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Enhancing the effectiveness of γδ T cells by mRNA transfection of chimeric antigen receptors or bispecific T cell engagers

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Adoptive cell therapy (ACT) utilizing γδ T cells is becoming a promising option for the treatment of cancer, because it offers an off-the-shelf allogeneic product that is safe, potent, and clinically effective. Approaches to engineer or enhance immune-competent cells for ACT, like expression of chimeric antigen receptors (CARs) or combination treatments with bispecific T cell engagers, have improved the specificity and cytotoxic potential of ACTs and have shown great promise in preclinical and clinical settings. Here, we test whether electroporation of γδ T cells with CAR or secreted bispecific T cell engager (sBite) mRNA is an effective approach to improve the cytotoxicity of γδ T cells. Using a CD19-specific CAR, approximately 60% of γδ T cells are modified after mRNA electroporation and these cells show potent anticancer activity *in vitro* and *in vivo* against two CD19-positive cancer cell lines. In addition, expression and secretion of a CD19 sBite enhances γδ T cell cytotoxicity, both *in vitro* and *in vivo*, and promotes killing of target cells by modified and unmodified γδ T cells. Taken together, we show that transient transfection of γδ T cells with CAR or sBite mRNA by electroporation can be an effective treatment platform as a cancer therapeutic.

INTRODUCTION

Immunotherapies are revolutionizing cancer treatment by harnessing the immune system to target cancerous cells. Adoptive cell therapy (ACT) offers a promising direction as an effective cancer therapeutic by using immune-competent cells in either an autologous or allogeneic setting.[1] Traditionally, ACT utilizes autologous αβ T cells that are isolated from the patient, engineered to improve their cytotoxicity, and then re-infused into the patient. Although these therapies are effective, off-the-shelf allogeneic products have advanced into clinical testing and have many advantages over autologous strategies.[2,3] γδ T cells are a small subset of lymphocytes that contributes to the body's innate and adaptive immunity and are involved in immune surveillance, rapid immune response, and modulating other immune cells.[4–7] γδ T cells are quickly becoming a promising option for ACT because they are non-alloreactive with limited risk of causing graft versus host disease, thus allowing their use in allogeneic settings.[8,9] They also exhibit several characteristics that make them favorable candidates for use in adoptive cell therapy. One of the major advantages of γδ T cells is their ability to recognize antigens in an MHC-independent manner, which means they do not require MHC-peptide priming for activation. They recognize several unique ligands and stress markers that direct their killing toward cancer cells, including butyrophilin via phosphoantigen activation,[10,11] Fas,[12] heat shock proteins,[13,14] and MHC class I-related molecules MICA, MICB, and ULBPs 1–6.[15,16] In addition, they perform antibody-dependent cellular cytotoxicity through expression of CD16 and show promising anticancer activity when used in combination with therapeutic antibodies.[17–19] Importantly, γδ T cells can be expanded *ex vivo* from peripheral blood with a serum-free protocol for clinical use.[20–22]

The development of chimeric antigen receptor (CAR) T cell therapy is among the most promising anticancer therapeutic and has improved immunotherapies by allowing for a more targeted treatment approach compared with chemotherapeutics. CAR T cells utilize the specificity of antibodies and the cytotoxic capabilities of T cells to target cancer cells. The most successful application of CAR T cells is the treatment of B-cell malignancies using CD19 CARs with complete remission rates reaching about 60% for children and young adults.[23] Despite its early success, there are obstacles and limitations that must be addressed to improve patient outcomes and safety, including cytokine release syndrome, neurotoxicity, acquired resistance to CAR T cell therapy, and health of the expanded T cell product.[24–26] Additionally, the development of bispecific antibodies has shown some success in preclinical and clinical studies.[27–29] Bispecific antibodies are a type of engineered antibody containing two binding regions, allowing for multiple applications including bringing

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immune cells in close contact with target cells, blocking immune checkpoints, and modulating inflammatory and other signaling pathways.[30] There are two major types of bispecific antibodies, immunoglobulin (Ig)G-like and non-IgG-like, with the major difference being the incorporation of the Fc fragment.[31] Blinatumomab is a type of non-IgG-like bispecific antibody known as a bispecific T cell engager that is specific to CD19. Bispecific T cell engagers are typically composed of a CD3-specific scFv linked to an scFv specific to a tumor antigen, a design that promotes T cell-cancer cell interactions to improve T cell cytotoxicity, serial killing, and proliferation.[32]

Immunotherapies utilizing unmodified or engineered γδ T cells have the potential to be effective cancer treatments. We have previously published our efforts to optimize the expansion and handling of γδ T cells, as well as identify successful donor characteristics to predict a more potent cellular product.[21,33,34] These optimizations have resulted in a Food and Drug Administration cleared γδ T cell product candidate for clinical testing against neuroblastoma (NCT05400603). We have also shown that γδ T cell cytotoxicity can be improved by upregulating stress antigens on cancer cells through combination therapy with chemotherapeutics such as temozolomide and bortezomib.[35–37] Another approach to improve γδ T cell cytotoxicity is to genetically engineer these highly potent immune-competent cells. However, engineering γδ T cells has been variable and inefficient.[38] Advances in mRNA design and transfer over the past several decades have allowed for increased stability, higher transfection efficiencies, and rapid expression of proteins.[39] In addition, transient engineering of γδ T cells offers a number of advantages over stable engineering and can reduce some of the risks associated with CAR T cell therapy. For example, in the event of toxicity, treatment can be halted quickly, and specified doses of cells can be administered for individual cases. Also, the use of transient engineering strategies can reduce the risk and duration of cytokine release syndrome because the finite length of expression limits the overactivation and excessive cytokine release of CAR T cells. Here we test whether transient engineering of γδ T cells with CAR or secreted bispecific T cell engager (sBite) mRNA can be an effective cancer treatment platform and an alternative to the traditional stable CAR expression in αβ T cells.

RESULTS

CD19 CAR expression in electroporated γδ T cells

Several electroporation strategies were tested using the BioRad Gene Pulser Xcell Electroporator or Lonza Nucleofector IIB device. Although both can be optimized for engineering *ex vivo* expanded γδ T cells, here we show optimization of the BioRad Gene Pulser Xcell Electroporator using a bicistronic CD19 CAR-GFP construct (Figure S1). Successful electroporation was determined by GFP expression (Figure 1A). To determine the optimal conditions for electroporation, increasing cell numbers and mRNA concentrations were tested for each individual reaction as described in materials and methods. Cell yield, which we define as the proportion of live cells remaining 24hrs after electroporation to the starting number of cells used for the electroporation reaction, is an important factor when considering downstream applications. Twenty-four hours after electroporation, reactions containing between 1 x 10^6 and 1 x 10^7 γδ T cells showed increasing cell yield with increasing cell number (Figure 1B). To measure transfection efficiency, three parameters were examined: GFP mean fluorescence intensity (MFI), GFP^+ percentage, and CAR^+ percentage (Figures 1C and 1D). The MFI for GFP increases with increasing amounts of mRNA per reaction for all cell numbers. Interestingly, the percentage of GFP^+ cells and CAR^+ cells was similar for all reaction conditions and was found to be around 90% and 60%, respectively. Since reactions with 30μg of mRNA did not improve any of the tested parameters compared to 15μg, 15μg of mRNA was used for functional studies. CD19 CAR-expressing γδ T cells from reactions with 5 x 10^6 and 1 x 10^7 were cocultured for 4 h with 697 cells, a $CD19^+$ B cell leukemia cell line, and the percent cytotoxicity was determined. All effector:target (E:T) ratios tested resulted in the same cytotoxicity, suggesting that varying the number of cells per transfection reaction, while keeping the amount of mRNA constant at 15μg, does not affect the cytotoxicity of the engineered γδ T cells (Figure 1E).

As γδ T cells are considered candidates for off-the-shelf ACT, a freezing step is anticipated. Therefore, these cells can be genetically engineered either before or after freezing. γδ T cells were electroporated on day 12 of expansion with the CD19 CAR-GFP construct and were examined before freezing and after a freeze/thaw cycle. The GFP^+ percentage was similar (around 90%) prior to freezing and after freeze/thaw whereas the CAR^+ percentage prior to freezing was approximately 60% and decreased to 20–40% after freeze/thaw. (Figure 2A). Cell viability was also measured and found that the viability decreased after thawing, compared to after electroporation/before freezing (Figure S2). To test whether freezing engineered γδ T cells also affected their ability to kill target cells, a cytotoxicity assay was conducted with γδ T cells that were either engineered before freezing or engineered after freezing (Figure 2B). Both groups performed similarly at low E:T ratios of 0.5:1 and 1:1. However, differences between the groups were more substantial at the higher E:T ratios of 2:1 and 5:1, with a lower average cytotoxicity of cells engineered before freezing, compared to engineered after freezing. Based on these studies, we found electroporating 5x10^6 to 1x10^7 thawed cells with 15μg of mRNA was the optimal conditions for γδ T cell electroporation.

Electroporation of CD19 and CD22 CAR mRNA enhances the effectiveness of γδ T cells

We then engineered an mRNA construct that (1) did not include GFP and (2) was codon optimized for expression in γδ T cells, as described in materials and methods. Comparing the codon optimized construct and the initial GFP-containing construct showed they both resulted in similar CAR expression and cytotoxicity against 697 cells (Figure S3). All subsequent functional experiments were conducted with the codon optimized/non-GFP construct. The efficacy of the engineered cells was then tested using *in vitro* cytotoxicity assays against two B-ALL cell lines, 697 and Nalm6. First, mock-electroporated γδ T cells or CD19 CAR-expressing γδ T cells were cocultured with 697 cells for 4 h at E:T ratios of 0.5:1, 1:1, 2:1, and 5:1 and the percent

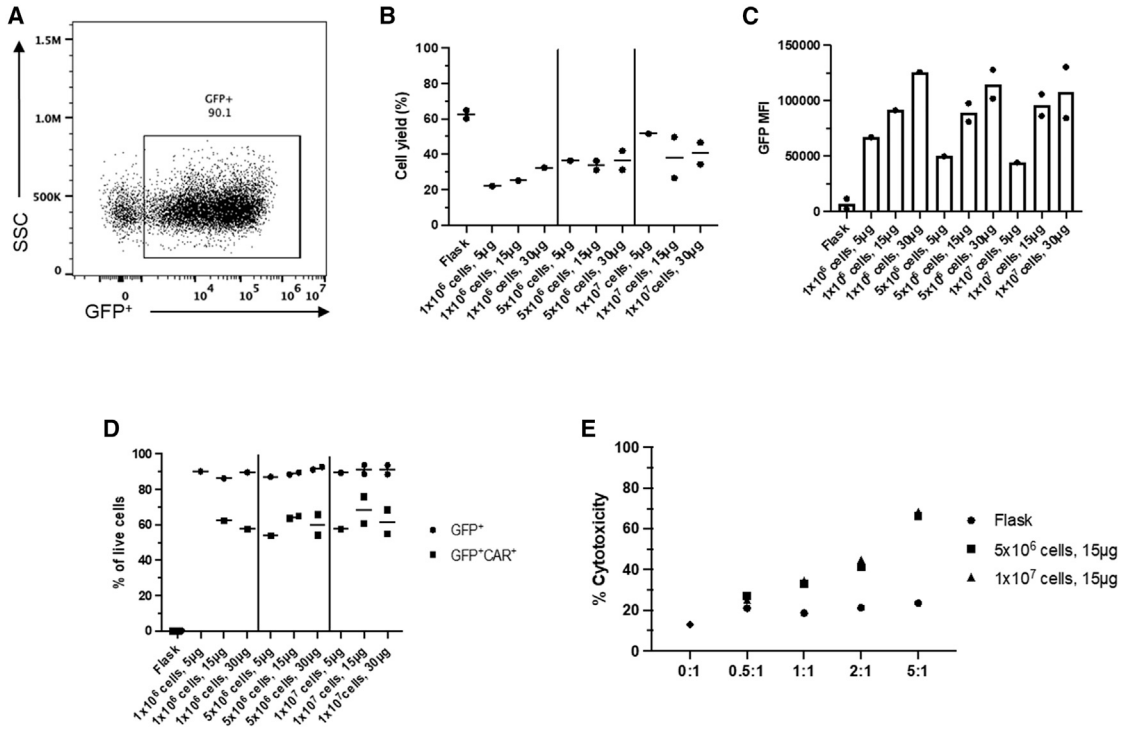

Figure 1. Optimization of γδ T cell electroporation with bicistronic CD19 CAR-GFP mRNA

(A) γδ T cells express GFP after mRNA electroporation using the BioRad Gene Pulser Xcell Electroporator. γδ T cell electroporation was optimized by testing varying cell numbers and mRNA amounts in each reaction. (B) Cell yield, calculated by determining the proportion of live cells remaining 24 h after electroporation to the starting number of cells used for the electroporation reaction, was calculated for all reaction conditions and increased as the cell number increased. (C) GFP mean fluorescence intensity (MFI) was determined by flow cytometry and increased with increasing amounts of mRNA. (D) The percentage of live cells expressing GFP and the CD19 CAR was similar for all conditions and was found to be about 90% and 60%, respectively. (E) γδ T cell cytotoxicity was determined by flow cytometry to test two promising electroporation reaction conditions and found no difference when comparing different cell numbers in each reaction.

cytotoxicity was measured by flow cytometry (Figure 3A). The percent cytotoxicity of the CD19 CAR-expressing γδ T cells increased with increasing effector cells, reaching 85% at the 5:1 E:T ratio, while the mock-electroporated γδ T cells remained constant at <20%. To further examine the effect of engineering γδ T cells, cytotoxicity assays were performed using γδ T cells engineered with a CD22 CAR against the same cell line (Figure 3B). Similar to the CD19 CAR-expressing γδ T cells, the cytotoxicity of the CD22 CAR-expressing γδ T cells increased with increasing E:T ratios, reaching 82% at the 5:1 E:T ratio.

To further confirm the efficacy of the engineered γδ T cells, CD19 CAR- and CD22 CAR-expressing γδ T cells were tested against a second B-ALL cell line, Nalm6 (Figure 3C). Mock-electroporated γδ T cells again had a constant cytotoxicity percentage across all E:T ratios and averaged approximately 6%. In contrast, CD19 CAR- and CD22 CAR-expressing γδ T cells exhibited a dose-dependent increase in cytotoxicity, reaching 76% and 43% at the 5:1 ratio, respectively, demonstrating (1) the CD19 CAR-engineered γδ T cells effectively kill B-ALL cell lines *in vitro* and (2) CD19 CAR-engineered γδ T cells are slightly more effective than CD22-based CARs against Nalm6 cells. This difference in cytotoxicity can be explained by lower CD22 expression in Nalm6 cells, compared to CD19 expression.[40]

Electroporation of sBite mRNA enhances the effectiveness of γδ T cells

Co-administration of γδ T cells with bispecific T cell engagers have shown great promise in preclinical cancer models.[41–43] To test whether γδ T cells engineered to secrete a CD19 bispecific T cell engager would enhance cytotoxicity toward CD19$^+$ tumors, we first developed an mRNA construct using the scFv portion of the CD19 CAR and linked it to an scFv specific to CD3 (Figure S1). sBite secreted by γδ T cells electroporated with 3–15 μg of CD19 sBite mRNA was measured by ELISA (Figure 4A). γδ T cells secrete 15 ng/mL of the sBite with as little as 3 μg mRNA and reached 80 ng/mL when using our standard 15 μg of mRNA. Western blot analysis of media conditioned by sBite mRNA transfected γδ T cells indicated the sBite was of the expected molecular weight and was detected after as little as 4 h of culture (Figure S4). To test whether engineering γδ T cells with CD19 sBite mRNA increases their cytotoxic capability, unmodified and sBite-modified γδ T cells were cocultured with several CD19-positive B-ALL and lymphoma cell lines in a cytotoxicity assay. As expected, the unmodified γδ

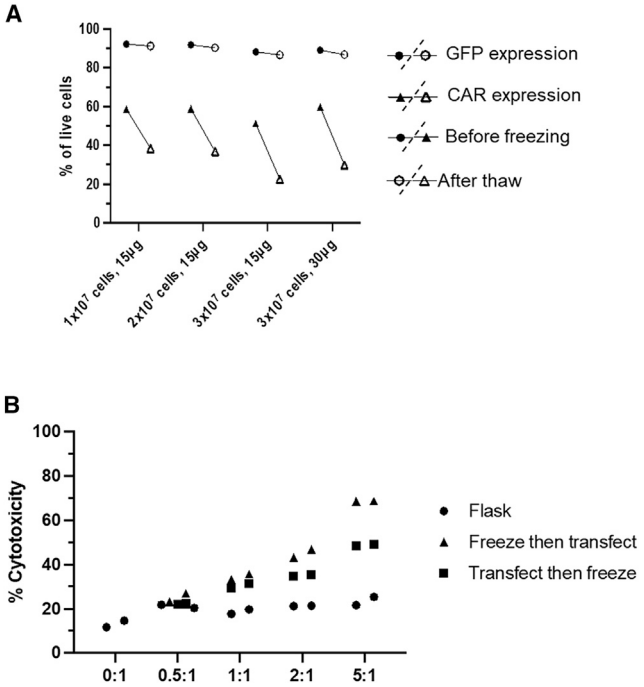

Figure 2. Electroporation of γδ T cells before freezing results in lower CAR expression and reduced cytotoxicity

γδ T cells were electroporated on day 12 of expansion and were analyzed before and after freezing. (A) While GFP expression (circles) remained constant at around 90% before and after freezing, the CAR percentage (triangles) decreased from about 60% before freezing to about 30%–40% after thawing. Closed data points denote before freezing and open data points denote after thawing. (B) The cytotoxicity of CD19 CAR-expressing γδ T cells before and after freezing was also measured to determine if a freeze/thaw cycle effects the cytotoxicity of the engineered cells. Similar cytotoxicity was observed at low effector to target (E:T) ratios; however, there was a reduction at higher E:T ratios for the thawed engineered cells.

T cells had a modest increase in cytotoxicity with increasing E:T ratios. In contrast, CD19 sBite-modified γδ T cells exhibited increased cytotoxicity for all cell lines and every E:T ratio (Figure 4B). Next, we tested the specificity of the CD19 sBite using the 697 cell line and a CRISPR-generated CD19KO 697 cell line. The CD19 sBite-secreting γδ T cells showed greater cytotoxicity toward $CD19^+$ 697 cells compared with unmodified γδ T cells, a difference not observed with CD19KO 697 target cells (Figure 4C). Therefore, the CD19 sBite secreted by the γδ T cells enhanced γδ T cell anti-tumor efficacy in a CD19-specific manner.

One of the major advantages of engineering γδ T cells with sBites rather than CARs is that sBites can bind to and activate unmodified T cells. To test this concept, conditioned media was collected from unmodified and CD19 sBite-modified γδ T cells approximately 16 h after mRNA electroporation. The conditioned media was then mixed with unmodified or sBite-modified γδ T cells and cocultured with 697 cells (Figure 4D). As expected, the CD19 sBite-modified cells exhibited increased cytotoxicity regardless of the conditioned media. Notably, mixing sBite-conditioned media with unmodified cells improved their cytotoxicity compared with mixing unmodified cells with unmodified conditioned media. These results indicate that γδ T cells electroporated with CD19 sBite mRNA secrete CD19 sBite that enhances the cytotoxicity of modified, as well as unmodified γδ T cells.

In vivo trafficking and growth of 697 cells

The 697 cell line provides a reasonable model for *in vivo* testing of CD19-based CARs and sBites, as CD19 expression is high (data not shown). Although 697 cells expand robustly in NSG mice, we show they (1) rapidly leave the blood stream after infusion, (2) home to the bone marrow, and (3) form avascular tumor nodules, especially in the liver (Figure 5A). Tissues were collected from NSG mice 3 weeks after intravenous injection with 697 cells. Samples from blood, bone marrow, spleen, and liver were analyzed for the presence of cancer cells using flow cytometry and histopathology. There were substantial numbers of $CD45^+CD3^-$ populations (i.e., 697 cells) in the bone marrow and a low percentage in the spleen and negligible numbers in the blood (Figure 5B). In addition, hematoxylin and eosin staining of tissues revealed sheets of neoplastic lymphocytes in the brain, liver, lungs, and kidneys, with avascularized nodules found within the liver (Figure 5C). Gross pathological examination found miliary patterns with white foci on the liver (Figure S5A).

In contrast to 697 growth *in vivo*, flow cytometry analysis of samples from mice administered γδ T cells showed limited $CD45^+CD3^+$ (i.e., γδ T cells) infiltration in the bone marrow, compared with the blood and spleen (Figure S5B). Taken together, these results show 697 cells form non-vascularized pockets of cancer cells within a wide range of organs. Once seeded in these peripheral compartments, it may be challenging for engineered γδ T cells to penetrate the 697 tumor nodules. In general, (1) cellular therapies require vascularized tumors and (2) γδ T cells do not efficiently migrate to the mouse bone marrow, so it would be predicted that the timing of γδ T cell administration is critical.[44,45] Also, it can be predicted that early treatment could be effective, but treatments administered after seeding would be less successful, as engineered γδ T cells would be unable to control cancer progression once the cancer cells leave circulation.

Engineered γδ T cells reduce tumor burden and improve survival in NSG B-cell leukemia mouse models

To test the efficacy of engineered γδ T cells *in vivo*, the 697 B-ALL mouse model was first used. Luciferase-expressing 697 cells were intravenously injected into the tail vein of NSG mice. The mice were treated twice a week for 2 weeks with CD19 CAR-expressing γδ T cells starting 1 day after cancer cell injection. Bioluminescence imaging was performed over the course of the experiment. Treating mice with CD19 CAR-expressing γδ T cells delayed tumor progression and significantly lowered tumor burden, as seen in the bioluminescence images and measured by raw flux values, compared with control mice (Figures 6A and 6B). In addition to reducing tumor burden, treating with the CD19 CAR-engineered γδ T cells also improved survival (Figure 6C). In contrast, as predicted, treatment of mice 7 days after tumor administration had no effect on overall tumor burden (Figure S6).

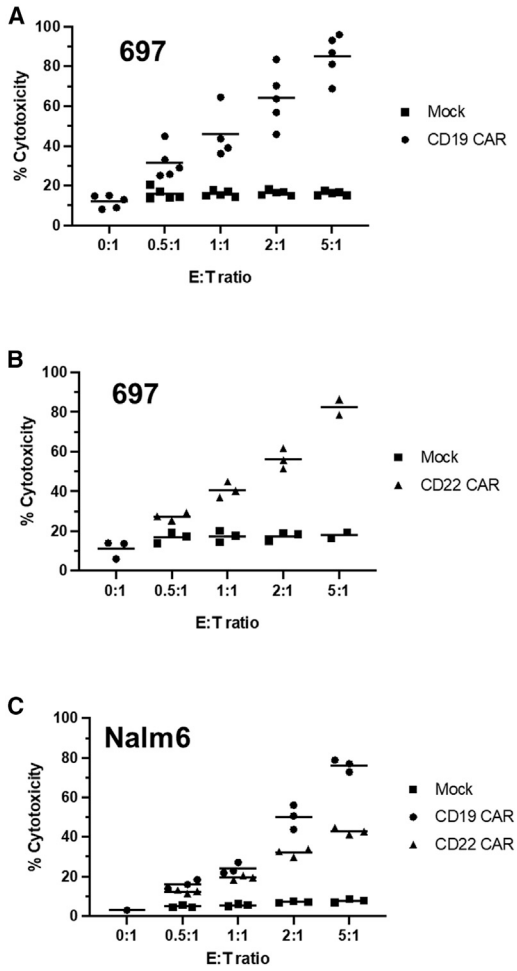

Figure 3. CD19 CAR- and CD22 CAR-expressing γδ T cells enhances cytotoxicity against two B-ALL cell lines

Effector and target cells were cocultured at the specified E:T ratio for 4 h and the percent cytotoxicity was determined by flow cytometry. Target cells were stained with VPD450 to differentiate effector and target cell death. Mock-electroporated, CD19 CAR-, and CD22 CAR-expressing γδ T cells were tested against the B-ALL cell lines 697 (A and B) and Nalm6 (C). While the cytotoxicity of mock-electroporated γδ T cells remained constant over all E:T ratios, CD19 CAR- and CD22 CAR-expressing γδ T cells exhibited a dose-dependent increase in cytotoxicity.

To further test the efficacy of CD19 CAR-engineered γδ T cells and compare CD19 sBite-engineered γδ T cells *in vivo*, a second *in vivo* model was established using luciferase-expressing Nalm6 cells. Mice were treated twice a week for 2 weeks and started 1 day after cancer cell injection. Treatments included either unmodified γδ T cells, CD19 CAR-expressing γδ T cells, or CD19 sBite-modified γδ T cells. Prior to administering the engineered cells, CAR expression was about 60%, as determined by flow cytometric detection of CD19Fc binding to γδ T cells (Figure 7A). Interestingly, γδ T cells engineered with CD19 sBite mRNA also bound the CD19Fc with about 40% of cells CD19Fc^{+}. In addition, the engineered cells were also tested for their cytotoxic capability against the Nalm6 cell line using an *in vitro* cytotoxicity assay at E:T ratios of 1:2 and 2:1, which showed consistent killing with our previous *in vitro* experiments (Figure 7B). Mice treated with unmodified γδ T cells exhibited a high tumor burden as early as 1 to 2 weeks after cancer cell injection. Mice treated with CD19 CAR- or CD19 sBite-engineered γδ T cells exhibited significantly delayed tumor progression and reduced tumor burden (Figures 7C and 7D). A survival benefit was also observed with mice treated with CD19 CAR and CD19 sBite γδ T cells, compared with unmodified γδ T cells (Figure 7E). Dual CAR T cells are becoming a promising direction for immunotherapies and have been shown to improve CAR T cell killing and limit acquired resistance as they target two different antigens on cancer cells. To examine the effectiveness of dual CAR γδ T cells, mice bearing Nalm6 cancer cells were also treated twice a week for 2 weeks with γδ T cells expressing both the CD19 CAR and CD22 CAR. Compared with the CD19 CAR alone or CD19 sBite, no added benefit was observed by co-expressing CD19/CD22 CARs based on bioluminescence imaging and survival (Figure S7). The results from these two *in vivo* B-ALL models show engineering γδ T cells with either CD19 CAR or CD19 sBite mRNA effectively delays tumor progression, decreases tumor burden, and improves survival.

Increasing the dose and frequency of treatments does not enhance survival

To determine if increasing the frequency and duration of CD19 sBite-engineered γδ T cell administration would further reduce tumor burden and increase the survival benefit, mice were injected with luciferase-expressing Nalm6 cells and treated with three doses of CD19 sBite γδ T cells per week for the first 2 weeks, compared with the previous twice a week for 2 weeks regimen. In addition, this was followed by two doses per week during weeks 2 and 3 and finally one dose per week for the final 2 weeks of treatment (Figure 8A). Even with increasing the number of doses in the first 2 weeks and adding additional doses, the *in vivo* tumor growth was similar compared with the previous Nalm6 experiment (Figures 8B and 8C). A slight increase in survival was observed, but the difference was not significant compared with the less aggressive treatment regimen (Figure 8D).

DISCUSSION

Developing novel immunotherapies that are effective and safe is a critical step in advancing cancer therapeutics. ACT is among the most promising developments for treating cancer, as these treatment strategies provide the ability to repopulate the patient's immune system with functional and potent anticancer immunocompetent cells. γδ T cells are well-suited for ACT, as they bridge the gap between the innate and adaptive immune system. In fact, based on a large pan-cancer molecular profiling study, γδ T cell infiltration was identified as the best prognostic marker for favorable outcomes.[46] Their ability to detect antigens in an MHC-independent manner gives them advantages over αβ T cells because they are able to be used in allogeneic settings, and they target cancer through endogenous stress markers and phosphoantigen

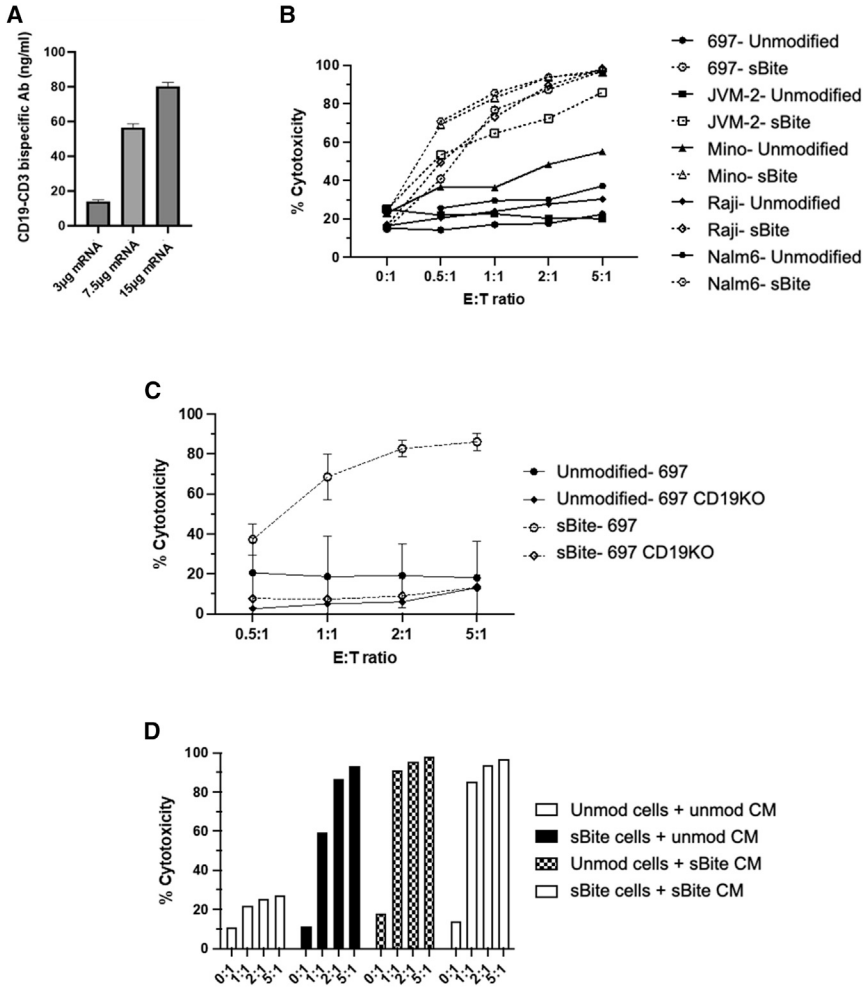

Figure 4. γδ T cells express and secrete CD19 sBite after mRNA electroporation

(A) γδ T cells were electroporated with 3 µg, 7.5 µg, and 15 µg of mRNA and the amount of CD19 sBite in the conditioned media was determined using an ELISA. (B) Unmodified and CD19 sBite-modified γδ T cells were cocultured with several $CD19^+$ cancer cells lines to test their cytotoxic capabilities. sBite-modified γδ T cells showed increased cytotoxicity at all E:T ratios and reached about 90% at the 5:1 ratio. (C) A CD19KO 697 cell line was generated using CRISPR to test the specificity of the secreted CD19 sBite. As expected, the sBite-modified γδ T cells showed improved cytotoxicity against the naive 697 cell line; however, no increase in cytotoxicity was seen when the CD19KO 697 cell line was used as target cells. (D) To test whether the CD19 sBite can induce killing of unmodified cells, conditioned media from unmodified and sBite-modified γδ T cells was collected after 16 h of culture and mixed with either unmodified or sBite-modified γδ T cells. As expected, the sBite-modified cells showed improved cytotoxicity, regardless of the conditioned media. The unmodified cells cultured with the sBite-conditioned media showed improved cytotoxicity, compared with unmodified cells with unmodified conditioned media.

expression that are typically upregulated in cancer cells.[47] In addition to their rapid immune response capabilities, γδ T cells are also involved in recruiting and priming other immune cells that can increase anticancer responses. For these reasons, γδ T cells are a promising ACT platform with great potential to improve cancer therapeutics.

Engineering γδ T cells has traditionally been an important, albeit challenging, step toward developing more effective γδ T cell therapies. Here, we test a therapeutic platform to engineer γδ T cells using mRNA electroporation to improve their cytotoxicity. First, mRNA electroporation of γδ T cells was optimized using a CD19 CAR-GFP construct. Based on the measurement of several parameters, we found rational conditions for modifying γδ T cells and confirmed the engineered cells were functional in cytotoxicity assays against B-ALL cell lines. We then tested whether this platform can be used to engineer γδ T cells to secrete a functional bispecific T cell engager through mRNA electroporation, and indeed showed sBites are secreted and significantly improved γδ T cell cytotoxicity. In addition, sBites can improve the cytotoxicity of unmodified γδ T cells. We then showed, using two B-ALL mouse models, that the engineered γδ T cells are effective at prolonging tumor progression, reducing tumor burden, and improving survival.

mRNA technologies are rapidly improving for a wide range of applications, especially in light of the recent success of COVID vaccines, and mRNA electroporation has emerged as a promising option for genetically engineering immune-competent cells.[48,49] The transient nature of electroporation in combination with γδ T cells offers several advantages over stable integrating vectors and may even alleviate some of the limitations of CAR T cell immunotherapies. For example, cytokine release syndrome is a significant obstacle for CAR T cell patients and leads to harmful side effects and early treatment termination. Transient engineering of γδ T cells offers a solution as the transient nature of the modification would limit the risk of the immune system's overactivation.[50] Also, γδ T cells do not form memory responses so their persistence is typically limited to weeks, which means treatment can be halted if treatment-related adverse effects were induced. Another limitation to traditional CAR T cell therapies is the development of acquired resistance through antigen escape. The ability for γδ T cells to detect stressed cells and phosphoantigen expression allows for added cytotoxic capabilities beyond CAR activation. This characteristic of γδ T cells can also be used in the context of combination therapy with chemotherapeutics that have been shown to upregulate some of the stress markers on cancer cells that are detected by γδ T cells.[37]

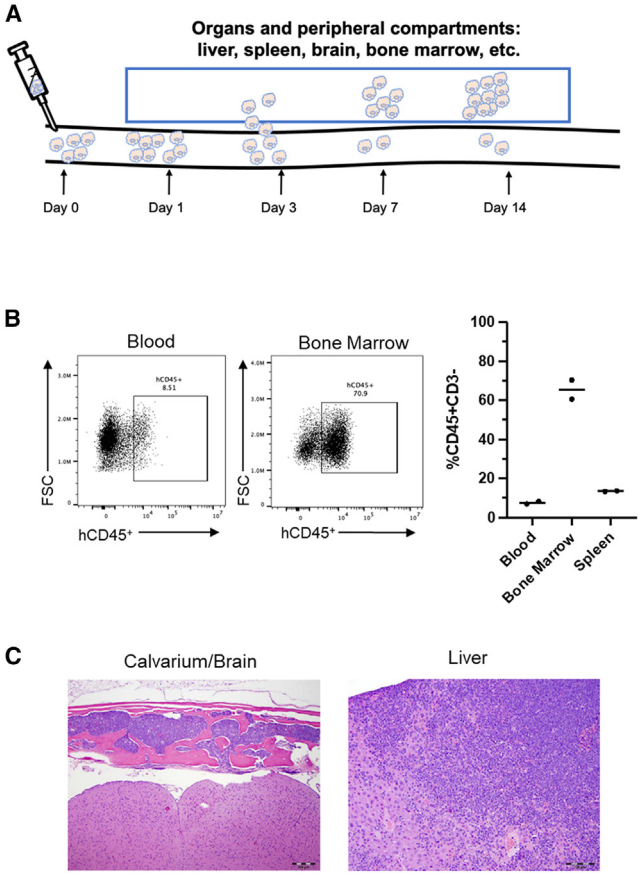

Figure 5. γδ T cells are not able to kill cancer cells once they extravasate from circulation

(A) In this cancer model, cancer cells are injected i.v. and gradually leave the circulation and form non-vascularized nodules in various organs and other compartments, leaving few cancer cells in circulation by days 3 and 7. Based on this model, it can be predicted that the timing for γδ T cell treatment is important in treating mice bearing the 697 cancer cell line. (B) Tissue samples from blood, bone marrow, and spleen were collected 3 weeks after cancer cell injection to detect the presence of cancer cells in each compartment (left flow plots are representative). A substantial number of $CD45^{+}CD3^{-}$ 697 cells were detected in the bone marrow, while limited numbers were found in the blood and spleen. (C) Representative hematoxylin and eosin staining images showing the presence of cancer cells with no vasculature around the cancer cells. Histopathological analysis revealed the presence of cancer cells in the brain, liver, lungs, and kidneys, with avascularized nodules found within the liver.

Although this therapeutic platform alleviates many of the side effects and obstacles of traditional CAR T cells, a limitation of the transient engineering technique is the short duration of CAR and sBite expression, which may limit the length of therapeutic efficacy. The duration of expression after mRNA electroporation in hematopoietic cells has been widely studied and has been found to last between 5 and 7 days, with peak expression occurring after 24–48 h.[51–53] Despite being short compared with stable integrating vectors, this is less of an issue when using γδ T cells because their persistence is limited *in vivo* as they do not mount long-term memory responses. The short half-life of mRNA expression and limited persistence of γδ T cells will need to be addressed clinically but can be countered by increasing the number of doses.

Currently, the most common engineering platform for immune-competent cells is the introduction of complementary DNA to express CARs. However, treatment and combination therapy utilizing bispecific T cell engagers is effective for several cancer indications.[54] They use the specificity of the scFv portion of an antibody to bridge T cells and cancer cells by binding to the CD3ε fragment of T cells and an antigen on cancer cells. This binding activates T cells, increases cytotoxicity, and induces immunocompetent cellular proliferation. There have been some studies investigating the combination of γδ T cells and bispecific T cell engagers, which have shown improvement in cytotoxicity against several types of cancer.[41–43,55,56] This study tested a novel technique of expressing sBites in this immune-competent cell, instead of co-administration. Having the cells secrete the sBites offers several advantages over co-infusing the cells with recombinant bispecific T cell engager protein. An obstacle for these infused therapies, and other non-IgG-like bispecific antibodies, is their short half-life and need for multiple (indeed sometimes continuous) infusions. Engineering γδ T cells using electroporation with sBite mRNA allows for continuous expression as long as the mRNA is within the cells. In addition, having the immune-competent cells secrete the sBite allows for a more targeted treatment approach because, in contrast to systemic administration, the sBite is secreted locally where it can be most utilized by immune cells. This concept can be coupled with modulation of chemokine receptor expression on the T cells. For example, modifications to overexpress chemokine receptors on T cells can enhance their migration to the site of the cancer.[57–59] This can be done in a targeted approach, as certain cancers are known to express certain chemokines and receptors.

Despite showing signs of success, a limitation of this platform is induction of survival benefits without complete cures. Although we think this is specific to the *in vivo* models, we did thoroughly pursue treatment timing strategies, for example, long durations of treatment and aggressive upfront regimens were tested but provided little improvement. For example, sBite-modified γδ T cells performed similarly in the protracted or extended regimens, showing that increased treatments over longer periods did not improve survival. A priori, this was predicted, as the cancer cells quickly leave the circulation and seed in compartments that are not easily reached by the γδ T cells. γδ T cells are most abundant in the blood followed by the spleen and bone marrow. In contrast, our cancer cell lines are most abundant in the bone marrow with very few in the blood. To investigate this hypothesis, tissue samples from mice bearing 697 cancer were collected and analyzed. We found increased presence of cancer cells in the bone marrow and spleen, compared with the blood. In addition, based on a histopathological examination, neoplastic sheets of lymphocytes were found in many organs, including the brain, liver, lungs, and kidneys. No presence of vasculature was found surrounding the cancer cells, which suggests the

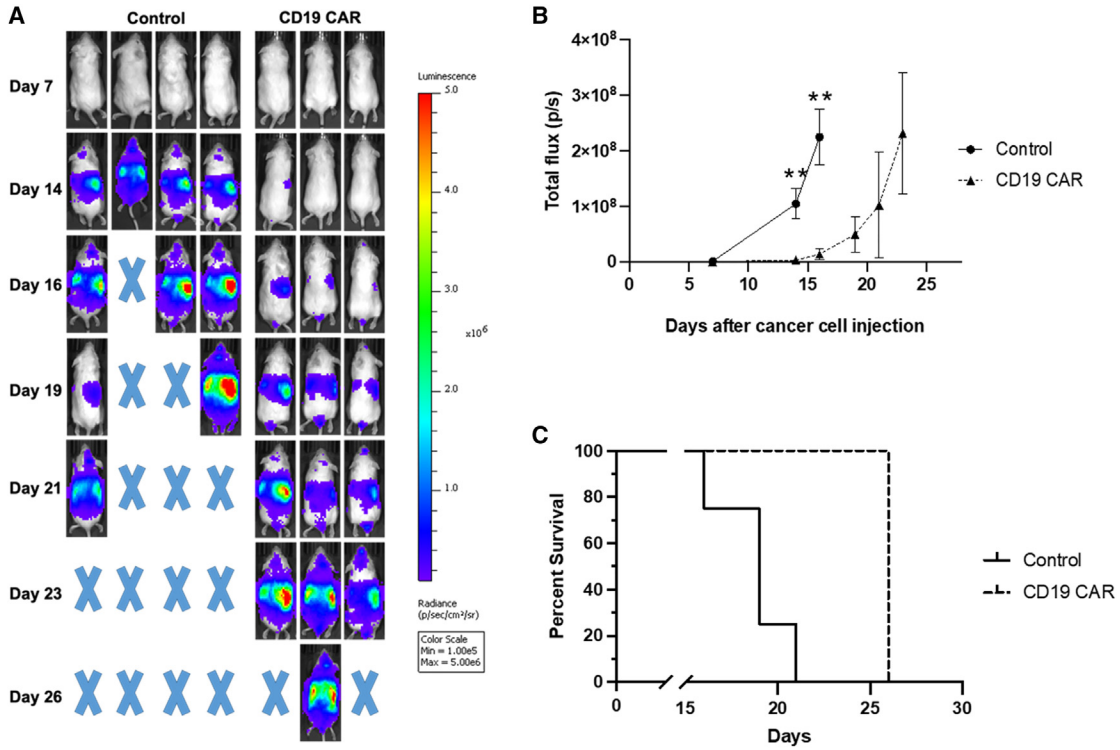

Figure 6. Engineered γδ T cells expressing a CD19 CAR reduce tumor burden and improve survival in the 697 model

(A) NSG mice were injected with 2 × 10^6 luciferase-expressing 697 cells and bioluminescence images were captured during the course of the experiment. Mice treated with CD19 CAR-expressing γδ T cells on day 1 of the experiment showed a reduction in tumor burden compared with control mice. (B) Raw total flux values were calculated and showed delayed tumor progression and significantly reduced tumor burden for mice treated with the CD19 CAR-expressing γδ T cells (triangles), compared with the control mice (circles). Statistics were performed using a 2-tailed Student's t test to compare experimental groups at each given time point. (C) Kaplan-Meier survival curves showed significantly increased survival in mice treated with CD19 CAR-expressing γδ T cells (dashed line), compared with control mice ($p = 0.02$ by log rank test). Control: n = 4; CD19 CAR: n = 3; error bars indicate standard deviation; **$p < 0.01$.

γδ T cells do not efficiently access these sites. As mentioned above, the expression of specific chemokine receptors on γδ T cells can be used to improve the migratory pathways of the cells *in vivo*. For example, this concept can be utilized to express CXCR4 to enhance the migration of γδ T cells to the bone marrow.

This study investigated the potential of mRNA electroporation as a therapeutic platform to engineer γδ T cells with either CARs or sBites. We utilized CD19 as the target antigen, the most commonly studied target for immunotherapy. However, it can be anticipated that this therapeutic platform can be applied to many cancers and may be especially beneficial to those where long-term CAR T persistence is detrimental, such as targeting antigens that are not cancer specific. Overall, these results show γδ T cells can be modified with CAR or sBite mRNA through electroporation and the engineered γδ T cells have improved cytotoxicity against cancer, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

The luciferase-expressing 697 cell line was kindly provided by the laboratory of Dr. Douglas Graham (Emory University) and the luciferase-expressing Nalm6 cell line was kindly provided by the laboratory of Dr. Christopher Porter (Emory University). The CD19 knockout 697 cell line was developed at Expression Therapeutics, Inc using CRISPR that was directed by a CD19-directed guide RNA. All cell lines were cultured in RPMI (Corning) with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator.

γδ T cell expansion

γδ T cell expansions were performed based on our previously published technique.[20,21] Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from healthy donor blood through the Children's Clinical Translational Discovery Core at Emory University under the core's approved institutional review board protocol or ordered directly from AllCells. PBMCs were isolated from fresh blood using Ficoll-Paque Plus density centrifugation. To preferentially expand γδ T cells, PBMCs were cultured in OpTmizer containing OpTmizer supplement, 1% penicillin/streptomycin, and 2 mM L-glutamine (complete OpTmizer). Cells were counted and resuspended at 2 × 10^6 cells/mL in fresh media every 3 days. On days 0 and 3 of expansion, 5 μM Zoledronate and 500 IU/mL

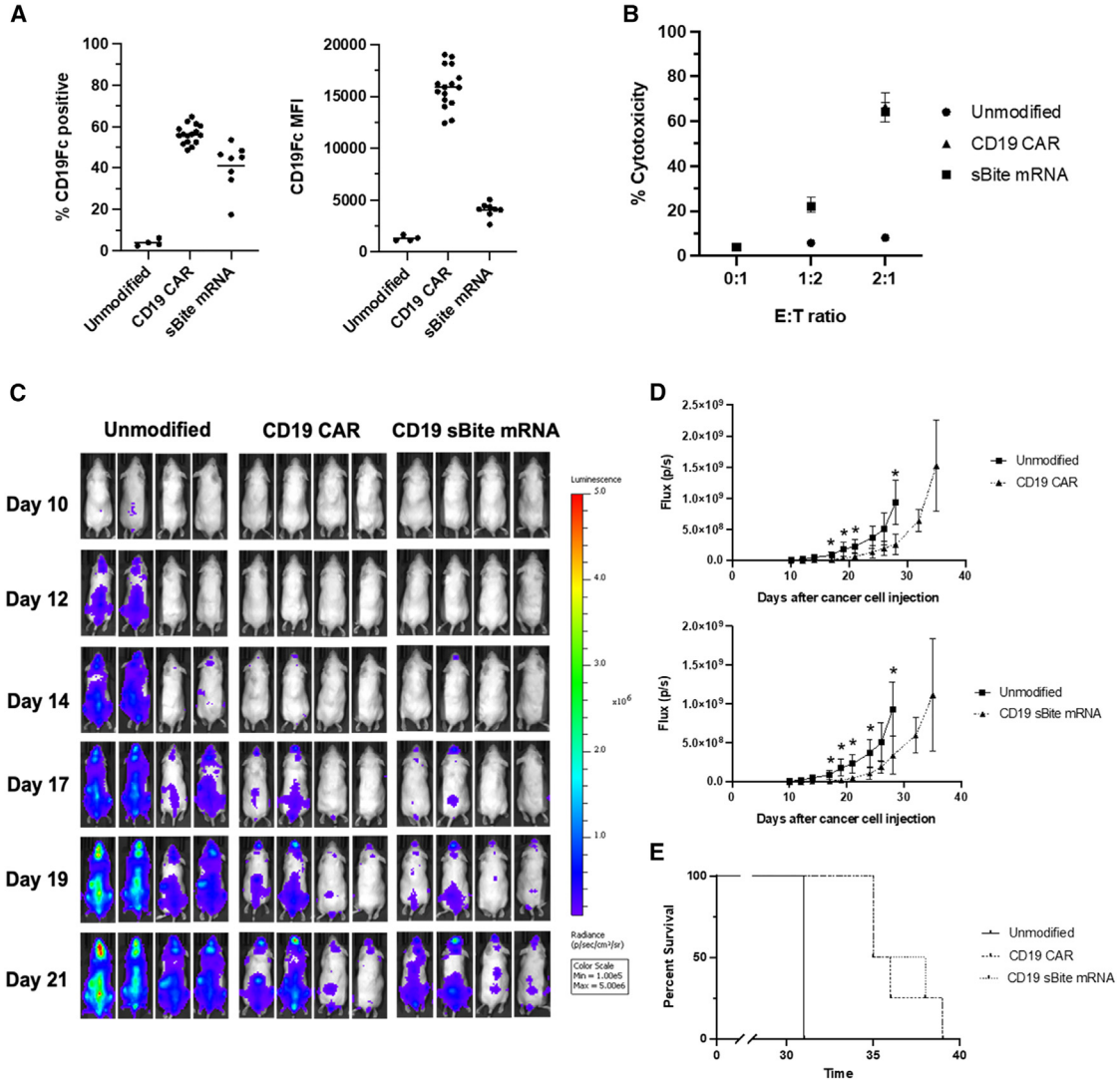

Figure 7. Engineering γδ T cells with CD19 CAR or sBite mRNA reduces tumor burden and improves survival in the Nalm6 model

NSG mice were injected with 2×10^6 luciferase-expressing Nalm6 cells and were treated with unmodified, CD19 CAR-modified, or CD19 sBite-modified γδ T cells on day 1 of the experiment with a treatment regimen of twice a week for 2 weeks. (A) Before injection, unmodified or modified γδ T cells were analyzed for CAR expression and MFI using flow cytometry. CAR expression was about 60% for CD19 CAR-expressing γδ T cells and, interestingly, the CD19 sBite-modified γδ T cells bound to the CD19Fc, with an average of about 40% CD19Fc positive (left graph). Despite the CD19 sBite-modified γδ T cell binding to the CD19Fc, the MFI was minimal compared with the CD19 CAR (right graph). (B) The cytotoxicity of the unmodified and modified γδ T cells were also examined before injection at E:T ratios of 1:2 and 2:1. The CD19 CAR- and sBite-modified γδ T cells exhibited increased cytotoxicity compared with the unmodified γδ T cells. (C) Bioluminescent imaging was performed during the experiment and mice treated with unmodified γδ T cells showed a high tumor burden as early as 1 or 2 weeks after cancer cell injection. (D) Raw total flux was determined for each image and graphed over time to compare treatment with unmodified γδ T cells and CD19 CAR-expressing (top graph) or CD19 sBite-expressing γδ T cells (bottom graph). Treatment with modified γδ T cells resulted in delayed tumor progression and reduced tumor burden. Statistics were performed using a 2-tailed Student's t test to compare experimental groups at each given time point. (E) Kaplan-Meier survival curves were generated to compare survival for each treatment group to treating with unmodified γδ T cells. Treatment with CD19 CAR- and CD19 sBite-expressing γδ T cells resulted in a significant survival benefit compared with treating with unmodified γδ T cells ($p = 0.01$ for CAR and sBite by log rank test). n = 5; error bars indicate standard deviation; *$p < 0.05$.

interleukin (IL)-2 were added to the media. On days 6 and 9, 1,000 IU/mL IL-2 was added to the media. In addition, on day 6 of expansion, an αβ depletion step was performed, as previously published.[18] On day 12 of expansion, γδ T cells were either used fresh for experiments or frozen in PBS containing 5% human serum albumin (HSA) and 10% DMSO. Flow cytometry was performed on days 0, 6, 9, and 12 to confirm successful expansion and αβ depletion. Successful expansions resulted in cultures containing about 90% γδ T cells and 10% natural killer cells (Figure S8).

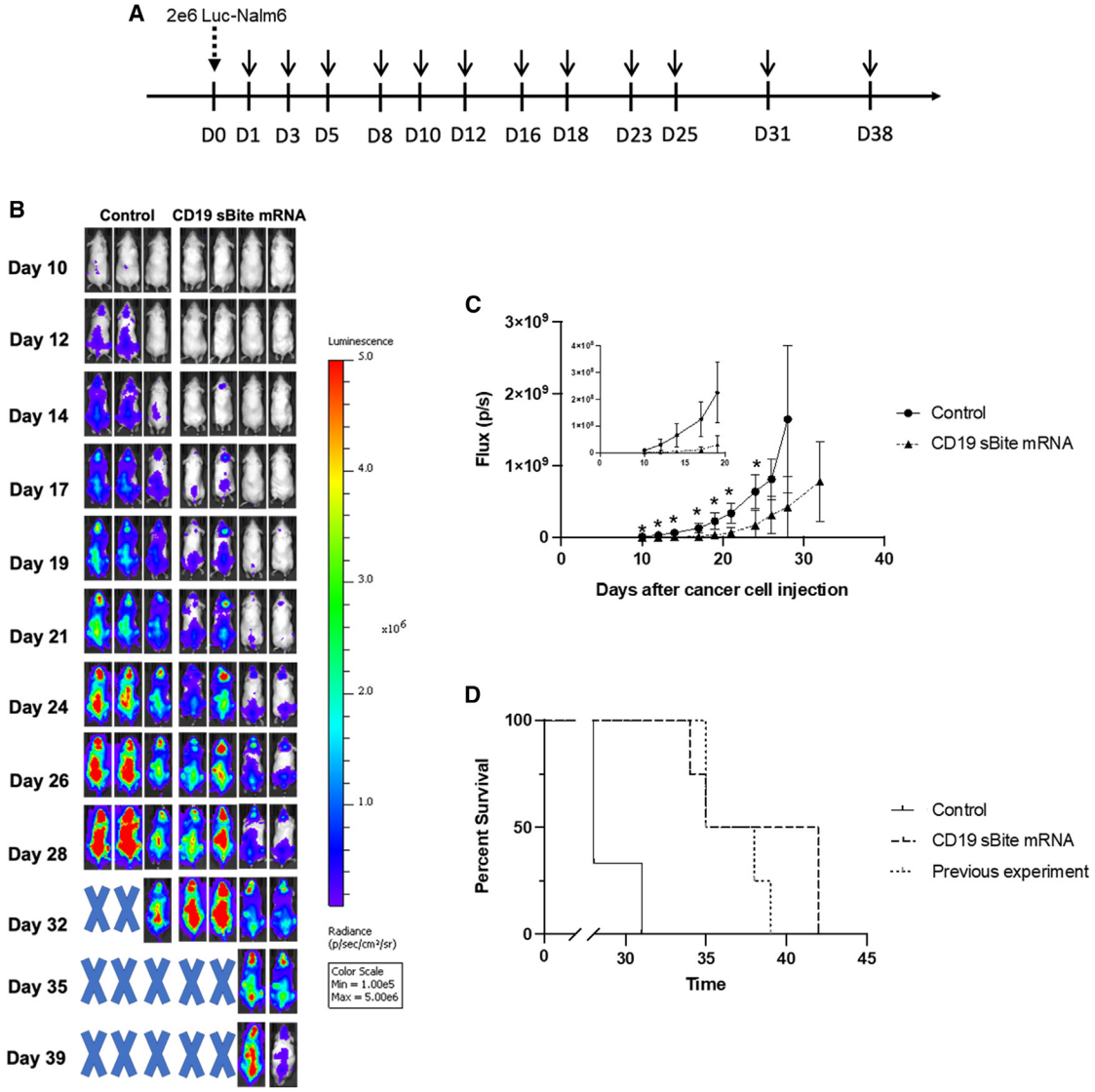

Figure 8. Longer treatment regimen does not lengthen survival benefit for Nalm6 model

Despite being significant, the survival benefit for the previous *in vivo* experiments was not as robust as the *in vitro* data would suggest. (A) To test whether a more extensive treatment regimen of three doses for the first 2 weeks, two doses for the next 2 weeks, and one dose for the final 2 weeks could further improve the survival benefit. (B) NSG mice were injected with 2×10^6 luciferase-expressing Nalm6 cells and treated with CD19 sBite-modified γδ T cells using the more extensive treatment regimen. Bioluminescent images were taken and again showed reduced tumor burden for the sBite-treated group, compared with the control group. (C) Graph of raw total flux shows the more extensive treatment regimen delayed tumor progression and reduced tumor burden, compared with control mice. The inset shows an expansion of the first 20 days of treatment. Statistics were performed using a 2-tailed Student's t test. (D) Kaplan-Meier survival curves were generated for the control group and more extensive treatment regimen of CD19 sBite-expressing γδ T cells. As expected, the more extensive treatment regimen resulted in a significant survival benefit compared with the control group ($p = 0.01$ by log rank test); however, there was no difference in survival when comparing the more extensive treatment regimen with the previous regimen of twice a week for 2 weeks ($p = 0.39$ by log rank test). Control: n = 3; sBite: n = 4; error bars indicate standard deviation; *$p < 0.05$.

Construction of mRNA expression vectors

To construct the mRNA expression vectors, plasmid DNA constructs were first cloned with the T7 promoter. The CD19 CAR and CD22 CAR consisted of the variable heavy and variable light regions of the FMC63 and M971-L7 antibodies, respectively. In addition to the scFv portion, the CAR constructs included a CD8 hinge, a CD28 costimulatory and transmembrane domain, and a CD3ζ signaling domain. The CD19 sBite plasmid consisted of the scFv portion of the FMC63 antibody for the CD19-specific region and the OKT3 for the CD3-specific region. Codon optimization was performed as previously published.[60] For mRNA production, DNA plasmids were first linearized, and the

mRNA was prepared using the mMessage mMachine T7 Ultra Kit (Life Technologies).

Electroporation

γδ T cells were either electroporated fresh on day 12 of expansion or from thawed cells that were frozen on day 12 of expansion. Cells were thawed in 5% HSA in PBS and were centrifuged at 250 × *g* for 10 min at room temperature. The cells were cultured at 4×10^6 cells/mL for 2 h in complete OpTmizer media with 1,000 IU/mL IL-2. Cells were then counted and the appropriate cell number for each reaction was aliquoted, washed twice with PBS, and resuspended in 100 μL OptiMEM (Life Technologies). The appropriate amount of mRNA was added to the tube and the mix was transferred to a 4-mm cuvette (Fisher Scientific). Electroporations using the BioRad's Gene Pulser Xcell Electroporator were conducted at 500 V for 5 ms using a square wave. Cells were collected from the cuvette and cultured overnight at 2×10^6 cells/mL in complete OpTmizer media with 1,000 IU/mL IL-2. Flow cytometry was used to confirm and analyze CAR expression after electroporation by labeling cells with a CD19-Fc fusion protein (AcroBiosystems) and an anti-IgG Fc secondary antibody (Jackson Immunoresearch Laboratories).

Cytotoxicity assay

A flow cytometry-based cytotoxicity assay was used to determine the cytotoxic capabilities of effector cells. Target cells were first stained with Violet Proliferation Dye 450 (BD Biosciences), in order to differentiate target cells from effector cells. Effector cells and target cells were cocultured at the specified effector to target ratios for 4 h at 37°C in 5% CO_2. The cells were then washed and resuspended in Annexin binding buffer (BioLegend) containing the early apoptosis stain Annexin V-APC (BioLegend). Right before analysis, 7AAD (BD Biosciences), a late apoptosis and necrosis marker, was added to differentiate live and dead cells. Cytotoxicity was calculated by adding the 7AAD and Annexin V single positive with the double-positive population of target cells.

ELISA and western blot

To measure and detect the presence of the CD19 sBite in culture media, an ELISA and western blot was performed. First, γδ T cells were electroporated and cultured overnight (∼16 h) and the conditioned media was collected. To perform the ELISA, streptavidin-coated plates (Fisher) were coated with biotinylated human CD3ε and CD3δ heterodimer protein with His/Avitag (Acro Biosystems). Conditioned media samples were then added to the plate, with an anti-CD19-anti-CD3 bispecific antibody (BPS Biosciences) used as a standard. Next, a CD19Fc fusion protein (R&D Systems) was added to the plates, followed by horseradish peroxidase (HRP) anti-human Fc (Jackson Labs). Finally, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Fisher) was added to the plate and the absorbance was measured at 450 nm.

To perform the western blot, γδ T cell conditioned media and anti-CD19-anti-CD3 bispecific antibody standards (BPS Biosciences) were prepared under reducing conditions. Next, separation by SDS-PAGE and transfer to a nitrocellulose membrane was performed. The blocked membrane was incubated with an anti-His antibody (R&D Research), followed by an HRP goat anti-mouse IgG secondary antibody (Abcam).

In vivo B-ALL models

All animal studies were conducted in accordance with Institutional Animal Care and Use Committee regulations. Eight-week-old NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Jackson Laboratory and housed in a pathogen-free facility. To establish the B-cell leukemia models, 2×10^6 luciferase-expressing 697 or Nalm6 cells were intravenously (i.v.) injected through the tail vein. Treatments with unmodified or engineered γδ T cells started 1 day after cancer cell inoculation and followed one of two treatment regimens, twice a week for 2 weeks or a more extensive treatment regimen as seen in Figure 7A. Each dose included 1×10^7 unmodified or engineered γδ T cells. Frozen γδ T cells were thawed and prepared for electroporation as described above. After electroporation, cells were cultured for 2.5 h in complete OpTmizer with 1,000 IU/mL IL-2. The cells were then washed twice with PBS, resuspended in fresh PBS at 1×10^7 cells/100 μL, and administered i.v. For *in vivo* imaging, mice were anesthetized with 2% inhaled isoflurane and bioluminescence images were taken with the IVIS Spectrum imaging system (PerkinElmer).

Tissue collection and analysis

Mouse tissue collection was performed at endpoint of the specified experiment. Mouse blood was collected via submandibular or retro-orbital veins in tubes containing 0.5M EDTA. Samples were centrifuged at 2,400 × *g* for 15 min at 4°C. The plasma layer was discarded, the pellet resuspended in 100 μL PBS, and three RBC lysis steps were performed. RBS lysis was conducted by adding 3 mL of RBC lysis buffer. The samples were then vortexed and incubated at room temperature for 10 min. The samples were centrifuged at 300 × *g* for 10 min and the supernatant was discarded. Samples were resuspended in 100 μL PBS and were stained for flow cytometry. Mouse spleens were processed by first pressing the tissue through a 40-μm mesh. The samples were then centrifuged at 300 × *g* for 10 min at 4°C. One RBS lysis step was performed as described above. Mouse livers were processed as previously published.[61] Briefly, livers were collected and placed in dishes containing PBS. The livers were pressed through a 70-μm mesh and then centrifuged at 30 × *g* for 3 min. The supernatant was collected and centrifuged again at 320 × *g* for 5 min. The cells were resuspended in 33% Percoll in PBS and centrifuged at 500 × *g* for 15 min with the break off. The cell pellet was resuspended in RBC lysis buffer and incubated at room temperature for 5 min. The samples were centrifuged at 300 × *g* for 10 min and resuspended in PBS.

Statistical analysis

All statistics were performed on GraphPad Prism 9. Unpaired two-tailed Student's t tests were used for statistical significance. A log rank (Mantel-Cox) test was performed on the Kaplan-Meier survival curves to determine significance between curves. Sample size is shown

on graphs as individual data points or specified for that experiment. Error bars represent standard deviation and statistical significance was defined as $p < 0.05$, unless otherwise stated.

DATA AVAILABILITY

All raw data are available upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.05.007.

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AUTHOR CONTRIBUTIONS

S.A.B. drafted the manuscript. S.A.B., B.P., H.B., S.S.R., C.D., and H.T.S. contributed to the conception, design of experiments, and data analysis. S.A.B, B.P., B.Y., and K.K. acquired the data. All authors reviewed, edited, and approved the final version.

DECLARATION OF INTERESTS

B.P., B.Y., and H.B. are employees of Expression Therapeutics, which is developing cancer treatments based on engineered γδ T cells. H.T.S. and C.D. have equity in Expression Therapeutics.

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