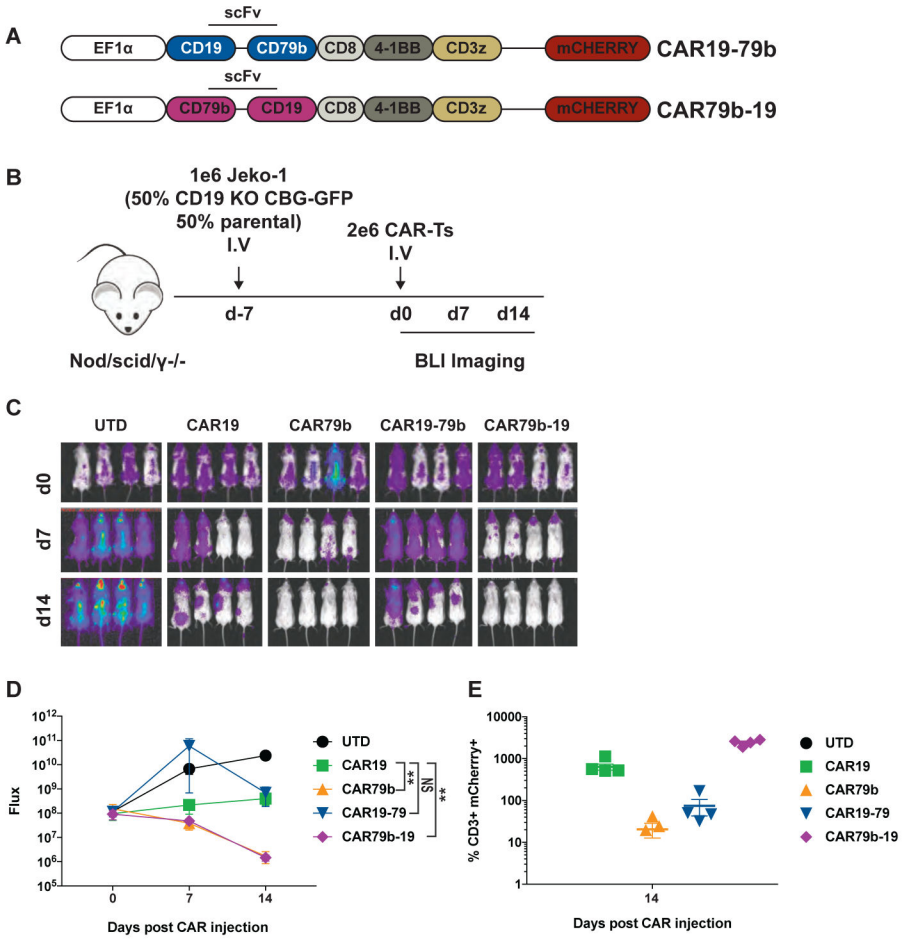

Figure 4: CD79b or CD79b-CD19 tandem CAR T cells show *in vivo* efficacy against heterogeneous tumors with variable CD19 expression

(A) Two second-generation chimeric antigen receptors were constructed, targeting both CD79b and CD19 in either a CD19-CD79b (CAR19-79b, top) or a CD79b-CD19 (CAR79b-19, bottom) configuration. (B) Schematic illustration of the experiment: NSG mice received an IV injection of a total of $1e^6$ Jeko-1 CD19-negative (F12 clone 5) and parental Jeko-1 tumor cells, mixed in a 1:1 ratio, into the tail vein. Tumor engraftment was established with BLI. At day 0, mice were grouped according to BLI and given a single dose of $2e^6$ CAR19-79b, CAR79b-19, CAR19, CAR79b (L/H), or UTD control cells. (C) Representative bioluminescent images of tumor growth over time from one experiment. (D) The average radiance [p/s/cm^2/sr] FLUX, of whole mice in different treatment groups over time (n=8 mice per group, mean±SEM shown, **<0.01, p=NS, two-way ANOVA). (E) Persistence of CD3+mCherry+ cells in peripheral blood 14 days post CART injection (n=4 mice per group, mean±SEM shown). Graphs are based on experiments with T cells from two different healthy donors, one shown as representative. NS; non-significant.

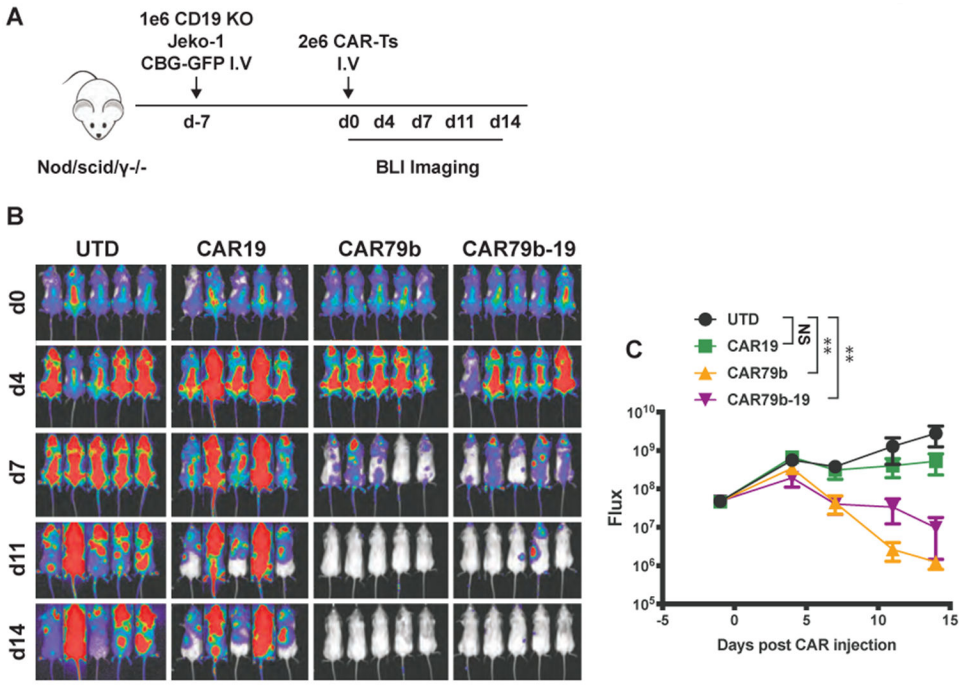

Figure 5: Both CAR79b and CAR79b-19 T cells eradicate CD19- "relapsed" tumors *in vivo*

(A) Schematic representation of the experimental design: NSG mice received an IV injection of $1e^6$ CD19 negative Jeko-1 tumor cells and tumor engraftment established by BLI. 7 days after initial tumor injection, mice received a single dose of CAR19, CAR79b, CAR79b-19, or untransduced control T (UTD) cells. (B) Representative bioluminescent images of tumor growth over time. (C) The average radiance [p/s/cm^2/sr] FLUX, of whole mice in different treatment groups over time (n=8 mice per group, mean±SEM shown, p=NS, **<0.01, two-way ANOVA).

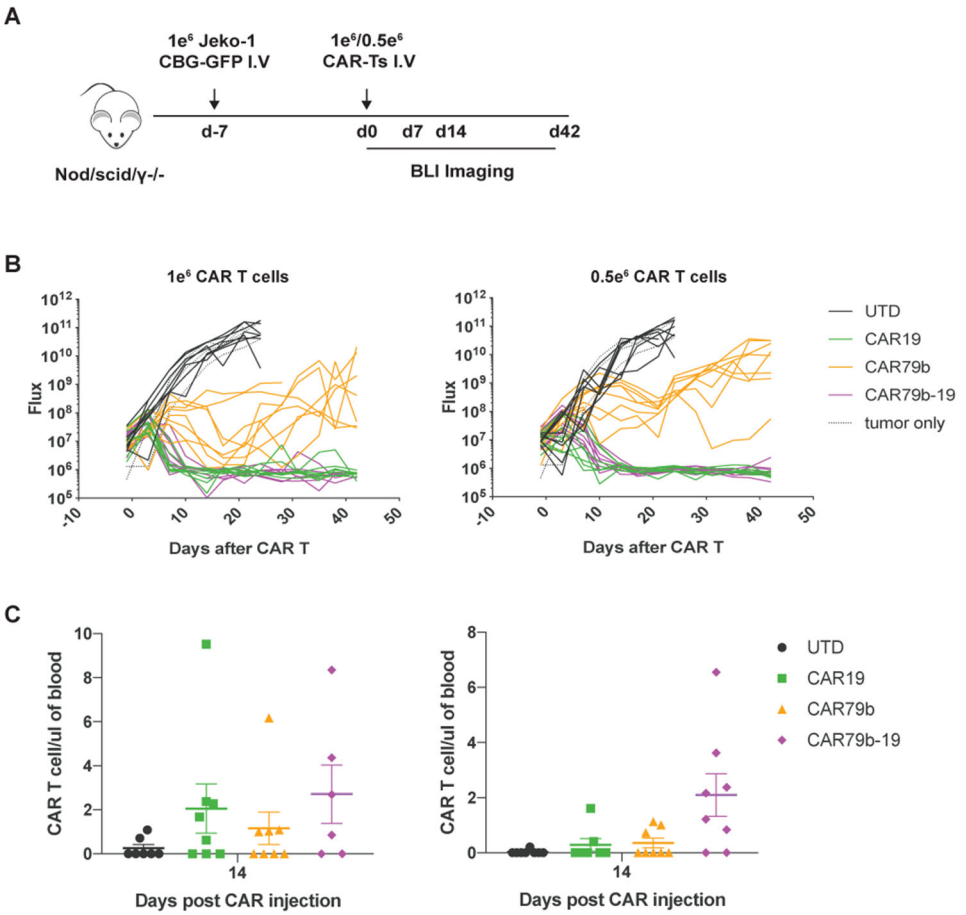

Figure 6: CAR79b-19 T cells eradicate MCL tumors at a lower dose compared to CAR79b.

(A) Schematic representation of the experimental design: NSG mice received an IV injection of $1e^6$ Jeko-1 tumor cells and tumor engraftment established by BLI. 7 days after initial tumor injection, mice received a single dose of $1e^6$ or $0.5e^6$ of CAR19, CAR79b, CAR79b-19, or untransduced control T (UTD) cells. (B) Tumor burden shown as bioluminescent signal quantified per animal every week over time. Each line represents one mouse (n=8 mice per group). (C) Detection of CD3+mCherry+ cells in whole blood at day 14 post CAR T cell injection (n=8mice per group, mean±SEM shown).