Switchable control over in vivo CAR T expansion, B cell depletion, and induction of memory

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Chimeric antigen receptor (CAR) T cells with a long-lived memory phenotype are correlated with durable, complete remissions in patients with leukemia. However, not all CAR T cell products form robust memory populations, and those that do can induce chronic B cell aplasia in patients. To address these challenges, we previously developed a switchable CAR (sCAR) T cell system that allows fully tunable, on/off control over engineered cellular activity. To further evaluate the platform, we generated and assessed different murine sCAR constructs to determine the factors that afford efficacy, persistence, and expansion of sCAR T cells in a competent immune system. We find that sCAR T cells undergo significant in vivo expansion, which is correlated with potent antitumor efficacy. Most importantly, we show that the switch dosing regimen not only allows control over B cell populations through iterative depletion and repopulation, but that the "rest" period between dosing cycles is the key for induction of memory and expansion of sCAR T cells. These findings introduce rest as a paradigm in enhancing memory and improving the efficacy and persistence of engineered T cell products.

CAR T cell | immunotherapy | cancer | control | memory

Clinical experience with CD19-targeted CARs has demonstrated that the fate of engineered cells is a critical factor to therapeutic efficacy. CAR T cell products that skew toward a more naive, persistent central memory (i.e., TCM) phenotype have been correlated with sustained remissions in patients with acute lymphoblastic leukemia (6, 9, 10). Accordingly, significant research has focused on achieving CAR T cell populations with this phenotype in vivo through costimulatory domain engineering or by presorting TCM subsets from patients before transplantation (10–15). However, memory responses in native T cells are formed through acute, high-load antigen stimulation followed by a "rest" phase, which cannot be recapitulated in current CAR T designs that are constitutively in the "on" state by constantly engaging antigen. The rest phase is important to allow T cell populations to undergo a 1–2-wk contraction in which effector cells that were expanded during stimulation go through apoptosis, resulting in a relative enrichment of the TCM subset. As such, understanding how rest can be leveraged to improve memory formation and engraftment in the context of CAR T cells is expected to lead to more efficacious products.

Significance

Chimeric antigen receptor (CAR) T cell therapy represents a powerful strategy in immuno-oncology. Nevertheless, associated life-threatening toxicities and chronic B cell aplasia have underscored the need to control engineered T cells in the patient. To address these challenges, we have previously developed a switchable CAR (sCAR) T cell platform that allows dose-titratable control over CAR T cell activity by using antibody-based switches. Here, we demonstrate in a syngeneic murine model that the switchable platform can impart antitumor efficacy while dissociating long-term persistence from chronic B cell aplasia. Further, the functional reversibility of the switchable platform can be leveraged to incorporate "rest" phases through cyclical dosing of the switch to enable the induction of a robust central memory population for in vivo, on-demand expansion of sCAR T cells.

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Here, we exploit a unique feature of the switchable CAR T cell platform that allows cells to rest to recapitulate a physiological period of T cell stimulation that induces robust memory formation (16–20). This work was carried out in a competent immune host as a clinically relevant model to study scAR T cell efficacy not only against CD19+ B cell lymphoma but also against normal B cells. We developed a syngeneic murine-based switchable CAR T cell platform with murine 4-1BB-based second- and third-generation costimulatory domains that match the human homologs used clinically to promote persistence (1, 10, 21). Costimulatory domains harboring murine 4-1BB were found to be essential in establishing persistence compared with more commonly used CD28-based costimulatory domains and were required to study long-term control aspects. By using these tools, we demonstrate that the switchable system exhibits hallmarks of classical immune regulation in its ability to undergo expansion and contraction of the effector memory compartment when dosed with an anti-CD19 switch. Further, we show that the dosage and timing of the anti-CD19 switch administration can control the amplitude and phenotypic distribution of scAR T cell expansion. This dynamic, functionally reversible system afforded antitumor control, repopulation of B cells, and physiological-like T cell responses.

**Results**

**Development of the Anti-Murine CD19 Switchable CAR T Cell System.** In our previous work (7), we described the development of the switchable system by engrafting a peptide neoepitope (PNE) in the antigen-binding fragment (Fab) region of a targeting antibody (Fig. 1A, gray) and creation of a corresponding scAR with an scFv that recognized the PNE (Fig. 1A, green). We demonstrated that the switch design, and in particular the site of fusion of the PNE in the Fab, defined the engagement of the immunological synapse between the engineered cell and target cell. This was critical to the activity of human scAR T cells in a xenograft model. Here, to create a surrogate murine scAR T cell system, we used an MSGV retroviral vector to transduce mouse splenocytes with an scAR subcloned into a previously described backbone (8, 22, 23) harboring a murine CD28 costimulatory domain and a modified murine CD3ζ domain with the first and third immunoreceptor tyrosine-based activation motifs (ITAMs) knocked out [28ζ(1-3)]; Fig. 2A].

The anti-murine CD19 switch was developed from the Fab (lacking the Fc domain) of the rat clone 1D3. To determine the optimal switch design, the PNE was fused to the N terminus or C terminus of the heavy or light chains of the 1D3 Fab to fuse a library of six designs (Fig. 1B) (7). In vitro cytotoxicity assays with the use of 1 nM of switch in the presence of an effector-to-target cell ratio of 10:1 of 28ζ(1-3) sCAR T cells to CD19+ Myel target cells demonstrated that the light-chain N-terminally grafted switch (LCNT) exhibited the greatest level of cytotoxicity compared with other switch designs (Fig. 1C). C-terminally grafted switches (HCCNT, LCCT, CTBV) exhibited weaker activity, illustrating that the murine system faithfully recapitulated the empirical design concepts of the human sCAR system. Further, LCNT + sCAR 28ζ(1-3) activity was comparable to that of a conventional CAR T cell [1D3 28ζ(1-3)], demonstrating that the murine switchable system was capable of conventional CAR T cell level potency in vitro. Therefore, the anti-murine CD19 switch LCNT was used for further studies.

**Generation of Murine scAR Constructs and in Vitro Potency.** We next optimized the hinge, costimulatory, and activation domains of the sCAR with the intention of developing an scAR platform that used the 4-1BB-based costimulatory domain similar to those used clinically (10, 24). Nine additional scAR variants were created and transduced into C5H mouse splenocytes for systematic comparison by in vitro cytotoxicity and cytokine release against the syngeneic CD19+ B cell lymphoma cell line 38c13 (Fig. 2A). The conventional CAR [1D3 28ζ(1-3)] was used as our starting point based on our previous work (8) and served as a baseline control throughout.

First, the 36-aa CD28 hinge domain was compared with the shorter 12-aa IgG4m hinge shown to improve activity in human sCAR constructs (7, 25, 26). As shown in Fig. 2B, the IgG4m hinge [Ig-28ζ(1-3)] afforded significantly higher levels of cytotoxicity and cytokine release (IFN-γ, TNF, and IL-2) in response to specific antigen stimulation in comparison with the CD28-based hinge [28ζ(1-3)].

Next, the murine CD28-based costimulatory domain was compared with a murine 4-1BB [Ig-BBζ(1-3)] or third-generation murine CD28 + 4-1BB [Ig-28BBζ(1-3)]-based costimulatory domains by using the IgG4m hinge (Fig. 2C) in the context of CD3ζ with mutated ITAMs. Interestingly, cytotoxicity and cytokine production of the 4-1BB construct were significantly impaired (Fig. 2C). We hypothesized that this may result from the loss of two of three functional ITAMs of the CD3ζ domain. This modification was previously reported to decrease T cell apoptosis and increase in vivo expansion in the context of the CD28 costimulatory domain (27). However, the impact, if any, of a mutated CD3ζ signaling domain in the context of 4-1BB second-generation CAR T cells has not been reported.

To determine if activity could be recovered with an intact (i.e., WT) CD3ζ, the ITAMs were restored to create the Ig-28ζ, Ig-BBζ, and Ig-28BBζ constructs (Fig. 2A). Indeed, cytotoxicity and cytokine release were recovered in the 4-1BB-based construct (Ig-BBζ) and cytotoxicity increased in the 28BB construct (Ig-28BBζ).
Fig. 2. In vitro assessment of sCAR designs. (A) Description of the sCAR designs. Conventional CART-19 is designated as 1D3 28z(1–3). The corresponding sCAR construct 28z(1–3) was based on the same backbone except that the scFv recognizes PNE instead of murine CD19. Second-generation sCAR constructs contained murine CD28 or murine 4–1BB as a costimulation molecule, and third-generation constructs incorporated both. The murine CD28-based hinge was replaced with IgG4m hinge or murine CD8-based hinge. The murine CD3ζ signaling domain was incorporated in its WT form with all three ITAMs intact or with the first and third ITAMs inactivated [CD3ζ(1–3); asterisk]. (B–E) Comparison of sCAR T cells based on their cytotoxicity as determined by flow cytometry against 38c13 target cells in the presence of 1 nM anti-mouse CD19 switch and on their cytokine secretion as measured by CBA assay. 1D3 28z(1–3) conventional CAR T cells and nontransduced cells (NT) are used as a reference and a control, respectively. (B) IgG4m [Ig-28z(1–3)] vs. CD28-based hinge [28z(1–3)] comparison. (C) CD28 [28z(1–3)] vs. 4–1BB [8-28BBz(1–3)] vs. CD28+4–1BB [Ig-28BBz(1–3)] costimulation domain. (C and D) WT (z) vs. mutated ITAMs [z(1–3)] in CD3ζ signaling domain. (E) Third-generation sCAR comparison based on hinge: IgG4m [Ig-28BBz(1–3) and Ig-28BBz] vs. CD8-based hinge (8-28BBz). All graphs depict the data from two pooled experiments performed in triplicate. Statistical analyses were performed by Kruskal–Wallis test completed with Dunn’s nonparametric multiple comparisons test. Means and SEM are shown (*P < 0.05, **P < 0.01, and ***P < 0.001; ns, not significant).
We next asked whether the switch dosing regimen could induce more T cells than the 1-wk low-dose group (Fig. 4A) and more T cells than the 3-wk high-dose group (Fig. 4B). Anti-murine CD19 switch dosing was initiated 4 h after sCAR T cell inoculation and continued every other day for 2 wk, followed by a rest period of 2 wk. The rest period is defined as the time when switch dosing is withheld. Although B cells are being continuously produced, the sCAR T cells are not being activated by switch during this time. This dosing regimen was repeated for three cycles (Fig. 2D).

Three sCAR T cell constructs, all bearing the IgG4m hinge and CD28, 4–1BB, or CD28 and 4–1BB costimulatory domains (Ig-28z, Ig-28BBz, and Ig-28BBz), were compared in vivo to assess how the costimulatory domain affected efficacy, B cell depletion, and CAR T cell expansion. Ig-2BBz and Ig-28BBz constructs eliminated tumors in all mice, with no relapse up to 152 d (Fig. 3B and SI Appendix, Fig. S3B). Notably, neither the Ig-28z nor the 1D3-28z (1–3) CAR effectively controlled tumor growth, despite their superior cytokinetic activity compared to BBz or 28BBz constructs in vitro (Fig. 2D). This concurs with previously reported results demonstrating the improved in vivo efficacy of CARs with 4–1BB relative to those bearing CD28 (32, 33).

Analysis of the peripheral blood by flow cytometry revealed that sCAR T cell-treated and control groups (CTX, tumor + CTX, 1D3 28z(1–3)) exhibited B cell depletion 1 wk after CTX treatment (day 15), which rebounded in control groups by day 35, in agreement with previous reports (Fig. 3C and SI Appendix, Fig. S3D) (8, 28, 34). Switch dosing in sCAR T cell treatment groups induced complete elimination of B cells after the first cycle of the switch (day 22). B cell populations rebounded when switch dosing was stopped (rest phase to day 35) but were iteratively depleted through the next two switch dosing cycles (days 36–50 and 64–78), thereby demonstrating functional reversibility of activity for the Ig-2BBz and Ig-28BBz constructs. B cells returned to levels of untreated controls by day 138 (60 d after last switch dose; Fig. 3C and SI Appendix, Fig. S3H) and were accompanied with the restoration of humoral immunity for Ig-2BBz and Ig-28BBz by day 153, 2 wk following immunization with ovalbumin (OVA; SI Appendix, Fig. S3H). Of note, OVA-specific IgG1 was detectable in the plasma of Ig-2BBz and Ig-28BBz mice, but the levels were low compared with CTX-treated mice, probably because of the plasma cells not yet normalized, as B cell levels were comparable.

To understand the impact of switch dosing on Ig-2BBz and Ig-28BBz sCAR T cell populations, we also examined sCAR+ T cells in peripheral blood at these time points. Low numbers of sCAR+ T cells were detected in the peripheral blood of Ig-2BBz and Ig-28BBz groups through the end of the first dosing and rest cycle (day 35; Fig. 3D and SI Appendix, Fig. S3E), putatively as a result of recruitment and recirculation in the secondary lymphoid tissues following CTX-induced lymphopenia and tumor lysis (31). Interestingly, sCAR T cells markedly expanded into a T CD8+ effector/effecter memory (T_EEM) phenotype after the second and third dosing cycles (Fig. 3E and SI Appendix, Fig. S3F). These populations underwent contraction during the rest phase, but persisted at detectable levels until day 138 (Fig. 3D and SI Appendix, Fig. S3E).

To compare the IgG4m vs. CD8-based hinge and the impact of the inactivated ITAMs in CD3ζ, Ig-28BBz was compared with 8–28BBz and Ig-28BBz(1–3), respectively. Both Ig-28BBz and Ig-28BBz(1–3) eliminated tumors in all animals, whereas 8–28BBz and 8–BBz conferred weak antitumor activity (Fig. 3F and SI Appendix, Fig. S3B and G, respectively), in agreement with our prior results demonstrating inferiority of the longer CD8-based hinge (7). However, Ig-28BBz(1–3) and Ig-28z(1–3) failed to eliminate B cells in the second and third dosing cycles (Fig. 3G and SI Appendix, Fig. S3D and G, respectively), which corresponded with an absence of sCAR+ T cells in peripheral blood (Fig. 3H and I and SI Appendix, Fig. S3E). Thus, the costimulatory domain, IgG4m hinge, and CD3ζ (WT) domains were critical to establishing a persistent sCAR T cell population fully controllable by switch dosing.

Induction and Recall of Memory Responses Using the Switch Dosing Regimen. We next asked whether the switch dosing regimen could modulate the sCAR T cell phenotype after sCAR T cell engraftment. To create a model in which switch dosing mimicked acute or chronic periods of T cell activation (16, 35), we devised two independent regimens centered on a 28-d cycle: 3 wk of every-other-day dosing followed by 1 wk of rest or 1 wk of every-other-day dosing followed by 3 wk of rest. We compared these with our reference condition of equal periods of dosing and rest (2 wk of dosing, 2 wk of rest). Each of the three regimens was tested with low (0.2 mg/kg) or high (5 mg/kg) doses of the switch (Fig. 4A). Analysis was focused on sCAR T cell phenotype and B cell depletion in the absence of tumor as a readout of sCAR T cell efficacy. The Ig-28BBz sCAR was used because this construct afforded favorable results in the tumor model and most accurately mimicked our human clinical candidate currently in development.

All dosing regimens with Ig-28BBz exhibited B cell depletion after the first week of dosing (day 7; SI Appendix, Fig. S4A) in agreement with the aforementioned results. Analysis after 3 wk (day 25) showed that B cells were depleted in 3-wk low and high dosing groups, whereas B cells in the 1- and 2-wk treatment groups (SI Appendix, Fig. S4A, Bottom and Top) exhibited a rebound as a result of the extended rest period between final switch dose and analysis for these groups. Each group was then resynchronized by the start of the second cycle, on day 28, at which time dosing was initiated in each group. Again, B cells were depleted 1 wk into the second cycle (day 35) with the exception of the 3-wk low-dose group, which exhibited low but detectable levels of B cells, indicating a potentially weaker response for this regimen (SI Appendix, Fig. S4A). Remarkably, in the second cycle, the 1-wk high-dose group expanded 385-fold more sCAR+ T cells than the 3-wk high-dose group (Fig. 4B, day 35). At this time point, 1-wk and 3-wk groups had received equal amounts of switch during the second cycle, with the only difference being the duration of dosing in the first cycle and rest period. Expansion in the 1-wk group was further correlated with switch dose level, as the 1-wk high-dose group exhibited 2.6-fold more sCAR+ T cells than the 1-wk low-dose group (Fig. 4B).

Assessment of T cell phenotypes showed that the expanded population was predominantly CD8+ T_EEM cells at day 35 (SI Appendix, Fig. S4B). This population underwent a contraction by day 53 (3 wk after the last switch dose for this group) hallmarkled by an important reduction in T_EEM but a relative retention of T_CM phenotype (Fig. 4C and D). Notably, the retention of this T_CM subset continued to remain higher in the 1-wk group than the 2- or 3-wk treatment groups 40 d after the second cycle was completed (day 74), even without additional switch dosing (Fig. 4D and SI Appendix, Fig. S4B). Neither the 3-wk high- nor low-dose groups exhibited detectable peripheral sCAR T cell levels, indicating that
Fig. 3. In vivo efficacy and persistence of sCAR designs in a syngeneic murine tumor model. C3H mice were implanted with 38c13 cells at day (D) 0 and preconditioned with CTX at D7. The following day (D8), sCAR/CAR T cells were injected i.v. Anti-murine CD19 switch doses (or PBS solution) were started at D8, D36, and D63/64 for eight doses (gray shading) every other day at 1 mg/kg in a 2-wk on/off (rest) cycle. (B–E) This study compared the efficacy of Ig-28z, Ig-BBz, and Ig-28BBz through the evaluation of murine CD28 vs. murine 4–1BB costimulatory molecule. (F–I) In this study, Ig-28BBz(1–3), Ig-28BBz, and 8–28BBz were compared to assess IgG4m vs. CD8-based hinge and CD3ζ (WT) vs. CD3ζ(1–3). The 1D3 28z(1–3) was used as a control (n = 5–6). (A) Experimental design. (B and F) Tumor growth kinetics of tumor-bearing mice that received no treatment, received CTX only (“Tumor + CTX”), or were administered sCAR/CAR T cells and switch/PBS solution following CTX. The number of tumor-free mice in each treatment group is reported in parentheses. The asterisk indicates, at days 73 and 76, when one mouse each day, in the Ig-28BBz(1–3) group was found dead for unknown reasons. (C and G) Number of B cells per microliter of peripheral blood over time as determined by flow cytometry. (D and H) Number of CD45+ cells (naive and CTX groups) and sCAR/CAR T cells per microliter of peripheral blood over time as determined by flow cytometry. (E and I) sCAR/CAR T cell phenotype analysis in the peripheral blood by flow cytometry at days 53 and 78/81: CD4+ or CD8+ T effector/effector memory (E/FM; CD44+CD62L−) and central memory (CM; CD44+CD62L+) subsets are indicated. A duplicate experiment combining these two experiments is shown in SI Appendix, Fig. S3. Means and SD are shown. Statistical analyses with Mann–Whitney test indicated significant differences at 95% CI (**P < 0.01).
the duration of the rest phase was more critical to the expansion of the sCAR T cells than the switch dose level.

**Trafficking of the sCAR T Cells in Vivo.** To understand where the sCAR T cells traffic during the expansion and contraction phase, and the extent of B cell depletion in our in vivo studies, we enumerated sCAR T cells present in the spleen, inguinal tumor-draining lymph node (TDLN), and bone marrow (BM) in animals in which tumor was efficiently cleared with Ig-28BBz sCAR T cells and switch (SI Appendix, Fig. S5). In this study, we observed an expansion of sCAR T cells (>3 × 10^5 sCAR T cells per microliter) that correlated with undetectable B cells in peripheral blood immediately after the second cycle of switch dosing (day 68; Fig. 5A and B and SI Appendix, Fig. S5B). B cells were also depleted in the spleen, TDLN, and BM, indicating that sCAR T cells and switch were capable of distribution and activity outside peripheral blood. The phenotype of sCAR T cells in blood, spleen, and BM at day 68 was skewed toward an activated CD8^+ T_EEM phenotype (Fig. 5C). sCAR T cells in the TDLN were mostly CD8^+ T_CM cells, consistent with migration of T_CM cells to secondary lymphoid tissues (35) (Fig. 5C and SI Appendix, Fig. S5C). Importantly, the global pool of sCAR T cells and specifically the number of cells in the sCAR^+ CD8^+ T_CM subset stayed relatively constant in the tissue during the T_EEM contraction phase between day 68 and day 82 (Fig. 5B and C) compared with the peripheral blood, where the most drastic changes were observed. These findings were consistent with 2-wk and 4-wk rest periods (Fig. 5 and SI Appendix, Fig. S5).

**Discussion**

In this study, we demonstrated the design and engraftment of a switchable, persistent sCAR T cell population with recallable activity that exhibits classical T cell expansion and contraction behavior. To enable the study, we first developed the PNE-based switch and sCAR in a syngeneic murine platform. Consistent with our prior report in the human system (7), the N-terminally designed switch molecule (i.e., LCNT) improved in vitro cytotoxicity and the short IgG4m hinge increased in vivo persistence. These components are expected to shorten the distance between the sCAR T cell and target cell and thereby improve
**Fig. 5.** sCAR T cell trafficking in tissues. 38c13 tumor-bearing C3H mice were treated with CTX before adoptive cell transfer with Ig-28BBz sCAR T cells. Anti-murine CD19 switch injections (or PBS solution) were initiated at day (D) D8 and D51 for eight doses (gray shading) every other day at 1 mg/kg with a 4-wk rest period. Peripheral blood and tissues (spleen, TDLN, BM) were analyzed for B and sCAR T cells at D15, D25, and D35 for blood only and at D68 and D82 for blood and tissues (n = 5). (A) The experimental design is shown, as well as a graph depicting the number of cells in the peripheral blood over time: B cells (left axis; naïve and CTX only-treated mice and Ig-BB28z mice; dotted line) and sCAR T cells (right axis) in the Ig-28BBz group. (B) Number of sCAR T cells (Top) and B cells (Bottom) per microliter of peripheral blood and in the spleen, TDLN, and BM of the Ig-28BBz–treated mice as determined by flow cytometry at D68 and D82. (C) sCAR T cell phenotype analysis in the peripheral blood, spleen, TDLN, and BM by flow cytometry at D68 and D82: CD4+ or CD8+ T effector/effector memory (E/EM; CD44+CD62L−) and central memory (CM; CD44+CD62L+) subsets are shown. The results from one study are depicted. Statistical analyses with Mann–Whitney test indicated significant differences at 95% CI. Data are shown as mean with SD (**P < 0.01 and ***P < 0.001; ns, not significant).
immunological synapse formation that can be decisive for in vivo antitumor activity (7, 26, 36). Because the anti-murine CD19 switch used in these studies was developed from a rat monoclonal antibody, there was a potential for an anti-switch antibody response. This was found in only two animals studied, shown in SI Appendix, Fig. S6, which corresponded with the inability to control B cell populations in these outlier animals. In clinical translation, humanization of the antibody is expected to mitigate this potential.

The murine 4-1BB costimulatory domain alone, or in conjunction with the murine CD28 costimulatory domain as a third-generation construct, was essential to achieving control of tumor burden and sCAR T cell persistence in our model. In vivo, sCAR T cell expansion of the 4-1BB-based constructs was predominantly sCAR* CD8+, which is consistent with reported 4-1BB-driven cytotoxic T cell expansion (37). Expansion was strictly dependent on a fully functional CD3ζ signaling domain in the sCAR construct. This was in contrast to a previous report that demonstrated that in-activation of the first and third ITAMs of CD3ζ can increase expansion of CD28-based constructs in vivo (27). Although CD28 and 4-1BB enhance TCR signaling (38, 39), CD28 stimulation is driven through PI3-kinase-Akt and 4-1BB through TRAF1-2 (40). As CD3ζ also signals through PI3-kinase (41), CD3ζ ITAM functionality could be dispensable in a CAR construct harboring CD28, but not in a 4-1BB-based construct. This is supported by the retention of activity in Ig-2BBBζ (1-3) in vitro compared with loss of activity for Ig-BBζ (1-3) (Fig. 2C). In addition, Ig-2BBBζ (1-3) sCAR T cells failed to expand in vivo upon repeated stimulation compared with Ig-2BBζ, underscoring the importance of CD3ζ ITAMs in the third-generation constructs.

We took advantage of the persistence of the Ig-2BBBζ sCAR T cells and the expression of CD19 on normal B cells as a target population during the second dosing phase (17). Although B cells remained depleted because the anti-murine CD19 antibody, there was a potential for an anti-switch antibody response. This was found in only two animals studied, shown in SI Appendix, Fig. S6, which corresponded with the inability to control B cell populations in these outlier animals. In clinical translation, humanization of the antibody is expected to mitigate this potential.

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To generate murine sCAR T cells, whole mouse splenocytes were submitted to red blood cell lysis (eBioscience) before being activated for 24 h with 1 μg/mL soluble anti-CD3ζ: NALE antibody (145-2C11), 1 μg/mL anti-CD28 NALE antibody (37.51; BD Biosciences), and 60 μ/mL of recombinant human IL-2 (R&D Systems). The activated sCAR T cells were then retrovirally transduced following a spinoculation protocol by using RetroNectin (Clontech). On the next day, transduced sCAR/CAR T cells were collected, sCAR expression was assessed by flow cytometry, and cells were directly seeded for expansion at 0.5 × 10^6 cells per milliliter with 60 μ/mL of recombinant human IL-2 or enriched for sCAR/CAR T cells (for cytotoxicity assays) before seeding. Cells were used for in vitro or in vivo assays the next day.

**Materials and Methods**

**Mice, Cell Lines, and Murine sCAR T Cells.** Six-week-old female C57BL/6J animals were obtained from Jackson Laboratory (strain 006644, and 6-7 wk-old female C57BL/6J mice were obtained from Charles River. C3H mice were housed in a vivarium with a 12-h light cycle and access to food and water ad libitum. All protocols were approved by the institutional animal care and use committee at the Calibar.

Myc5 CD19+ murine B-lymphoma cells (C57BL/6 origin; Myc5 cells over-expressing GFP and CD19) were obtained from Andrei Thomas-Tikhonenko (University of Pennsylvania, Philadelphia, PA). The 38c13, CD19-expressing B cell lymphoma (C3H/HeN origin) was a gift from Ronald Levy (Stanford University, Stanford, CA).

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**Cytotoxicity Assay by Flow Cytometry.** 38c13 target cells labeled with PKH67 according to the manufacturer’s procedure (Sigma-Aldrich) were cocultured at a ratio 1:10 with sCAR and conventional CAR T cells (1 × 10^6 targets with 1 × 10^6 effectors) previously CAR+ enriched and normalized (to a 50 ± 6% transduction efficiency with nontransduced cells; SI Appendix, Fig. S1B) and 1 nM anti-mouse CD19 LCNT switch for 6 h at 37 °C. Supernatants were frozen for further analysis, and cells were stained with the Zombie Red Fixable Viability kit (BioLegend) and frozen in 4% paraformaldehyde final (Electron Microscopy Sciences). Five microliters of CountBright Absolute Counting Beads (Molecular Probes) were added to the cells. A fixed volume of samples was acquired on an LSFortessa X-20 flow cytometer (BD Biosciences) and analyzed by using FlowJo software (version 10.1). The number of viable target cells remaining (Zombie Red+ PKH67+) and beads were used to calculate the number of cells per microliter following the CountBright manufacturer’s instructions. The cytotoxicity against the target cells (percentage target killed) was calculated as 100 × [number of live target cells per microliter in target + effector cells, no switch] – (number of live target cells per microliter in target + effector cells, switch)/[number of live target cells per microliter in target + effector cells, no switch]. Cytokines in the supernatants were quantified by using Cytometric Bead Array (CBA) Mouse Soluble Protein Flex Set kits (BD Biosciences) according to product manuals after acquisition on an Accuri C6 flow cytometer (BD Biosciences) and analysis with FCAP Array software (BD Biosciences).
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Materials and Methods

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