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Human CD19-specific switchable CAR T-cells are efficacious as constitutively active CAR T-cells but cause less morbidity in a mouse model of human CD19$^+$ malignancy

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ABSTRACT

Current Food and Drug Administration (FDA)-approved CD19-specific chimeric antigen receptor (CAR) T-cell therapies for B-cell malignancies are constitutively active and while efficacious, can cause morbidity and mortality. Their toxicities might be reduced if CAR T-cell activity was regulatable rather than constitutive. To test this, we compared the efficacies and morbidities of constitutively active (conventional) and regulatable (switchable) CAR (sCAR) T-cells specific for human CD19 (huCD19) in an immune-competent huCD19$^+$ transgenic mouse model. Conventional CAR (CAR19) and sCAR T-cells were generated by retrovirally transducing C57BL/6 (B6) congenic T-cells with constructs encoding antibody-derived single chain Fv (sFv) fragments specific for huCD19 or a peptide neoepitope (PNE), respectively. Transduced T-cells were adoptively transferred into huCD19 transgenic hemizygous (*huCD19*$^{Tg/0}$) B6 mice; healthy B-cells in these mice expressed *huCD19*^{Tg}. Prior to transfer, recipients were treated with a lymphodepleting dose of cyclophosphamide to enhance T-cell engraftment. In tumor therapy experiments, CAR19 or sCAR T-cells were adoptively transferred into *huCD19*$^{Tg/0}$ mice bearing a syngeneic B-cell lymphoma engineered to express huCD19. To regulate sCAR T cell function, a switch protein was generated that contained the sCAR-specific PNE genetically fused to an anti-huCD19 Fab fragment. Recipients of sCAR T-cells were injected with the switch to link sCAR effector with huCD19$^+$ target cells. Mice were monitored for survival, tumor burden (where appropriate), morbidity (as measured by weight loss and clinical scores), and peripheral blood lymphocyte frequency.

CAR19 and sCAR T-cells functioned comparably regarding in vivo expansion and B-cell depletion. However, sCAR T-cells were better tolerated as evidenced by the recipients' enhanced survival, reduced weight loss, and improved clinical scores. Discontinuing switch administration allowed healthy B-cell frequencies to return to pretreatment levels.

In our mouse model, sCAR T-cells killed huCD19$^+$ healthy and malignant B-cells and were better tolerated than CAR19 cells. Our data suggest sCAR might be clinically superior to the current FDA-approved therapies for B-cell lymphomas due to the reduced acute and chronic morbidities and mortality, lower incidence and severity of side effects, and B-cell reconstitution on cessation of switch administration.

INTRODUCTION

Chimeric antigen receptors (CARs) bind defined antigens (Ags) and elicit effector functions in engineered immune cells.[1–3] Ideal targets for CAR cancer immunotherapy are neo-Ags encoded by tumor-associated mutations absent on healthy cells. Unfortunately, few appropriate neo-Ags have been identified.[4] Instead, the best clinical success to date has come from CAR T-cells specific for cell-lineage Ags expressed by malignant cells, such as the pan-B-cell differentiation Ag CD19.[5]

In clinical trials of United States Food and Drug Administration (FDA)-approved human CD19 (huCD19)-specific CAR T-cell products, durable remission rates ranged from 40% to 90% for relapsed/refractory B-cell malignancies.[6–10] These successes, though, are often tempered by significant acute and chronic morbidities, and sometimes mortalities.[6–10] Acute side effects include cytokine release syndrome (CRS), hemophagocytosis histiocytic-like manifestations, and immune effector cell-associated neurotoxicity syndrome; chronic morbidities include hypogammaglobulinemia due to the on target/off tumor killing of healthy B-cells. These toxicities may relate to the inability to control constitutive CAR T-cell activation and expansion in vivo.

To address this, we created monoclonal antibody (mAb)-derived switch proteins to

regulate switchable CAR (sCAR) T- cells.[11–14] Switches are Ag-specific mAb fragments genetically linked to a peptide neoepitope (PNE) that connect effector sCAR and Ag$^+$ target cells. Because the sCAR binds only the PNE, sCAR T-cell activation and function are strictly switch-dependent. This orthogonal approach combines the potency of CAR T-cells with the titratability of mAbs as a means of potentially reducing toxicity by controlling sCAR T-cell activation.

Here, we compare huCD19-specific sCAR and conventional CAR (CAR19) T-cell function in a mouse model.[15] Our model has three components: (1) C57BL/6 (B6) mice whose healthy B-cells express a hemizygous huCD19 transgene (*huCD19$^{Tg/0}$*); (2) a B6-derived B-cell lymphoma engineered to express huCD19; and 3) congenic B6 T-cells retrovirally transduced with huCD19-specific constitutive or sCARs. We now report that huCD19-specific sCAR T-cells cure mice of a lethal B-cell tumor burden and are better tolerated than CAR19 cells. While CAR19 cells can cause significant morbidity and mortality, sCAR cells at the same T-cell dose do not. Moreover, discontinuing switch administration in sCAR recipients reverses B-cell aplasia. These data suggest sCAR could be clinically superior to the current FDA-approved therapies for B-cell lymphomas.

METHODS

Mice

B6 and B6.SJL-Ptprc^{a}Pepc^b/BoyJ (CD45.1) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The *huCD19$^{Tg/Tg}$* B6 mouse line TG-1 was described elsewhere.[16] We bred TG-1 and B6 mice to generate *huCD19$^{Tg/0}$* hemizygotes. We use hemizygotes because *huCD19^{Tg}* expression decreases B-cell frequency in a gene dose-dependent manner; peripheral blood B-cell frequencies in *huCD19$^{Tg/0}$* and *huCD19$^{Tg/Tg}$* mice are 50% and 25% those of B6 mice, respectively. Additionally, huCD19^{Tg} expression exceeds that of endogenous huCD19 observed in healthy B-cells in normal human volunteers.[15 16]

Tumor model

huCD19$^{Tg/0}$ mice were injected intraperitoneally (ip) with 1E+06 TBL12.huCD19 lymphoma cells 8 days before adoptive transfer of CAR19 or sCAR T-cells. TBL12.huCD19 cells express huCD19, green fluorescent protein, and luciferase and were generated by retroviral transduction of TBL12, a Burkitt's-like lymphoma triply transgenic for E-mu-MYC, hen egg lysozyme, and hen egg lysozyme-specific B cell receptor.[15 17]

Retroviral constructs and switch protein

The MP71 retroviral *CAR19* and *sCAR* constructs differ only by their Ag-specific single chain Fv (sFv) fragments (figure 1A).[11 15] *CAR19* encodes a sFv derived from the huCD19-specific mAb FMC63 while the *sCAR*-encoded

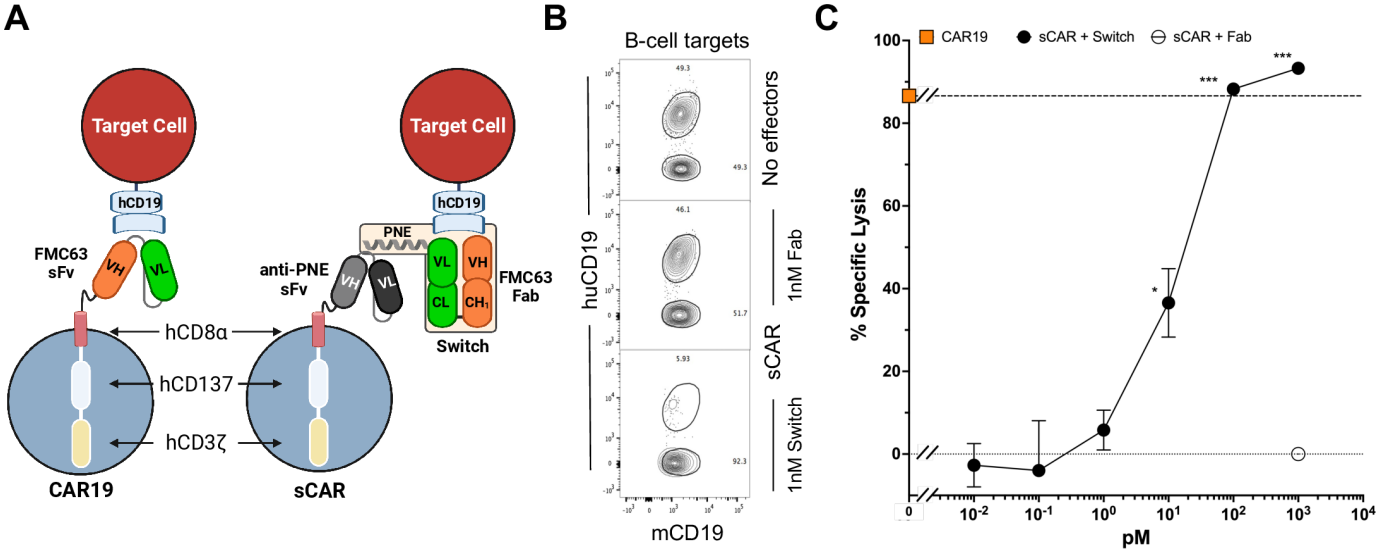

Figure 1 Switch requirement for huCD19-specific sCAR T-cell cytotoxicity. (A) CAR19 and sCar T-cells bind huCD19$^+$ cells directly and indirectly, respectively. (B) representative flow cytometry contour plots showing splenic B-cell (CD45R$^+$) target cell frequencies used to calculate % specific lysis in vitro. Ag negative (huCD19$^-$) and Ag positive (*huCD19$^{Tg/0}$*) B-cells were combined (50,000 each) and incubated for 21 hours with no effectors (top), or sCar effector T-cells (250,000) plus 1 nM Fab (middle) or switch (bottom). (C) The ratios of huCD19$^-$ to huCD19$^+$ targets for each sample (n=3) were calculated. Specific lysis (%) is defined as (1 – [control ratio/experimental ratio] × 100) where the control ratio equals (huCD19$^-$/huCD19$^+$) target cells with no effectors. The data are the mean±SEM and represent three independent experiments. The upper dotted line shows the % specific target lysis mediated by CAR19 cells (orange square) at 5:1 E:T. The lower dotted line shows the % specific lysis of sCAR T-cells (open circle) with 1 nM Fab control. *p≤0.05; ***p≤0.001 (two-tailed unpaired t-test with Welch's correction). E:T, effector:target; sCAR, switchable chimeric antigen receptor.

sFv binds a PNE derived from the yeast GCN4 protein.[11–14] Both CAR constructs are followed by a gene encoding the T2A peptide to permit translation of a separate, non-functional, truncated human epidermal growth factor receptor (hEGFR) reporter.[18] The switch contains the PNE genetically linked to the FMC63 Fab light chain.[11 12]

In vitro cytotoxicity

Splenic B-cells from wildtype (huCD19$^-$) and *huCD19$^{Tg/0}$* (huCD19$^+$) B6 mice were negatively enriched to ≈95% purity. A total of 50 000 cells of each were combined and added to 250 000 effector T-cells to yield a 5:1 effector:target ratio. PNE-FMC63 Fab (switch) or FMC63 Fab-only (negative control) proteins were added as indicated. After 21 hours, B-cells were analyzed by flow cytometry by gating on CD45R (B220), huCD19, and mouse CD19 (mCD19) expression. The ratios of huCD19$^-$ (B6) to huCD19$^+$ (*huCD19$^{Tg/0}$*) mCD19$^+$CD45R$^+$ B-cells (n=3) were calculated. Specific lysis (%) was defined as (1 – [control ratio/experimental ratio] × 100) where the control ratio equals target cells with no effectors.

Adoptive cell transfer

CD45.1 splenic naïve T-cells were negatively enriched and retrovirally transduced as described.[15] Transduction efficiencies were determined with the anti-hEGFR mAb AY13 (BioLegend, San Diego, CA).[15 18] Transduction efficiencies ranged from 22% to 79% with a median of 41%. Transduced cells were not enriched prior to use but were normalized for transduction efficiencies to model clinical use. Accordingly, mice in different experiments receiving the same number of transduced cells typically received different numbers of total cells due to variability in transduction efficiencies. The day before adoptive cell transfer (ACT), recipients were injected ip with 300 mg/kg body weight of cyclophosphamide (CY; Sigma Aldrich, St. Louis, Missouri, USA) in phosphate buffered saline (PBS) to improve CAR T-cell engraftment.[6–10] The next day (day 0) 3E+06 transduced T-cells were injected intravenously. Switches were injected iv at 1 or 5 mg/kg every day or every other day eight times per non-tumor-bearing recipient starting on day 0 and ending on day 7 or 14, respectively. Tumor-bearing recipients were injected with vehicle or switch at 0.5 mg/kg every other day a total of eight times from days 0 to 14.

In vivo readouts

Equal numbers of male and female *huCD19$^{Tg/0}$* mice (2–4 months of age) were randomized into groups (n=5/group) for all experiments. Tumor growth was measured by bioluminescence using an IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, Massachusetts, USA) after ip injection of 3 mg D-luciferin potassium salt (Gold Biotechnology, St. Louis, Missouri, USA) in PBS. Weights were plotted as percentages of pretreatment weights. Appearance and activity were recorded as one clinical score ranging from 0 (healthy) to 8 (moribund).[15 19] For peripheral blood analyses, blood was incubated with fluorophore-conjugated mAbs, erythrocytes lysed, and flow cytometry data were acquired.[15] Data were analyzed using FlowJo X 10.8.1 software (FlowJo, Ashland, Oregon, USA).

Statistical analyses

All in vivo experiments were replicated at least twice. Prism V.9.4.0 (GraphPad Software, CA) was used for the statistical tests.

RESULTS

sCAR T-cell cytotoxicity is Ag- and switch-dependent

CAR19 and sCAR T-cells bind huCD19$^+$ cells directly and indirectly, respectively (figure 1A). The CAR19 sFv binds huCD19 while the sCAR sFv binds a yeast-derived PNE. Coupling the PNE to the anti-huCD19 FMC63 Fab creates a switch that links sCAR and huCD19$^+$ target cells.[11 12] The sCAR system is orthogonal because the sCAR sFv does not cross-react with any known mammalian protein; it is modular because the PNE can be affixed to any mAb fragment to form an Ag-specific switch; and it is regulatable because the switch can be titrated or removed.[11 12]

An in vitro cytotoxicity assay illustrates these characteristics. Equal numbers of huCD19$^+$ and huCD19$^-$ targets (*huCD19$^{Tg/0}$* and B6 B-cells, respectively) were added to T-cell effectors and 21 hours later, target cell frequencies were determined. Specificity was evidenced by the killing of only *huCD19$^{Tg/0}$*, and not B6 B-cells, by sCAR T-cells plus switch (figure 1B). The orthogonal and titratable features of the sCAR system were evidenced by the requirement of ≥10 pM switch for significant, specific cytotoxicity; sCAR T-cells with ≥100 pM switch were equivalently cytotoxic to CAR19 cells (figure 1C).

sCAR T-cells cause less morbidity

Morbidity and mortality caused by CAR19 cells in *huCD19$^{Tg/0}$* recipients are cell-dose dependent.[15] 1E+06 CAR19 cells eradicate tumor but with less morbidity and mortality than 3E+06 CAR19 cells, which are uniformly fatal. While the exact cause of death remains unclear, we suspect it involves IL-6 driven CRS. Like clinical CAR T-cell-induced CRS, we showed that antibody-mediated neutralization of IL-6 signaling blunts morbidity and prevents mortality in CAR19 recipients.[15]

To test the hypothesis that sCAR cells are better tolerated than CAR19 cells, we adoptively transferred 3E+06 cells of each to separate cohorts. Within 5 days, *huCD19$^{Tg/0}$* recipients of CAR19 T-cells experienced significant peak weight loss of 22% from baseline (figure 2A) and uniform lethality occurred within 7 days (figure 2F). Significant weight loss occurred in sCAR recipients treated every day for 8 days with 5 mg/kg switch with a peak weight loss of 15% seen on day 5 (figure 2B); notably, all mice recovered (figure 2F). Significant peak weight losses of 12% and 15% occurred in sCAR recipients treated every other day with 1 or 5 mg/kg switch but they were delayed to day 6 (figure 2C); again, all mice recovered (figure 2F).

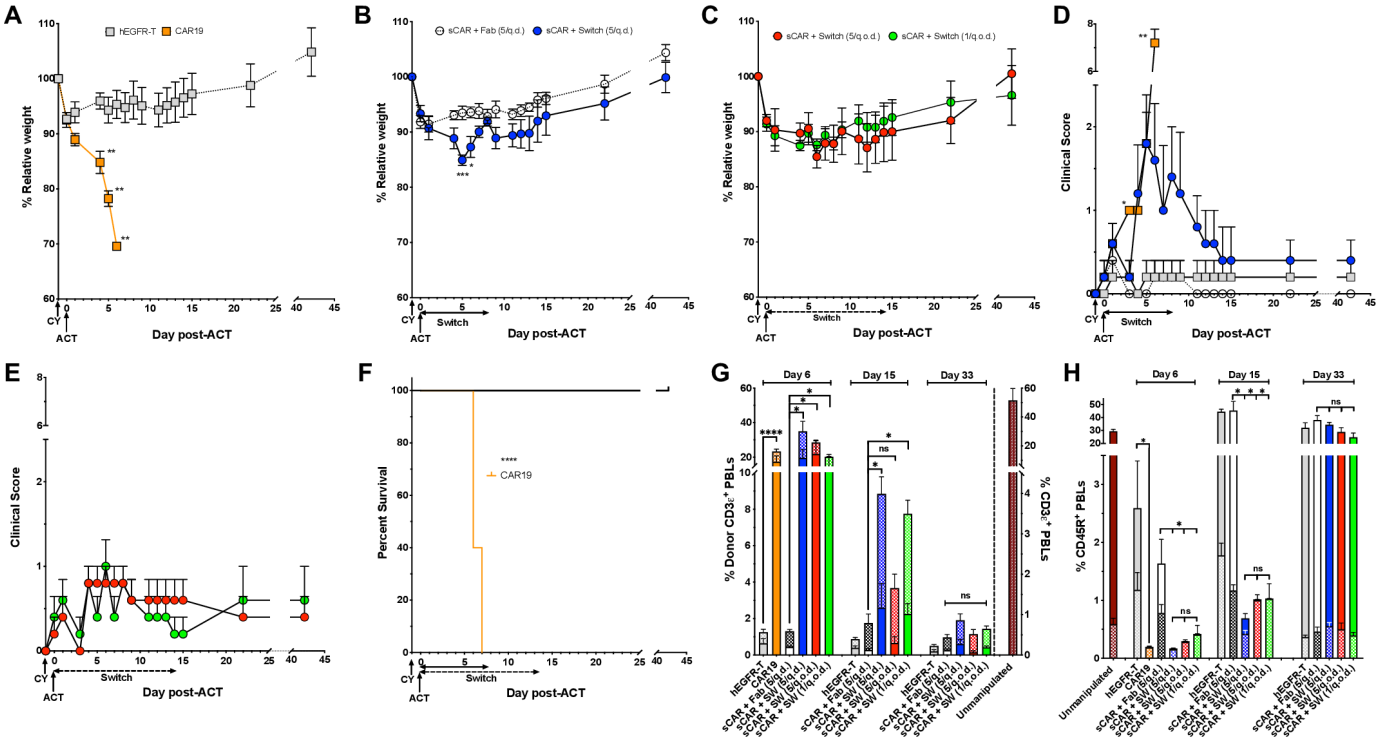

Figure 2 sCAR T-cells are less toxic than CAR19 cells in vivo despite comparable B-cell depletion and T-cell expansion. CY-lymphodepleted *huCD19*$^{Tg/0}$ mice received 3E+06 transduced T-cells (n=5/group). Select groups also received switch or Fab proteins at one or 5 mg/kg every day (solid horizontal arrow) or every other day (dotted horizontal arrow) for eight injections/mouse. The data are the mean±SEM and represent at least three experiments. (A–C) Weights were plotted as percentages of pretreatment weights. Mice were euthanized if their relative weights dropped below 70%. *p≤0.05, **p≤0.01, ***p≤0.001 (two-tailed unpaired t-test with Welch's correction). (D, E) Clinical scores of 0 and 8 indicate healthy and moribund mice, respectively. *p≤0.05, **p≤0.01; (two-tailed unpaired t-test with Welch's correction). (F) Kaplan-Meier survival curves. ****p≤0.0001 (Log-rank/Mantel-Cox). (G) Donor peripheral blood T-cell frequencies ([CD3e$^+$CD45.1$^+$]/CD45$^+$) were determined by flow cytometry. Hatched and filled bars represent untransduced (hEGFR$^-$) and transduced (hEGFR$^+$) donor T-cells, respectively. 'Unmanipulated' refers to the frequency of CD3e$^+$ (CD45.2 $^+$) PBLs in untreated, age-matched *huCD19*$^{Tg/0}$ mice. (H) Peripheral blood B-cell (CD45R$^+$/CD45$^+$) frequencies were determined by flow cytometry. Hatched and filled bars represent huCD19$^-$CD45R$^+$ and huCD19$^+$CD45R$^+$ B-cells, respectively. 'Unmanipulated' refers to the frequency of CD45R$^+$ cells in untreated, age-matched *huCD19*$^{Tg/0}$ mice. *p≤0.05, ****p≤0.0001; NS (not significant; p>0.05) (one-way ANOVA with Tukey's multiple comparison correction). ANOVA, analysis of variance; hEGFR, human epidermal growth factor receptor; sCAR, switchable chimeric antigen receptor.

Weight loss between sCAR recipients treated every other day with 1 or 5 mg/kg switch did not differ significantly on any given day (figure 2C).

Mice were monitored for appearance and activity (reported as clinical scores). Recipients of CAR19 or sCAR T-cells plus every day injections of 5 mg/kg switch had indistinguishably worsening clinical scores until day 5 (figure 2D). Despite continued switch injection, sCAR T-cell recipients then began to recover while the CAR19 recipients continued to decline. sCAR T-cell recipients treated with 1 or 5 mg/kg switch every other day fared slightly better than those treated with 5 mg/kg switch every day, but these improvements were not significantly different (figure 2E).

CAR19 and sCAR T-cell expansion and B-cell depletion in vivo

To determine if the reduced morbidity of sCAR recipients correlated with poor in vivo expansion or reduced on target/off tumor cytotoxicity, peripheral blood T-cell and B-cell frequencies were measured on days 6, 15, and 33 post-ACT. On day 6, CAR19 T-cells had expanded approximately 20-fold relative to controls, as had sCAR T-cells in mice receiving any switch dose (figure 2G). By day 15, the sCAR T-cell frequencies declined but remained significantly higher in mice receiving 5 mg/kg every day or 1 mg/kg switch every other day compared with Fab controls. In contrast, sCAR T-cell frequencies in the 5 mg/kg every other day group were intermediate between, and not significantly different from, the Fab controls or the other two switch-treated groups. All sCAR T-cell populations had contracted to Fab sCAR control levels by day 33 (figure 2G).

B-cell frequencies on day 6 were significantly reduced in recipients of CAR19 or sCAR T-cells (with any switch dose) relative to hEGFR-T or Fab controls, respectively (figure 2H). By day 15, B-cell frequencies in both control groups rebounded equivalently to frequencies higher than basal levels (≈45% vs 29%, respectively); in contrast, B-cell frequencies remained significantly reduced

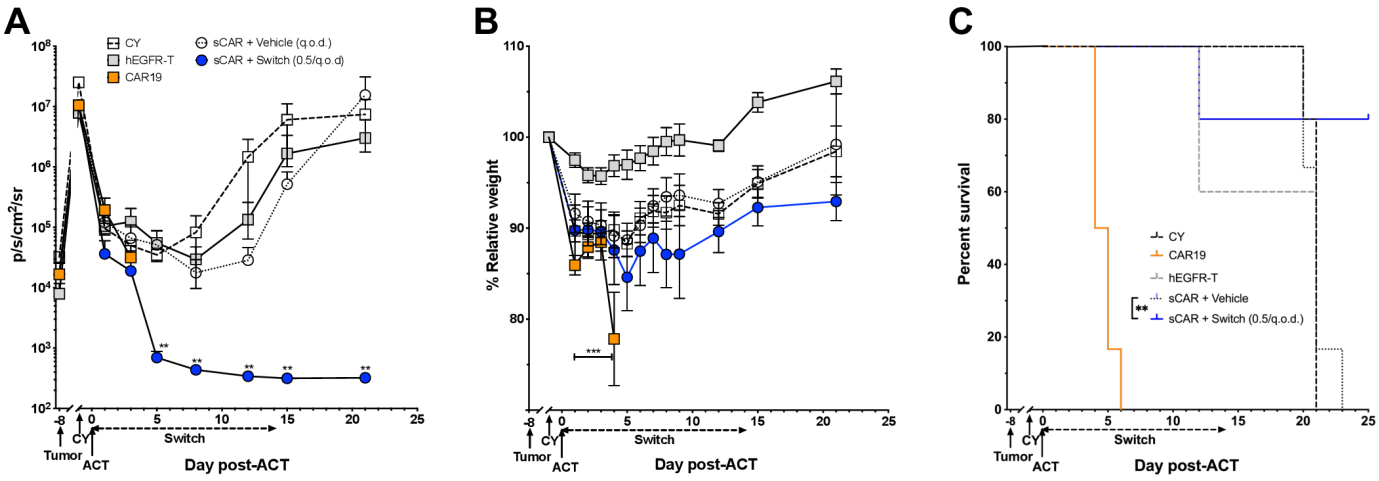

Figure 3 sCAR+switch eliminate huCD19⁺ B cell lymphoma. *huCD19*$^{Tg/0}$ mice received 1E+06 TBL12.huCD19 cells IP followed by CY (300 mg/kg) 7 days later and 3E+06 transduced T-cells the next day (0) (except for the CY group; n=5/group). Select groups also received switches at 0.5 mg/kg or vehicle every other day (dotted arrow) for eight injections/mouse. The data are the mean±SEM and represent two experiments. (A) Tumor growth was measured by bioluminescence intensity. (B) Weights were plotted as percentages of pretreatment weights. (C) Mice were euthanized once their relative weight dropped below 70%, their clinical score was ≥6, or their tumor burden exceeded 1E+07 p/s/cm^2/sr after day 8. *p≤0.05, **p≤0.01, ***p≤0.001 (two-tailed unpaired t-test with Welch's correction or log-rank/Mantel Cox). ACT, adoptive cell transfer; hEGFR, human epidermal growth factor receptor.

(<1%) in recipients of sCAR T-cells plus any switch dose (figure 2H). B-cell frequencies in all switch-treated sCAR T-cell recipients returned to pretreatment levels by day 33.

sCAR T-cells have in vivo antitumor efficacy

huCD19$^{Tg/0}$ mice were injected ip with 1E+06 TBL12.huCD19 tumor cells 7 days before CY. Mice in all but the CY-only group received 3E+06 transduced T-cells the next day (day 0) with sCAR recipients also being injected with switch (0.5 mg/kg) every other day from days 0 to 14. ACT of 3E+06 CAR19 cells to tumor-bearing *huCD19*$^{Tg/0}$ recipients was uniformly fatal in 7 days, consistent with non-tumor-bearing *huCD19*$^{Tg/0}$ recipients (figure 3A). However, 80% of mice receiving sCAR T-cells plus switch remained tumor-free with minimal morbidity (figure 3B, C). Tumor recurred in recipients of control transduced T-cells (hEGFR-T) or sCAR T-cells plus control (Fab) switch comparably to CY treatment alone. Together these data show orthogonal sCAR T-cells plus anti-huCD19 switches can cure mice bearing B-cell tumors without causing life-threatening morbidities.

DISCUSSION

Acute and chronic morbidities caused by sCAR T-cells are less severe than those caused by CAR19 cells in our mouse model. CAR19 and sCAR T-cells at the same dose (3E+06) function comparably in terms of in vivo expansion and depletion of healthy B-cells. sCAR-recipients given switches, however, had enhanced survival, reduced weight loss, improved clinical scores, and were cured of a lethal tumor burden. While it is not yet clear why sCAR T-cells cause less toxicity than CAR19 cells, we hypothesize it relates to the reduced severity of CRS. Preclinical and clinical studies show Ag-activated CAR T-cells produce proinflammatory cytokines (eg, IFN-γ and GM-CSF) that induce endogenous myeloid and endothelial cells to secrete IL-6, a driver of CRS.[15 20] We speculate that proinflammatory cytokine production and resultant CRS wax and wane in sCAR recipients due to the pharmacokinetics of switch protein cyclic dosing.

B-cell aplasia was reversed following cessation of switch administration. B-cell frequencies returned to pretreatment levels in ≤19 days in sCAR recipients treated with 1 or 5 mg/kg switch every other day and in ≤26 days in sCAR recipients treated with 5 mg/kg switch every day. These novel data contrast with our previous report of persistent B-cell aplasia (≥55 days) experienced by recipients of a sublethal dose (1E+06) of CAR19 cells.[15]

In addition to reduced toxicity, the sCAR system has improved efficacy over time. We previously showed that cycling switch administration to iteratively deplete and repopulate healthy B-cells also induced the robust formation and expansion of sCAR T-cell central memory (CD44⁺CD62L⁺) populations.[12] We speculated that the 'rest' phase (1–2 weeks) between dosing regimens allowed the sCAR T-cell population to contract and differentiate into memory cells, mimicking physiological T-cell responses to viral infections. Rest phases are not possible with constitutively active CAR T-cells, and may account for T-cell exhaustion that often limits their clinical efficacy.[21] Future work will investigate causes of reduced morbidity in sCAR T-cell *huCD19*$^{Tg/0}$ recipients (eg, whether or not IL6-driven CRS is reduced in sCAR+switch recipients) and whether cycling switch administration improves resistance to tumor challenge.

sCAR T-cells are one type of regulatable engineered immune cells. Other strategies include building synthetic

circuits that follow Boolean logic to signal immune effector functions under defined conditions (eg, SynNotch receptors that require an 'AND' signal delivered when two target Ags are engaged).[22–24] Preclinical models such as ours will be critical in directing which engineered cells should next be taken to clinical trials.

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Contributors Conceptualization, CAP, TSY, and BRB; methodology, CAP, HC, MDS, SB-W, MJO, YR, MJ and SV; investigation, CAP, HC, and MDS; writing–original draft, CAP and BRB; writing–review and editing, all authors; Funding acquisition, CAP, TSY and BRB.

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