CAR T cells targeting BAFF-R can overcome CD19 antigen loss in B cell malignancies

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CAR T cells targeting CD19 provide promising options for treatment of B cell malignancies. However, tumor relapse from antigen loss can limit efficacy. We developed humanized, second-generation CAR T cells against another B cell–specific marker, B cell activating factor receptor (BAFF-R), which demonstrated cytotoxicity against human lymphoma and acute lymphoblastic leukemia (ALL) lines. Adoptively transferred BAFF-R-CAR T cells eradicated 10-day preestablished tumor xenografts after a single treatment and retained efficacy against xenografts deficient in CD19 expression, including CD19-negative variants within a background of CD19-positive lymphoma cells. Four relapsed, primary ALLs with CD19 antigen loss obtained after CD19-directed therapy retained BAFF-R expression and activated BAFF-R-CAR, but not CD19-CAR, T cells. BAFF-R-CAR, but not CD19-CAR, T cells also demonstrated antitumor effects against an additional CD19 antigen loss primary patient–derived xenograft (PDX) in vivo. BAFF-R is amenable to CAR T cell therapy, and its targeting may prevent emergence of CD19 antigen loss variants.

INTRODUCTION
Chimeric antigen receptor (CAR) T cells directed against CD19 have shown excellent response rates for treatment of lymphomas and leukemia (1–5). Despite high initial efficacy, some patients relapse through different modes of disease recurrence. One mode is CD19-negative relapse with CD19 surface expression loss, likely resulting from consequent mutations and selection for alternatively spliced isoforms. Another involves CD19-positive relapse with CD19 surface expression retained, likely resulting from rapid disappearance or decreased function of the CAR T cells (6, 7). An estimated 20 to 30% of relapses after CD19-CAR T cell therapy involve antigen loss, pointing to the urgent need to identify alternative targets and improve efficacy and persistence of CAR T cells (6). We developed humanized, second-generation CAR T cells against another B cell–specific marker, B cell activating factor receptor (BAFF-R), a target for immune therapy of cancer that has not been fully realized. BAFF-R is a B lineage marker with expression restricted to B cells after the progenitor stage and before the plasma cell stage of development, including malignant B cell counterparts. Its function has been well characterized, and studies show that its expression is critically required for normal B cell survival (8–12). These characteristics may, thus, limit the ability of malignant B cells to evade BAFF-R–directed therapies by down-regulation. We tested BAFF-R–directed CART cells against human lymphoma and acute lymphocytic leukemia (lines) in vitro and in mouse models in comparison with CD19-directed CAR T cells. Specifically, we tested BAFF-R-CAR T cell activity against CD19-negative targets.

RESULTS
Generation and characterization of BAFF-R-CAR T cells
A humanized, single-chain variable fragment (scFv) derivative of a mouse anti-human BAFF-R antibody (J3) was engineered onto a second-generation CAR construct containing 4-1BB costimulatory and CD3ζ intracellular signaling domains. Healthy donor T cell subpopulations were selected, transduced by BAFF-R-CAR, enriched, expanded, and tested for specific activation by BAFF-R–expressing targets (Fig. 1A). Considerable amounts of tumor necrosis factor–α (TNF-α), interferon-γ (IFN-γ), and granzyme B were released by CD4 and CD8 CAR T cells coincubated with human BAFF-R–expressing mouse fibroblast L cells and human mantle cell lymphoma (MCL) lines (JeKo-1 and Z-138), compared with BAFF-R–negative parental (L) cells. Allogeneic reactions were excluded by including corresponding nontransduced T cells from the same donors (non-CAR; fig. S1). CD8– and, to a lesser extent, CD4-derived BAFF-R-CAR T cells also elicited specific cytotoxicity against a panel of human lymphoma cell lines and leukemia cell lines (Fig. 1B and fig. S2). In addition, CD8– and, to a lesser extent, CD4-derived BAFF-R-CAR T cells elicited significant specific cytotoxicity against multiple primary human lymphoma subtypes (P < 0.0001 versus controls; Fig. 1C).

BAFF-R-CAR T cell subpopulations demonstrated robust in vivo antitumor effects
Next, we tested CAR-transduced human T cells for therapeutic efficacy against 10-day preestablished human Burkitt lymphoma (Raji) xenografts in immunocompromised mice. Because T cell phenotypes can affect CAR T cell function, we tested different mixtures of T cells (14–19). Remarkable tumor regression and prolonged survival were
observed after treatment with CAR-transduced Pan T cells (fig. S3) or defined mixtures of CD4 naïve T cells (T_N) with either T_N-, or central memory (T_CM)-, or stem memory (T_SCM)-enriched CD8 CAR T cells, compared with either nontransfected T cells or phosphate-buffered saline (PBS) controls (Fig. 2A and fig. S3). Furthermore, at the minimum therapeutic dose of $2 \times 10^6$ cells (1:1 ratio of CD4:CD8 CAR T cells), we observed superior therapeutic effects mediated by CD8 T_N CAR T cells, as demonstrated by 80% long-term survival compared with 20 and 40% survival, respectively, by CD8 T_CM and T_SCM CAR T cells (Fig. 2B).

We also tested the therapeutic efficacy of T_N-derived BAFF-R-CAR T cells against an aggressive CD19-positive Burkitt lymphoma (Raji) line (Fig. 3A and fig. S4). Mice with previously established tumors were treated with a single dose of defined mixtures of T_N CD4 and CD8 BAFF-R-CAR or CD19-CAR T cells (20) (identical CAR backbone) on day 7. Compared with control mice treated with non–CAR T cells or PBS, mice treated with CD19-CAR T cells exhibited delayed but progressive lethal tumor growth. In contrast, mice treated with BAFF-R-CAR achieved complete tumor regression, with 100% long-term survival (Fig. 3B). As one...

Fig. 1. BAFF-R-CAR T cells activate and elicit specific cytotoxicity against multiple subtypes of human B cell malignancies. (A) Either CD4+ or CD8+ BAFF-R-CAR T cells were incubated with BAFF-R–expressing L cells, MCL lines JeKo-1, Z-138, or, as controls, parental L cells (mouse fibroblast) or T-activator CD3/CD28 beads. ELISA measurement of cytokines IFN-γ, TNF-α, and granzyme B supernatant concentrations were determined following 24 hours of incubation with targets. (B) Cytotoxic T lymphocyte assay measuring the specific lysis of target cells by $^{51}$Cr release after 4 hours. Various $^{51}$Cr-labeled BAFF-R–positive lymphoma and leukemia cell lines, BAFF-R–expressing L cells, or control BAFF-R–negative parental L cells were incubated with either CD4 or CD8 CAR T cells, as indicated at an E:T ratio of 3:1. B-CLL, B cell CLL. (C) Lymphomas from the same healthy donors were used as allogeneic controls (non-CAR). Data are shown as the means ± SD of one CAR T cell donor against triplicate tumors samples. All experiments were repeated with at least three different CAR T cell donors. **P < 0.001 versus corresponding non-CAR control with the following tests: (A) one-way ANOVA and Dunnett's multiple comparisons test; (B) two-way ANOVA and Dunnett's multiple comparisons test; and (C) two-way ANOVA and Tukey's multiple comparisons test.
potential explanation for the difference between BAFF-R-CAR and CD19-CAR T cell efficacy, we characterized respective surface antigen density on Raji and several other lymphoma and leukemia lines. Unexpectedly, BAFF-R surface antigen density was significantly lower than that of CD19 on all cell lines ($P < 0.0001$ BAFF-R versus CD19; Fig. 3C).
Therapeutic effects of BAFF-R-CARs against human CD19-negative B cell tumor lines in vitro and in vivo

One strategy to overcome the problem of antigen loss tumor escape variants emerging after successful CD19-targeted therapies is to target alternative cell surface molecules, such as BAFF-R. We modeled disease relapse due to the loss of CD19 by generating CRISPR CD19 gene knockout (KO) of multiple human B cell tumor lines, including MCL (Z-138), chronic lymphocytic leukemia (CLL) (MEC-1), and acute lymphoblastic leukemia (ALL) (Nalm-6), and a gRNA-silenced CD19 gene knockdown (KD) of an ALL primary patient–derived xenograft (PDX) (21–23) (Fig. 4, A and B, and fig. S5). CD19 expression on all resulting cell lines was absent by surface staining, whereas BAFF-R expression was not affected, as expected. We then tested CD8 Tn−-derived BAFF-R-CAR or CD19-CAR T cells for cytotoxicity against both wild-type (WT) and CD19-negative tumor cells in vitro. CD19-CAR T cells demonstrated cytotoxicity only against WT tumor cells, whereas BAFF-R-CAR T cells maintained cytotoxicity against both WT and CD19-negative tumors.
The therapeutic efficacy of BAFF-R-CAR T cells was tested against human MCL Z-138–CD19-deficient xenografts established in nonobese diabetic scid gamma (NSG) mice (Fig. 4C). A single dose of a defined mixture of TN CD4 and CD8 BAFF-R-CAR T cells infused on day 11 completely eliminated established tumors. In contrast, treatment with identical mixtures of CD19-CAR T cells or nontransduced T cells from the same donor were used as allogeneic controls (non-CAR). Similar results were also observed against ALL Nalm-6–CD19–KO xenografts in vivo (Fig. 4D).

We next challenged NSG mice with Z-138–CD19–deficient cells spiked into a background of WT Z-138 cells to determine whether CAR T cells could prevent the emergence of a preexisting CD19-negative tumor population. NSG mice were challenged with a mixture of $5 \times 10^4$ luciferase-expressing Z-138 (WT) plus $5 \times 10^4$ Z-138–CD19–KO tumor cells. Groups of five tumor-bearing mice each were then randomly assigned to treatment with either $2.5 \times 10^6$ CD4 TN CAR T + $10^6$ CD8 TN BAFF-R- or CD19-CAR T cells per mouse intravenously on day 8, as a single dose. Nontransduced CD4/CD8 T cells from the same donor were used as allogeneic controls (non-CAR).

**BAFF-R–specific CAR T cell activation by CD19 antigen loss primary human tumors**

Last, we tested BAFF-R-CAR T cells against a panel of cryopreserved primary CD19-negative tumor samples obtained from four patients at the time of relapse after CD19-targeted therapy [CD19/CD3 bispecific T cell engager antibody, BiTE, blinatumomab (24)]. Cell surface staining demonstrated CD19 and BAFF-R expression by corresponding tumors obtained before CD19-targeted therapy. However, posttreatment samples exhibited clear down-regulation of CD19 while retaining positive BAFF-R expression (Fig. 6A). After depletion of patient T cells from tumor samples, specific effector function of either CD19-CAR or BAFF-R-CAR T cells against paired pre- and post-CD19 BiTE therapy primary tumor cells was first determined by the expression of degranulation marker CD107a on CAR T cells. Activation of CD19-CAR T cells by all four CD19-negative posttherapy tumors was substantially reduced, compared with BAFF-R-CAR T cells and with corresponding available CD19-positive pretherapy tumors, whereas BAFF-R-CAR T cells were equally activated by pre– and post–CD19-targeted therapy tumors (Fig. 6B).

A fifth B cell ALL (B-ALL) patient sample obtained at relapse was used for in vivo PDX engraftment. These B-ALL blasts were CD19 negative and BAFF-R positive (Fig. 6C). B-ALL blasts were successfully engrafted, and by 26 days, peripheral blasts reached 1 to 5% of total cells (human/mouse; Fig. 6D). Mice were randomly assigned to treatment...
with BAFF-R-CAR or CD19-CAR T cells (5 × 10^6, 1:1 CD4:CD8 T_N ratio/mouse) on day 26. Nontransduced CD4/CD8 T cells from the same donor were used as allogeneic controls (non-CAR). Analysis on day 54 revealed significantly fewer circulating tumor cells in BAFF-R-CAR T cell–treated mice, compared with CD19-CAR T cell– or control-treated mice (P < 0.0001). BAFF-R-CAR T cell–treated mice also demonstrated significantly prolonged survival compared with all the other groups (P < 0.01; Fig. 6E).

**DISCUSSION**

BAFF-R is a highly expressed B cell lineage surface marker in various B cell malignancies, making it an appealing target for immunotherapy. Mechanistically, BAFF-R signaling activates nuclear factor κB pathways to promote tumor survival and proliferation (25, 26), and increased BAFF-R expression correlated with disease progression in patients with B cell lymphoma and pre–B-ALL (27–29). Furthermore, mouse strains expressing a mutant BAFF-R exhibit decreased B cell life...
spans (30), associated with a substantially reduced peripheral B cell compartment, and BAFF-R-null mice exhibit greatly reduced B cell numbers and are essentially devoid of marginal zone B cells (8, 31). Collectively, these reports suggest that BAFF-R signaling is a driver of B cell growth and survival. This critical feature may also limit the capacity of B cell tumors to escape therapy by down-regulation of BAFF-R expression (10, 32–34).

The BAFF/BAFF-R axis has been targeted successfully for autoimmune diseases, particularly with monoclonal antibodies (mAbs) against the BAFF ligand (35, 36); however, the promise for cancer therapy has not yet been realized. Most previously described mAbs against the receptor (37) failed to demonstrate efficacy against human B cell tumors (11, 12). More recently, targeting BAFF-R with mAbs showed efficacy against CLL in preclinical models, particularly when combined with a Bruton tyrosine kinase inhibitor (38), and this mAb has entered clinical trials (NCT03400176). A preliminary report targeting BAFF-R using a CAR platform was limited to in vitro studies of ALL (39).

Although CD19-CAR T cell therapy is effective in many patients with B-ALL or lymphoma, CD19-negative tumor cells are observed in 30% of post–CD19-directed BiTE or CAR T-cell therapy relapses, underscoring the urgent need to exploit alternative targets (6, 40). CAR T cells targeting the B lineage marker CD22 have been proposed as one alternative strategy to overcome relapse from CD19 antigen loss, as demonstrated by achievement of durable clinical responses in patients with CD19-negative B-ALL (41–43). This clinical trial also demonstrated comparable potency of CD22-CAR T cells to that of CD19-CAR T cells at biologically active doses. However, relapses were associated with diminished CD22 site density that likely permitted CD22-negative cells to escape killing by CD22-CAR T cells.

We modeled CD19 antigen loss by CD19 gene KO of multiple B-ALL and lymphoma cell lines and observed retention of susceptibility to cytotoxicity in vitro, and tumor eradication in vivo by BAFF-R-CAR but not CD19-CAR T cells. These in vitro findings were also verified in a primary PDX ALL tumor in which KD of CD19 expression had been achieved. We further modeled one mechanism of CD19-negative escape by demonstrating that BAFF-R-CAR, but not CD19-CAR, T cells could prevent the emergence of CD19-deficient tumor cells when spiked into a background of WT tumor in vivo. Last, using four primary CD19-negative ALL tumor escape variants emerging naturally from patients treated with prior CD19-directed therapies, we demonstrated retention of BAFF-R expression, specific activation of BAFF-R-CAR, but not CD19-CAR, T cells in vitro, and antitumor effects and prolonged survival specifically associated with BAFF-R-CAR T cell treatment against one additional primary PDX in vivo. Together, the findings suggest the potential effectiveness of BAFF-R-CAR T cells in the setting of CD19 antigen loss. Under the specific experimental conditions selected, the therapeutic effects of our BAFF-R-CAR T cells exceeded that of CD19-CAR T cells produced using the same second-generation CAR backbone and administered at identical doses, particularly in vivo. The specific reasons for this therapeutic discrepancy between BAFF-R-CAR and CD19-CAR T cells remain to be elucidated by additional investigation.

To our knowledge, given that there is no existing model of spontaneous CD19-negative antigen loss due to immunologic pressure of CD19-targeted therapy against human tumor xenografts in immunocompromised mice, the use of BAFF-R-CAR T cells to demonstrate true rescue of tumor escape variants must await the development of suitable models. Another potential limitation of our study is that although the critical role of BAFF-R signaling on normal B cell survival strongly suggests that antigen loss emerging from BAFF-R targeting is not likely, the definitive test of this potential mechanism of resistance to BAFF-R-CAR T cell therapy must await human trials, which are planned. As with most preclinical CAR T cell studies, recipient mice were immunodeficient, so side effects in an intact immune environment would not be detected.

Together, our data suggest that further development of BAFF-R-directed adoptive T cell therapies is warranted for B cell malignancies. Future strategies combining dual targeting of CD19 and BAFF-R may also be warranted.

**MATERIALS AND METHODS**

**Study design**

The overall objective of this study was to demonstrate that BAFF-R is a suitable target for CAR T cell therapy against CD19 antigen loss disease. A novel BAFF-R-CAR T cell was developed to test this hypothesis in vitro and in vivo. The in vitro experiments consisted of cell surface staining characterization of both target and CAR T cells and functional cytotoxic T lymphocyte (chromium release) assays. All in vitro assays were performed with at least triplicate samples. Because of the limited availability of primary patient samples, replicate testing was not always possible. In vivo studies were performed with n = 5 mice per group of 8- to 12-week-old mice. Experiments were repeated at least three times. On the basis of the previous studies to establish the tumor models, the survival data in two-sided log-rank tests have at least 80% power at an overall 0.05 significance level to detect a hazard ratio of 0.15. Tumor-challenged mice were randomized into treatment groups. Treatments were not administered blinded; however, mice were monitored for signs of distress and humane endpoints in a blinded manner. Veterinary staff, independent of the researchers and studies, monitored mice daily and alerted researchers when a humane endpoint had been reached. All studies were performed under approved Institutional Animal Care and Use Committees (IACUC) and institutional review board (IRB) protocols. Primary data are reported in data file S1.

**Animals**

NSG mouse breeding pairs were purchased from The Jackson Laboratory (stock no. 005557). The NSG breeding colony was maintained by the Animal Resource Center at the City of Hope. Mice were housed in a pathogen-free animal facility according to institutional guidelines. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC: 15020). NSG mice used in the in vivo PDX model were bred within the Sandra and Edward Meyer Cancer Center PDTX Animal Core under strict specific and opportunistic pathogen-free conditions (protocol number 2014-0024).

**Cell lines**

Malignant human hematologic cell lines including JeKo-1, Raji, OCI-LY10, RL, R5411, MEC-1, and Nalm-6 were purchased from either the American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Mouse fibroblast L cells, HT1080 human epithelial cells, and 293FT cells were obtained from the ATCC. The Z-138 cell line was provided by M. Wang (MD Anderson Cancer Center). Banks of all cell lines...
were authenticated for the desired antigen/marker expression by flow cytometry before cryopreservation, and thawed cells were cultured for less than 6 months before use in assays.

**Human blood and primary tumor samples**
Noncultured, primary human lymphomas were obtained as cryopreserved, viable single-cell suspensions in 10% dimethyl sulfoxide from the Lymphoma Satellite Tissue Bank at MD Anderson Cancer Center under an IRB-approved protocol (IRB: 2005-0656). Primary patient samples included peripheral blood mononuclear cells (PBMCs) from leukapheresis or blood from patients with MCL, and excised lymph nodes from patients with diffuse large B cell lymphoma (DLBCL) or follicular lymphoma (FL). Tumor cells in each sample ranged from 80 to 98% for leukapheresis or blood, and from 50 to 60% for lymph node biopsies. Primary human ALL samples were obtained from the Pathology: Liquid Tumor Core tissue bank at the City of Hope (IRB: 03162). PBMCs from healthy donors were provided by the Michael Amini Transfusion Medicine Center at the City of Hope (IRB: 15283).

**CAR T cell production**
A second-generation BAFF-R-CAR was generated consisting of the humanized H90 BAFF-R antibody scFv (13), CD8 transmembrane, 4-1BB, and CD3ζ intracellular signaling domains. The CAR cDNA was cloned into pLenti7.3 lentiviral vector (pLenti7.3/V5-DEST Gateway Vector Kit, Invitrogen). CD19-CAR was generated in the same way, replacing BAFF-R antibody scFv with CD19 antibody scFv (20). Lentiviruses were produced in 293FT cells, concentrated, and titered with HT1080 human epithelial cells.

CD4 and CD8 T\(N\) from the same healthy donor were isolated with Human Naïve CD4\(^+\) or CD8\(^+\) T Cell Isolation Kits, respectively (Stemcell Technologies). To isolate CD8 T\(SCM\), CD8 T cells prepared with the EasySep Human CD8\(^+\) T Cell Enrichment Kit (Stemcell Technologies) were stained with CD8 PerCP Cy5.5, CD45RO APC, and CD62L phycoerythrin (PE) and sorted for CD8\(^+\)/CD45RO\(^-\)/CD62L\(^+\) T\(SCM\). CD8 T\(SCM\)-like cells were generated by CD8 T\(N\) cultured in AIM-V medium supplemented with 5% human serum, interleukin-7 (IL-7) (5 ng/ml), and IL-21 (30 ng/ml). TWS119 (5 mM; GSK3 inhibitor) was supplemented to inhibit differentiation and retain the T\(SCM\)-like state. All T cells were activated with Human T-Activator CD3/CD28 beads (Life Technologies) for 24 hours followed by transduction with lentivirus encoding CAR/green fluorescent protein (GFP) at multiplicity of infection = 1. After 7 days in culture, GFP-positive CAR T cells were enriched by fluorescence-activated cell sorting (FACS) and further activated and expanded with CD3/CD28 bead stimulation for another 7 days. Only productions that yielded ≥95% GFP-positive CAR T cells were used for further studies.

**Cytokine production assay**
CAR T cells were coincubated for 24 hours with BAFF-R expressing target cells (tumor lines) at an effector-to-target (E:T) ratio of 2:1. Controls included BAFF-R-negative L cells, BAFF-R-positive B2D cells, CD3/CD28 beads (10 µl/10\(^6\) CAR T cells), and nontransduced T cells from the same donor. Supernatant was collected for enzyme-linked immunosorbent assay (ELISA) detection of cytokines (Human IFN-γ ELISA Set and Human TNF ELISA Set, BD Biosciences) and granule release (Human Granzyme B DuoSet ELISA Kit, R&D).

**Cytotoxic T lymphocyte assay**
A standard chromium-51 (\(^{51}\)Cr) release assay was used to calculate specific lysis by CAR T cells. Briefly, target cells (tumor cell lines or primary patient tumors) were radiolabeled with \(^{51}\)Cr (PerkinElmer). CAR T cells were coincubated with labeled target cells at E:T ratios ranging from 1:1 to 10:1 for 4 hours. Controls included nontransduced T cells from the same donor. Clarified supernatant was sampled for \(^{51}\)Cr detection in a Wizard Automatic Gamma Counter (PerkinElmer). Percent lysis was calculated by Specific Lysis (%)= \(\frac{\text{CPM}_{\text{SR}} - \text{CPM}_{\text{MR}}}{\text{CPM}_{\text{SR}} - \text{CPM}_{\text{MR}}} \times 100\%\), where CPM is the count per minute, SR is the CPM of spontaneous release, and MR is the CPM of maximum release. Individual experiments represent the means ± SD of triplicate samples from a single T cell donor. A paired Student’s t test was performed comparing experimental conditions with corresponding controls.

**Generation of KO cell lines**
FACS-sorted, stable Z-138–CD19–KO and Nalm-6–CD19–KO were generated using CD19-CRISPR-Cas9 and red fluorescent protein (RFP) reporter gene containing homologous directed repair (HDR) Plasmid Systems (Santa Cruz Biotechnology) according to the manufacturer’s directions. Briefly, DNA-In Transfection Reagent (MTI-GlobalStem) was used to transfect the plasmid system. RFP-positive cells were sorted by FACS and expanded. A stable CD19-deficient clone was verified by flow cytometry and Western blots for CD19 KO before banking for subsequent studies.

**CRISPR-mediated gene editing and CD19 gRNA cloning**
Patient-derived, pre–B-ALL cells (LAX7D, Müschen Lab) were transduced with lentivirus expressing Lenti-dCas9-KRAB-blast (Addgene, plasmid #89567). Blasticidin-resistant cells were subsequently transduced with lentivirus expressing gRNAs against human CD19 in a puromycin-RFP vector backbone. gRNA sequences against human CD19 were selected from the hCRISPRi-v2 library and purchased from Integrated DNA Technologies Inc. Sequences were cloned into plasmids and verified by Sanger sequencing before lentivirus production. Nontargeting gRNA was used as control. Ten days after CD19-gRNA transduction, cells were sorted on the basis of RFP using BD Fusion.

**In vivo tumor modeling**

**Tumor models**
Stable, luciferase-expressing tumor lines were established for bioluminescence imaging in mouse models. Briefly, a luciferase gene was introduced into tumor lines by a lentivirus gene delivery system (pLenti7.3/V5-DEST Gateway Vector Kit, Invitrogen). The minimum lethal dose was determined for each tumor cell line by dose titration [1.5 × 10\(^6\) JeKo-1, 1 days; 0.5 × 10\(^6\) Raji, 7 days; 5 × 10\(^4\) Z-138–CD19–KO, 11 days; 5 × 10\(^4\) Z-138–WT + 5 × 10\(^4\) Z-138–CD19–KO (1:1 mixture), 8 days; and 0.2 × 10\(^6\) Nalm-6–CD19–KO, 10 days]. Tumor cells were injected intravenously into 6- to 8-week-old NSG mice, and tumor development was monitored daily by in vivo bioluminescence imaging.

**Bioluminescence imaging**
Mice were anesthetized with isoflurane and administered d-luciferin (150 mg/kg; Life Technologies) via intraperitoneal injection 10 min before imaging. Imaging was performed on an AmiX imaging system (Spectral Instruments Imaging).
In vivo CAR T cell therapy
Eight- to twelve-week-old mice (n = 5 per group) were challenged with the minimum lethal dose of tumor cells administered intravenously and then treated with CAR T cells once tumor engraftment was confirmed via imaging. Treatments consisted of a single 300-μl intravenous injection with defined populations of CD4 and CD8 CAR T cells. Total CAR-positive cells infused ranged from 2 × 10^6 to 5 × 10^6 cells at a CD4:CD8 ratio of 1:1 to 2.5:1. Controls included nontransduced T cells (non-CAR) from the same donor and PBS. Imaging was performed weekly up to 80 days. Survival data are reported in Kaplan-Meier plots and analyzed by log-rank tests.

Postmortem tumor analysis
Spleens from mice challenged with Z-138–WT + Z-138–CD19–KO mixture (Fig. 3D) were harvested immediately postmortem. Spleens were prepared into a single-cell suspension by mechanical disruption and stained for human tumor cells with FACS antibodies for human CD45 + TdT + C-ALL, negative B-ALL leukemia (identified by human CD45 + CD19 + CD79a + CD22 -) were harvested immediately postmortem. Spleens | (Fig. S5). CD19-KO clone selection.

Primary sample immunophenotyping
T cells were removed from thawed primary patient samples with the Human CD3 Positive Selection Kit (Stemcell Technologies). Remaining cells were stained with CD20 fluorescein isothiocyanate, CD10 PE Cy7, CD22 PerCP Cy5.5, CD19 PE, and BAFF-R Alexa 647 antibodies (BD Biosciences). The average percentages of CD19-positive and CD19-negative expression in tumor cells were calculated.

Degranulation assay
T cell–depleted, primary patient samples (1 × 10^6 cells) were cocultured with 2 × 10^5 CAR T cells at an E:T ratio of 2:1 in complete media. Degranulation assay analyzed with the FlowJo software.

Intracellular cytokine production
A total of 2 × 10^5 CAR T cells were cocultured overnight with 1 × 10^5 T cell–depleted patient sample as blast cells in 96-well tissue culture plates in the presence of brefeldin A (BD Biosciences). The cell mixture was then stained using anti-CD8, anti-CD4, and biotinylated erbitux/streptavidin to analyze surface coexpression of CD8, CD4, and CAR, respectively. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences). After fixation, the T cells were stained with antibodies against IFN-γ and TNF-α. Cells were then analyzed using multicolor flow cytometry on MACSQuant and FCS Express software (Miltenyi Biotec Inc.). Nontransduced T cells from the same donors (non-CAR) were used as negative effector cells.

In vivo PDX model
Leukemic cells (1 × 10^6) isolated from a patient relapsing with CD19-negative B-ALL leukemia (identified by human CD45 + TdT + CD79a - CD22-) were injected into the tail vein of 6- to 8-week-old NSG mice. Successful engraftment was confirmed when the percentage of peripheral blood circulating B-ALL reached 1 to 5% of the total (human/mouse) cells. Mice received a single infusion of 5 × 10^6 CAR T cells (BAFF-R-CAR or CD19-CAR, CD4:CD8 ratio 1:1) by tail vein injection. Controls included nontransduced T cells (non-CAR) from the same donor and PBS. CAR T and leukemia cells were monitored by blood collections and FACS analysis for human CD45 + CD3 + CD4 + CD8 + and CD45 + CD22 + CD58-, respectively. Circulating murine cells were determined using anti-murine CD45 antibody (PE-Cy7–A). Data are reported as means ± SD and analyzed by a Student’s t test. Survival data are reported in a Kaplan-Meier plot analyzed by log-rank test.

Statistical analysis
All statistical analyses were performed with the GraphPad Prism 8 software. Depending on the experiment design, the following methods were used to calculate significance: Student’s t test, one-way analysis of variance (ANOVA), two-way ANOVA, two-way repeated-measures ANOVA, and log-rank tests. ANOVA tests were followed by an appropriate Tukey’s, Dunnett’s, or Sidák’s post hoc test for multiple comparisons between experimental groups. All testing was two sided with a testing level (α) of 0.05. Unless otherwise stated, data are presented as means ± SD, with n = 3 or 4 replicates.

REFERENCES AND NOTES

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CAR T cells targeting BAFF-R can overcome CD19 antigen loss in B cell malignancies

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Circumventing CD19 antigen loss

Chimeric antigen receptor (CAR) T cell treatment for B cell malignancies was pioneered with CD19-targeted CAR T cells. Despite robust clinical responses, relapse due to CD19 antigen loss is common. Qin et al. examined B cell activating factor receptor (BAFF-R) as an alternate CAR T cell target. BAFF-R-targeted CAR T cells could kill multiple human lymphoma and leukemia cell lines, either in vitro or in mice, as well as patient-derived samples. The BAFF-R-CAR T cells could also eradicate tumors lacking CD19. Their compelling preclinical results support the clinical development of BAFF-R-targeted CAR T cells for combating B cell malignancies.