Molecular Therapy
Methods & Clinical Development
Original Article

CAR T Cell Generation by *piggyBac* Transposition from Linear Doggybone DNA Vectors Requires Transposon DNA-Flanking Regions

David C. Bishop,[1,2,3,4] Lisa Caproni,[5] Kavitha Gowrishankar,[1,4] Michal Legiewicz,[5] Kinga Karbowniczek,[5] John Tite,[5] David J. Gottlieb,[1,2,3,4,6,7] and Kenneth P. Micklethwaite[1,2,3,4,6]

[1]Westmead Institute for Medical Research, Sydney, NSW, Australia; [2]Department of Haematology, Westmead Hospital, Sydney, NSW 2145, Australia; [3]Blood and Bone Marrow Transplant Unit, Westmead Hospital, Sydney, NSW, Australia; [4]Sydney Medical School, The University of Sydney, Sydney, NSW, Australia; [5]Touchlight Genetics, Hampton, United Kingdom; [6]Sydney Cellular Therapies Laboratory, Westmead Hospital, Sydney, NSW, Australia; [7]Department of Medicine, Westmead Hospital, Sydney, NSW, Australia

CD19-specific chimeric antigen receptor (CAR19) T cells, generated using viral vectors, are an efficacious but costly treatment for B cell malignancies. The nonviral *piggyBac* transposon system provides a simple and inexpensive alternative for CAR19 T cell production. Until now, *piggyBac* has been plasmid based, facilitating economical vector amplification in bacteria. However, amplified plasmids have several undesirable qualities for clinical translation, including bacterial genetic elements, antibiotic-resistance genes, and the requirement for purification to remove endotoxin. Doggybones (dbDNA) are linear, covalently closed, minimal DNA vectors that can be inexpensively produced enzymatically *in vitro* at large scale. Importantly, they lack the undesirable features of plasmids. We used dbDNA incorporating *piggyBac* to generate CAR19 T cells. Initially, expression of functional transposase was evident, but stable CAR expression did not occur. After excluding other causes, additional random DNA flanking the transposon within the dbDNA was introduced, promoting stable CAR expression comparable to that of using plasmid components. Our findings demonstrate that dbDNA incorporating *piggyBac* can be used to generate CAR T cells and indicate that there is a requirement for DNA flanking the *piggyBac* transposon to enable effective transposition. dbDNA may further reduce the cost and improve the safety of CAR T cell production with transposon systems.

INTRODUCTION

Chimeric antigen receptors (CARs) are synthetic proteins that can be expressed on T cells to redirect their specificity to a chosen target antigen. A CAR consists of a single-chain variable fragment (scFv) that determines its specificity, a spacer domain that projects the scFv from the cell surface, a transmembrane domain, and intracellular costimulatory and CD3ζ T cell-activating domains.[1] CD19-specific CAR (CAR19) T cells have been highly effective in the clinic, inducing remissions in the majority of patients with relapsed and refractory B cell malignancies.[2–15]

The cost of CAR T cell production remains a major barrier to their widespread use.[16] Most CAR T cells to date have been produced using γ-retroviral or lentiviral transduction. The generation of these vectors is expensive, and the availability of vector at clinical grade is a major barrier to widespread implementation of CAR T cell therapy, a problem that will worsen as demand increases when effective CAR T cell therapies are developed for common cancers.

Transposon systems, such as *piggyBac*, represent economical, nonviral alternatives for the production of CAR T cells.[17–20] The *piggyBac* system is conventionally used as two plasmids, one encoding the transposase and the other encoding a gene of interest within the transposon, which are electroporated into cells. When expressed, the transposase excises the transposon from the second plasmid and integrates it into the cellular genome. We have previously described a simple and inexpensive method for generating CAR T cells using the *piggyBac* system[21] and demonstrated that CAR19 T cells produced in this manner are capable of eradicating B cell acute lymphoblastic leukemia (B-ALL) xenografts *in vivo*[22] and $CD19^+$ malignancies in humans.[23]

Plasmids can be produced inexpensively at large scale, but their reliance on bacteria for amplification has several disadvantages for clinical translation. Regulatory bodies remain concerned about the potential for antibiotic-resistance genes, included in plasmids as selection markers, to be horizontally transferred to pathogenic bacteria.[24] While in bacteria, there is a small chance of recombination events damaging the gene of interest, necessitating quality control for molecular integrity of each batch of plasmid.[25] Endotoxin must be removed from each batch of plasmid to ensure only amounts below the clinically acceptable threshold are present.[26] Finally,

Received 9 October 2019; accepted 21 December 2019;
https://doi.org/10.1016/j.omtm.2019.12.020.

Correspondence: Kenneth P. Micklethwaite, Department of Haematology, Westmead Hospital, Hawkesbury Road, Westmead, NSW 2145, Australia.
E-mail: kenneth.micklethwaite@sydney.edu.au

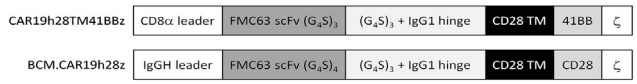

Figure 1. Schematic of Second-Generation CAR19 Constructs
Both CARs include an N-terminal leader sequence, an FMC63-derived scFv, an identical spacer domain, the CD28 transmembrane domain, a 4-1BB or CD28 costimulatory signaling domain, and CD3ζ. IgGH, IgG heavy chain; scFv, single-chain variable fragment; $(G_4S)_n$, a flexible linker comprised of Gly-Gly-Gly-Gly-Ser repeated n times; CD28 TM, CD28 transmembrane domain; ζ, CD3ζ signaling domain.

unmethylated CpG motifs in plasmid bacterial sequences can stimulate innate immune responses via Toll-like receptor 9 signaling pathways.[27–29] Each of these issues impacts the efficacy, cost, and risk associated with a clinical-grade plasmid.

Doggybones (dbDNA) are minimal DNA vectors that can be produced enzymatically *in vitro* at scale.[30–32] As production does not involve bacteria, the issues associated with plasmids are avoided. No origin of replication sequences or antibiotic-resistance genes is required, and the risk of recombination events within bacteria is eliminated. Large amounts of clinical-grade dbDNA can be produced more rapidly and for lower cost than an equivalent plasmid.

We therefore sought to transfer the *piggyBac* system from plasmid to dbDNA for production of CAR19 T cells. Although the *piggyBac* transposase was functional when expressed from dbDNA similar in structure to that used in other applications, our initial dbDNA encoding the *piggyBac* transposon required modification. Specifically, addition of random DNA sequences flanking the transposon within the dbDNA was required for effective transposition and CAR expression. After this modification, CAR19 T cells were successfully generated using *piggyBac* by electroporating primary human T cells with dbDNA encoding both components. We conclude that dbDNA is a viable alternative to a plasmid that even further reduces the risks and costs associated with *piggyBac*-generated CAR19 T cells for clinical applications.

RESULTS

CAR19 T Cells Can Be Generated Using *piggyBac* Transposase but Not Transposon Encoded by Nonoptimized, Standard dbDNA

Two second-generation CAR19 constructs, denoted BCM.CAR19h28z and CAR19h28TM41BBz to reflect their structure (Figure 1), were designed and used to evaluate the generation of CAR19 T cells with the *piggyBac* system in dbDNA. The *piggyBac* transposase utilized throughout was the hyperactive "*Super piggyBac*" variant. Linear dbDNA that included either *piggyBac* transposase or a CAR-containing transposon was produced enzymatically (see Materials and Methods) from parent plasmids (Figure 2).

We initially performed a pilot study to test the functionality of *piggyBac* components in dbDNA. Peripheral blood mononuclear cells (PBMCs), isolated from a single healthy donor, were coelectroporated with *piggyBac* transposase and transposon BCM.CAR19h28z, either in plasmid or dbDNA (equimolar amounts), such that all plasmid and dbDNA pairs were evaluated. The resulting cultures were selectively expanded for CAR19 T cells over 15 days via weekly CD19 stimulation and cytokine support.

From 8 days of culture onward, high levels of CAR expression could be detected on greater than 40% of T cells when either plasmid or dbDNA transposase was used in combination with the transposon plasmid. However, with transposon dbDNA there was minimal CAR expression, regardless of the transposase format utilized (Figures 3A and 3B). Similarly, culture expansion was noted with the transposon plasmid but not transposon dbDNA, regardless of the transposase format used (Figure 3C). These results indicated that there was a problem with the transposon dbDNA, rather than with expression of BCM.CAR19h28z itself.

Poor CAR Expression from Transposon in Standard dbDNA Is Not Due to Inability to Localize to the Nucleus

Nuclear entry of the transposon dbDNA is a critical requirement for both transient-nonintegrated CAR expression from dbDNA and also for integration of the CAR transposon into genomic DNA (gDNA) for long-term expression. We therefore investigated whether failure of the transposon dbDNA to enter the nucleus was the reason for poor CAR expression.

The Jurkat cell line is continually cycling, so dissolution of the nuclear membrane with mitosis offers an opportunity for nuclear entry of exogenous DNA.[33–36] Jurkat cells were electroporated with equimolar amounts of BCM.CAR19h28z transposon in either plasmid or dbDNA, with transposase plasmid. Despite an apparently equal opportunity for nuclear entry, clear CAR expression was observed with transposon plasmid but was lower with transposon dbDNA at both 24 h and 8 days postelectroporation (Figure 4A). The inferior expression from the transposon dbDNA in this setting suggested that the underlying problem was unrelated to nuclear entry.

To confirm that the transposon dbDNA is able to enter the nucleus in primary human T cells, we sought to detect CAR DNA in nuclear extracts. PBMCs from a healthy donor were electroporated with BCM.CAR19h28z transposon alone, in either plasmid or dbDNA, and were harvested after 24 h of culture. At this early stage following electroporation, flow cytometry confirmed expression of CAR in $CD3^+$ T cells only (Figure 4B). Because the elongation factor 1α (EF1α) promoter facilitates gene expression from a wide variety of cell types, the observed restriction of CAR expression to T cells confirmed that our previously optimized electroporation settings[21] remained selective for T cell transfection. PCR for a CAR-specific DNA sequence revealed the presence of CAR-transposon in whole-cell lysates and in both cytoplasmic and nuclear extracts, regardless of vector (Figure 4C). CAR-transposon is thus able to enter the T cell nucleus with either vector, excluding failure to enter the nucleus as the cause for poor CAR expression from transposon dbDNA.

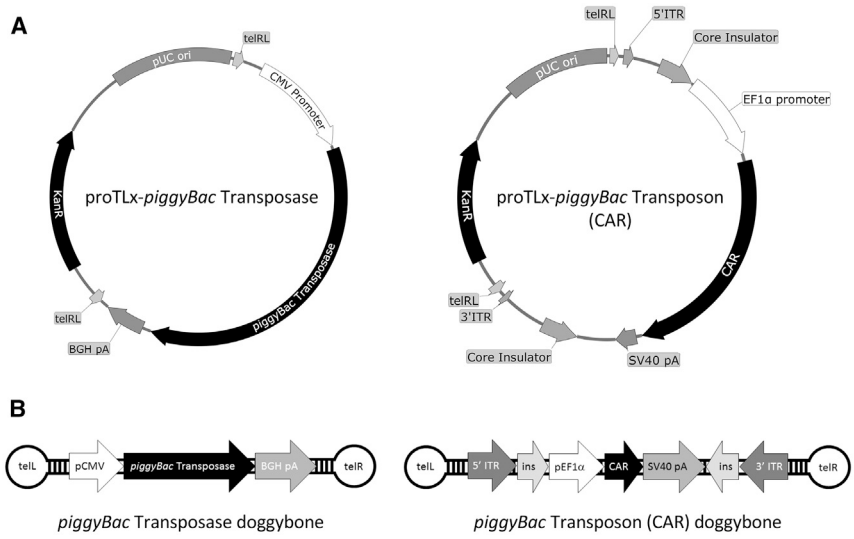

Figure 2. Parent Plasmids and dbDNA Constructs

(A) Maps of proTLx plasmids containing *piggyBac* transposase or transposon that were used as templates for enzymatic generation of dbDNA constructs. (B) Schematics of final linear double-stranded *piggyBac* transposase and transposon dbDNAs with terminal single-stranded hairpin loops. pUC ori, bacterial origin of replication sequence; CMV, cytomegalovirus; EF1α, elongation factor 1α; ITR, inverted tandem repeat; ins, core insulator; BGH, bovine growth hormone; SV40, simian virus 40; pA, polyadenylation sequence; KanR, kanamycin-resistance gene; CAR, chimeric antigen receptor; telRL, telomere resolution sites for TN protelomerase from prophage N15; telL and telR, covalently closed doggybone hairpin-like ends.

Increasing the Amount of Transposon dbDNA Electroporated Does Not Improve CAR Expression

We next investigated whether low CAR expression with transposon dbDNA could be overcome by increasing the amount of transposon dbDNA used. If this was possible, it could indicate poor efficiency in nucleofection or transposition. PBMCs were electroporated with a fixed amount of transposase plasmid and increasing amounts of BCM.CAR19h28z transposon dbDNA (up to 16-fold the amount equimolar to plasmid). BCM.CAR19h28z transposon plasmid alone (no transposase) was used as a control for nucleofection and nonintegrated CAR expression. The proportion of viable $CD3^+$ T cells on day 1 postelectroporation was noted to decrease with increasing amounts of transposon dbDNA, indicating a possible cytotoxic effect (Figure 5A). CAR T cells were selectively expanded in culture for 15 days, as previously outlined. At day 15, the proportion of T cells expressing CAR was negligible for all concentrations of electroporated transposon dbDNA other than that equimolar to the plasmid, where low levels of CAR expression were present (Figure 5B). Given previous results indicating that the transposon dbDNA is able to enter the nucleus, these findings suggested that failure of transposition from dbDNA was likely.

Protelomerase Binding Sites Do Not Impair Transposition

dbDNA contains protelomerase binding sites that are not present in the pVAX1 transposon plasmid (Figure 2), so it was important to exclude this difference as a possible cause of impaired transposition. Because the proTLx parent plasmid also contains protelomerase binding sites, additional failure of CAR expression from this DNA format would provide evidence that the protelomerase binding sites were problematic.

PBMCs were electroporated with BCM.CAR19h28z transposon in pVAX1 plasmid or in the proTLx dbDNA parent plasmid, with or without additional transposase plasmid, and CAR T cells were selectively expanded in culture. At day 8, cultures without transposase and hence, no CAR transposon genomic integration had negligible CAR expression. However, there was comparable CAR expression when transposon in either pVAX1 plasmid or dbDNA parent proTLx plasmid was used in combination with transposase (Figure 6).

Because the entire transposon dbDNA sequence is contained within the parent proTLx plasmid, these findings demonstrated that neither the protelomerase binding sites nor any other dbDNA sequence elements were impairing transposition. We hypothesized that either the short length of DNA flanking the transposon inverted tandem repeat (ITR) sequences or the loop structure of the dbDNA ends may be directly impairing transposase protein activity, preventing excision and integration of the transposon cassette. We reasoned that either of these could potentially be overcome through lengthening the DNA sequences flanking the transposon ITR.

CAR Is Stably Expressed from Transposon dbDNA with Longer Sequences Flanking the ITR

To investigate whether elongation of DNA sequences flanking the ITR in the dbDNA transposons would facilitate CAR expression, we introduced an extra 200 bp of random DNA sequence between the protelomerase binding site and ITR on either side of the transposon cassette. This increased the separation of these elements from <100 bp to approximately 230 bp. Because our previous work identified CAR19h28TM41BBz as our most effective construct,[22] this CAR was used for evaluation of the new dbDNA configuration. PBMCs were coelectroporated with *piggyBac* transposase (plasmid or dbDNA) and CAR19h28TM41BBz-transposon (plasmid or larger dbDNA), such that all plasmid and dbDNA pairs were evaluated. Equimolar amounts of plasmid and dbDNA were used.

After 15 days of selective culture for CAR T cells, CAR expression was detectable with each transposase-transposon format combination. The proportion of T cells expressing CAR was dependent on the combination of the transposase-transposon format ($n = 3$ donors, $p = 0.0035$), with the transposase plasmid and transposon dbDNA combination having a significantly lower proportion of CAR^+ T cells

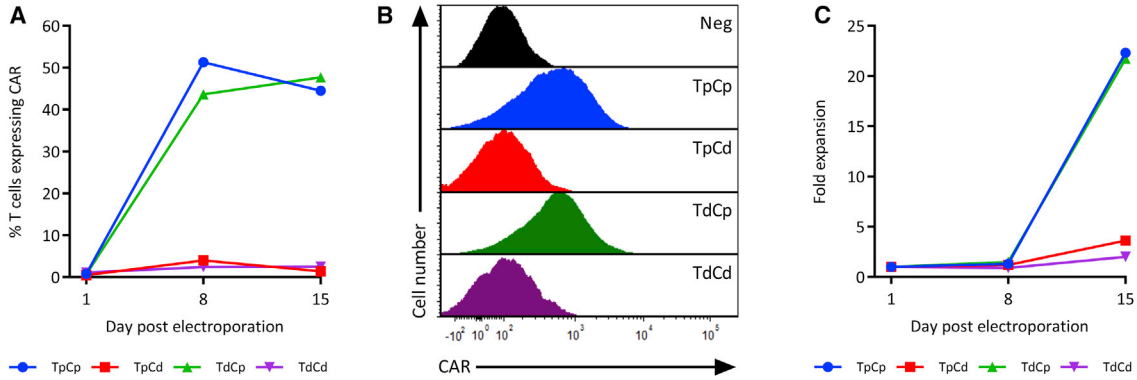

Figure 3. Pilot Study Generating CAR T Cells with Plasmid and dbDNA Components

All plasmid and dbDNA combinations of *piggyBac* transposase and transposon-BCM.CAR19h28z were used to generate CAR T cells. Tp, transposase plasmid; Td, transposase dbDNA; Cp, transposon-CAR plasmid; Cd, transposon-CAR dbDNA; neg, nontransfected T cells. (A) Proportion of $CD3^+$ T cells expressing CAR, as assessed by flow cytometry on a weekly basis. (B) Histograms showing CAR expression on $CD3^+$ T cells after 15 days of culture. (C) Expansion of CAR T cells over 15 days.

compared to all other combinations (12.9% versus 44.0%–78.3%, n = 3 donors, $p < 0.05$ for each pairwise comparison) (Figures 7A and 7B). The degree of CAR expression on T cells, as assessed by median fluorescence activity (MFI), was also dependent on the combination of the transposase-transposon format (n = 3 donors, p = 0.046), with the transposase plasmid and transposon dbDNA combination having a significantly lower intensity of CAR^+ T cell expression compared to combinations utilizing the transposon plasmid (1,208 versus 3,124 − 3,455, n = 3 donors, $p < 0.05$ for each pairwise comparison) (Figure 7C). For CAR T cells generated using entirely plasmid and entirely dbDNA components, there was no statistically significant difference in the proportion of T cells expressing CAR (n = 3 donors, p = 0.0885; Figure 7A), the MFI of CAR expression (n = 3 donors, p = 0.5807; Figure 7C), the cytotoxic effect of transfected nucleic acid on T cells (n = 3 donors, p = 0.3313; Figure 7D), or the transgene integration copy number, as assessed by droplet digital PCR (ddPCR; n = 2 donors, p = 0.8862; Figure 7E).

DISCUSSION

Clinical trials have demonstrated the efficacy of CAR19 T cells against relapsed and refractory B cell malignancies.[2–15] However, their widespread application is limited, in part, by the cost and complexity of production processes utilizing viral vectors.[16] Transposon systems are nonviral vectors that are traditionally plasmid based and provide the opportunity to significantly reduce the cost of CAR T cell production. Both the *piggyBac* and *Sleeping Beauty* transposon systems have been used to generate CAR19 T cells that have potent activity against B cell malignancies.[7,21,22,37–39] Nevertheless, plasmids have several undesirable qualities that include bacterial genetic elements, antibiotic resistance genes, and the requirement for expansion in bacteria with subsequent endotoxin removal.

In this study, we demonstrate that the *piggyBac* system can be based in linear, covalently closed, minimal DNA constructs, known as dbDNA, rather than plasmids and used to generate CAR19 T cells. Because dbDNAs are amplified enzymatically in a bacteria-free system, the issues associated with traditional plasmid-based transposon systems are avoided. Unlike typical open-ended linear DNA, which has a propensity for genomic integration, linear DNA with covalently closed ends, like dbDNA, in fact, has a lower frequency of natural integration than plasmid.[40] Furthermore, whereas a double-strand break in plasmid leaves it free to integrate, a double-strand break in covalently closed dbDNA that leads to integration results in chromosomal disruption, with separation of the centromere from the telomere, and apoptosis.[40,41] Therefore, dbDNA also minimizes the risk of propagating undesirable cells with genomic integration of a sequence encoding the *piggyBac* transposase.

We designed two second-generation CAR19 constructs with varying leader sequences and costimulatory domains and cloned these individually into the plasmid-based *piggyBac* transposon. The *piggyBac* transposase coding sequence and transposons containing the different CAR constructs were then cloned individually into the dbDNA parent plasmid. The resulting plasmid served as a template for bacteria-free *in vitro* enzymatic amplification of dbDNA that lacked antibiotic-resistance genes and bacterial sequences. We investigated generation of CAR19 T cells using all combinations of plasmid- and dbDNA-based *piggyBac* transposon and transposase and examined potential issues underlying the varying success in production.

Simply cloning the *piggyBac* transposase into a dbDNA permitted its normal function. However, this was not true of the *piggyBac* transposon, which initially could not be transposed from dbDNA into gDNA. We demonstrated that the reason for this problem was not due to failure of dbDNA to localize to the nucleus; the presence of genetic elements, such as the protelomerase binding sites; or inefficiencies related to transfection or dbDNA transposase activity. Instead, we found that transposition could occur when the amount of random DNA flanking the transposon within the dbDNA was increased.

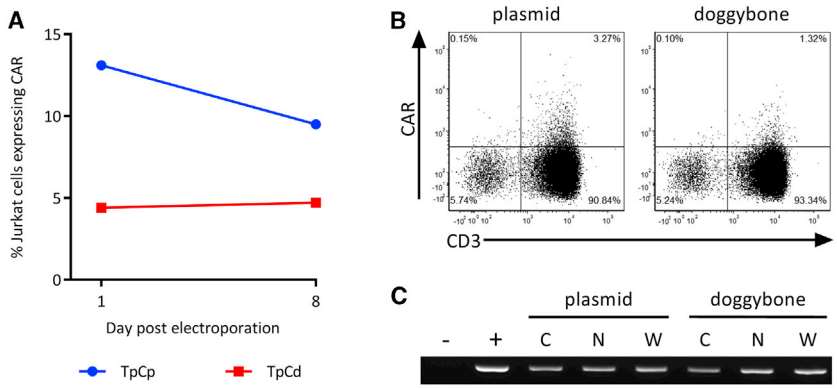

Figure 4. CAR Expression from Transposon dbDNA Is Poor Despite Nuclear Entry

(A) Expression of BCM.CAR19h28z on Jurkat cells after electroporation with transposase plasmid and transposon-CAR plasmid or dbDNA. Tp, transposase plasmid; Cp, transposon-CAR plasmid; Cd, transposon-CAR dbDNA. (B) Expression of BCM.CAR19h28z. (C) Detection of the CAR gene in cell fractions, 24 h after transient transfection of T cells with *piggyBac*-transposon plasmid or dbDNA alone. −, no template control; +, plasmid template; C, cytoplasmic fraction; N, nuclear fraction; W, whole cell lysate.

Our findings indicate that in linear vectors, the *piggyBac* transposase has a requirement for a minimum amount of DNA external to the transposon in order to mediate effective transposition. The reason for this is unclear and has not been previously explored, but we speculate that it may relate to the ability of transposase to bind and spatially manipulate DNA. In plasmid vectors, it has been demonstrated that a shorter amount of DNA external to the transposon ITRs leads to more efficient transposition with a variety of transposases, including *piggyBac*.[42–44] However, the effect of this configuration in circular plasmid is to physically bring the ITRs closer together, thereby facilitating paired-end complex formation, the initial step in transposition where the transposase aligns both ends of the transposon.[43] The benefit of this transposon ITR configuration in circular vectors is not applicable to linear vectors, where different factors appear to be important.

Ultimately, we were able to generate CAR19 T cells using dbDNA-based *piggyBac* components. CAR19 T cell cultures generated using transposase plasmid and transposon dbDNA had a lower proportion of T cells expressing CAR and lower CAR surface expression on positive cells compared to other cultures. Although not statistically significant, there was also a trend toward a lower proportion of T cells expressing CAR in CAR19 T cell cultures generated using only dbDNA. These findings are consistent with previous reports of a trend toward reduced expression from dbDNA compared to an equimolar amount of plasmid[30] and that *piggyBac* transposition occurs more efficiently from circular than linear donor vectors.[45] Transfection of an equal mass of dbDNA to plasmid leads to equivalent levels of expression,[30,31] and this approach might also improve CAR expression in this setting. Further optimization might also be possible using strategies, such as increasing the length of random DNA flanking the ITRs, adjusting the ratio of transposase-to-transposon dbDNA, and altering electroporation conditions. However, we elected not to pursue this, as the proportion of CAR^+ T cells in products generated using only dbDNA components was similar to products used in CAR T cell trials that have shown massive expansion postinfusion and demonstrated anti-tumor efficacy.[15,46] Optimization that increases the number of *piggyBac* integrations per cell may therefore be unnecessary and has the potential to increase the risk of insertional mutagenesis.

A functional assessment of CAR19 T cells generated using entirely dbDNA components was not performed in this study. We previously reported that CAR19 T cells expressing a second-generation, CD19-specific CAR, denoted CAR19h28TM41BBz, had specific and potent activity against $CD19^+$ cell lines *in vivo* and were able to eradicate patient-derived chemorefractory $CD19^+$ B-ALL xenografts in mice.[22] These same CAR19 T cells have demonstrated activity against $CD19^+$ malignancies in a phase I first-in-human clinical trial.[23] CAR19 T cell activity occurred, despite production utilizing plasmid-based *piggyBac* components rather than traditional viral vectors and together with other studies, demonstrated that if sufficient CAR is expressed on the T cell surface, then vector choice does not appear to dictate anti-tumor activity.[7,22,23,39,47] In the current study, CAR19 T cells generated using dbDNA-only *piggyBac* components had similar surface expression of CAR19h28TM41BBz to those generated with plasmid-only components, and we expect that CAR19h28TM41BBz T cells produced with dbDNA-only *piggyBac* components should retain potent activity against $CD19^+$ targets.

With the recognition of the issues associated with using plasmids for transfection purposes, others have explored minicircles as alternative minimal DNA vectors that lack bacterial elements and antibiotic-resistance genes.[48] Minicircles incorporating the *Sleeping Beauty* transposon system have been used to generate CAR19 T cells with equivalent *in vitro* and *in vivo* functional characteristics to those generated with a lentiviral vector.[47] Because minicircles are generated within bacteria, the potential for undesirable recombination events and the requirement for endotoxin removal remain issues. Importantly, the use of dbDNA avoids these problems, as bacterial culture is only required for propagation of the parent plasmid; amplification of the final dbDNA product from this occurs entirely by a bacteria-free enzymatic process *in vitro*.

In conclusion, minimal dbDNA vectors lacking bacterial sequences and antibiotic-resistance genes show great potential as alternatives to plasmid for clinical application. Uniquely, dbDNA technology offers the advantage of bacteria-free enzymatic amplification, streamlining the production and improving the safety of a clinical-grade vector. We have demonstrated that dbDNA incorporating the *piggyBac* transposon system can be used to generate CAR19 T cells when

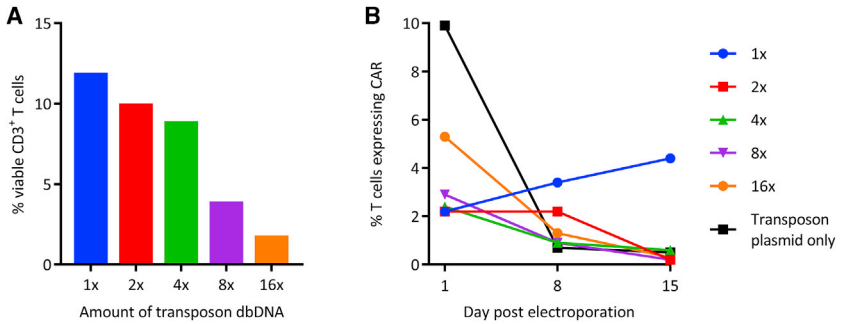

Figure 5. CAR Expression Is Not Improved with Increasing Amounts of Transposon dbDNA

(A) Proportion of viable $CD3^+$ T cells on day 1 post-electroporation with increasing amounts of dbDNA transposon. (B) Expression of BCM.CAR19h28z on T cells after electroporation with plasmid transposase and increasing amounts of dbDNA transposon. The amount indicated is that relative to an equimolar amount of transposon plasmid. T cells were also transfected with transposon plasmid alone as a reference for transient expression from nonintegrated plasmid.

additional random DNA flanking the transposon is included. We predict that this hybrid technology will further reduce costs and improve the safety of CAR T cells generated with nonviral vectors.

MATERIALS AND METHODS

Cell Lines

Jurkat (DSMZ; no. ACC 128) cell lines were kindly provided by Dr. Linda Bendall (The University of Sydney, Australia) and were cultured in complete RPMI (cRPMI): RMPI 1640 (Lonza, Basel, Switzerland) with 10% heat-inactivated fetal bovine serum (FBS; Serana, Bunbury, Australia) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA).

CD19-Specific CAR Constructs

Two second-generation CAR19 constructs were used (Figure 1). CAR19h28TM41BBz has been previously described, and *in vivo* efficacy against B-ALL xenografts was established in murine studies.[22] It incorporates an N-terminal CD8α leader sequence. BCM.CAR19h28z differs from CAR19h28TM41BBz in that it instead uses the N-terminal immunoglobulin G (IgG) heavy-chain leader sequence and FMC63-derived scFv, both from the previously described CAR19.28z[49,50] (kindly provided by Profs. Malcolm Brenner, Gianpietro Dotti, and Matthew Wilson, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA), and substitutes the cytoplasmic domain of CD28 (UniProt: P10747) for that of 4-1BB (CD137; UniProt: Q07011).

Plasmids

The plasmids pVAX1SPBase, pVAX1PB-CAR19.28z, pVAX1PB-CAR19h28z, and pVAX1PB-CAR19h28TM41BBz have been previously described.[22,49,50] To generate pVAX1PB-BCM.CAR19h28z, pVAX1PB-CAR19.28z was first digested with BsmBI (New England BioLabs, Ipswich, MA, USA) to excise DNA encoding the CAR spacer, transmembrane, and CD28 intracellular domains. DNA fragments encoding the CAR spacer, transmembrane, and intracellular costimulatory domains were amplified by PCR from pVAX1PB-CAR19h28z using primers, including 15 bp extensions overlapping with 5′ and 3′ sequences of the BsmBI-digested pVAX1PB-CAR19.28z backbone. The PCR fragments were then fused separately to the pVAX1PB-CAR19.28z backbone using the Cold Fusion cloning kit (System Biosciences, Palo Alto, CA, USA) to create the final plasmids.

DNA encoding the *piggyBac* transposase and CAR-containing transposons was cloned separately from pVAX1 plasmids into the dbDNA parent proTLx plasmid between telomere resolution (telRL) sites (Figure 2A) to form proTLx SPBase and proTLx PB-BCM.CAR19h28z. proTLx PB-CAR19h28TM41BBz was formed in a similar fashion but included an extra 200 bp of random DNA sequence between the protelomerase binding site and ITR on either side of the transposon cassette.

dbDNA Production

Linear dbDNAs were enzymatically produced from proTLx plasmids, as described previously.[30] Briefly, plasmid template DNA was first amplified into concatamers by rolling-circle replication using the phi29 DNA polymerase. The telN protelomerase, from *Escherichia coli* prophage N15, was then used to cleave concatamers at the telRL sites and covalently close the ends with short hairpin loops (telR and telL) to form individual linear vectors. Each resulting dbDNA construct consisted of a section of linear double-stranded DNA encoding either *piggyBac* transposase or CAR-containing transposon, flanked by single-stranded telomere ends (Figure 2B). Residual plasmid DNA was selectively digested with restriction enzymes and then exonuclease III.

Generation and Expansion of CAR T Cells

Ethics approval was obtained from the Sydney West Local Health District Human Research Ethics Committee for collection of PBMCs from healthy donors that had provided informed consent in accordance with the Declaration of Helsinki. Isolation of PBMCs, electroporation using the Neon Transfection System (Life Technologies), stimulation with autologous-irradiated PBMCs and human recombinant interleukin-15 (IL-15; Miltenyi Biotec, Bergisch Gladbach, Germany), and expansion in Falcon 24-well tissue-culture plates (BD Biosciences, San Jose, CA, USA) was performed, as described previously.[22] Plasmids were electroporated at 50 μg/mL and dbDNAs at equimolar concentrations to their plasmid counterpart. Cultures were harvested 15 days postelectroporation or as otherwise stated.

Transfection of Jurkat Cells

Jurkat cells were washed twice with PBS and resuspended in buffer T of the Neon Transfection System (Life Technologies) at a concentration of 20×10^6/mL. *PiggyBac* transposase and BCM-CAR19h28z

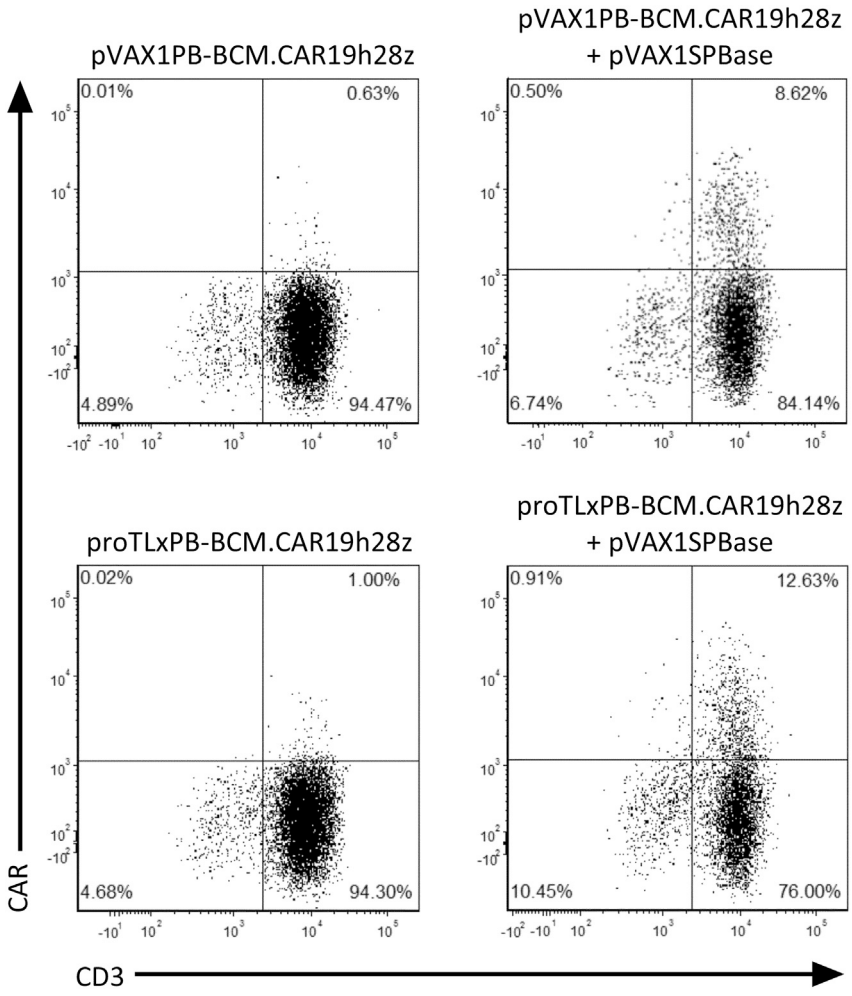

Figure 6. Protelomerase Binding Sites Do Not Impair Transposition or CAR Expression

Expression of BCM.CAR19h28z on T cells 8 days after electroporation with pVAX1PB or proTLxPB transposon plasmids, with or without the additional pVAX1 transposase plasmid (pVAX1SPBase). proTLxPB plasmids contain protelomerase binding sites, whereas pVAX1PB plasmids do not. The presence of pVAX1SPBase facilitates transposition of the CAR gene into gDNA, whereas its absence permits transient transfection only.

transposon were each added either in plasmid or dbDNA format. Plasmids were each at a concentration of 50 μg/mL, whereas dbDNAs were at equimolar concentrations to their plasmid counterpart. Electroporation was performed in 100 μL aliquots using the Neon Transfection System and following the manufacturer's instructions, with the following settings: 3 pulses, 10 ms, and 1,350 V. Transfected Jurkat cells were cultured in Falcon 24-well tissue-culture plates (BD Biosciences) in cRPMI at 37°C and 5% CO_2, with media exchanges as required. Cells were analyzed by flow cytometry on days 1 and 8 postelectroporation.

Phenotypic Analysis

Transfected cells were phenotyped at weekly intervals. The following fluorochrome-conjugated anti-human monoclonal antibodies (mAbs) were used: CD3-Pacific Blue and CD3-phycoerythrin (PE) (BD Biosciences). Surface CAR was detected using a CAR19 scFv-specific mAb (clone no. 136.20.1)[51] (kindly provided by Drs. Bipulendu Jena and Laurence Cooper, MD Anderson Cancer Center, Houston, TX, USA), which was labeled using the Molecular Probes Alexa Fluor 647 Antibody Labeling Kit (Life Technologies). Cell staining, acquisition using FACSCanto II (BD Biosciences) flow cytometers, gating, and analysis with FACSDiva (BD Biosciences) were performed as previously described.[22] FCS Express version 4 Research Edition (*De Novo* Software, Los Angeles, CA, USA) was used for more detailed analysis and graphic representation.

Transposon dbDNA Cellular Localization

PBMCs were transfected with BCM-CAR19h28z transposon alone in either plasmid or dbDNA format, as described above. After 24 h, cells were harvested and washed twice in PBS, and an aliquot of whole cells was set aside. Remaining cells were fractionated by incubation in hypotonic buffer (20 mM Tris [Astral Scientific, Sydney, Australia]–HCl [Ajax Finechem, Sydney, Australia], pH 7.4, 10 mM NaCl [Astral Scientific], and 3 mM MgCl [Ajax Finechem]) on ice for 15 min, addition of 5% (v/v) Nonidet P-40 (Astral Scientific) with vortex mixing for 10 s, and centrifugation at 13,000 rpm for 30 s at 4°C. The supernatant was set aside as the cytoplasmic fraction, and the nuclear pellet was washed twice in hypotonic buffer. DNA was extracted from whole cells, cytoplasmic fractions, and nuclear fractions using the Wizard SV gDNA purification system (Promega, Madison, WI, USA). PCR for the BCM-CAR19h28z gene was performed using 50 ng template and the following primers: 5′-ACGGTGGTAGCTA TGCTATG-3′ and 5′-CCGCCATCTTACTTTCTGC-3′. Thermocycling conditions consisted of initial denaturation (95°C for 2 min), followed by 30 PCR cycles (95°C for 30 s, 59°C for 30 s, 72°C for 30 s) and final extension (72°C for 5 min). PCR products were analyzed by gel electrophoresis.

Integration Copy Number by ddPCR

gDNA was extracted from CAR T cells after 15 days of culture using the QIAamp DNA Micro kit (QIAGEN, Hilden, Germany). ddPCR was performed using the QX200 system (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Briefly, ddPCR reaction mixes were set up to contain 1 × ddPCR Supermix for Probes (no 2′-deoxyuridine 5′-triphosphate [dUTP]; Bio-Rad), 900 nM/250 nM RPP30 primers/probe (hexachloro-fluorescein [HEX]), 900 nM/250 nM

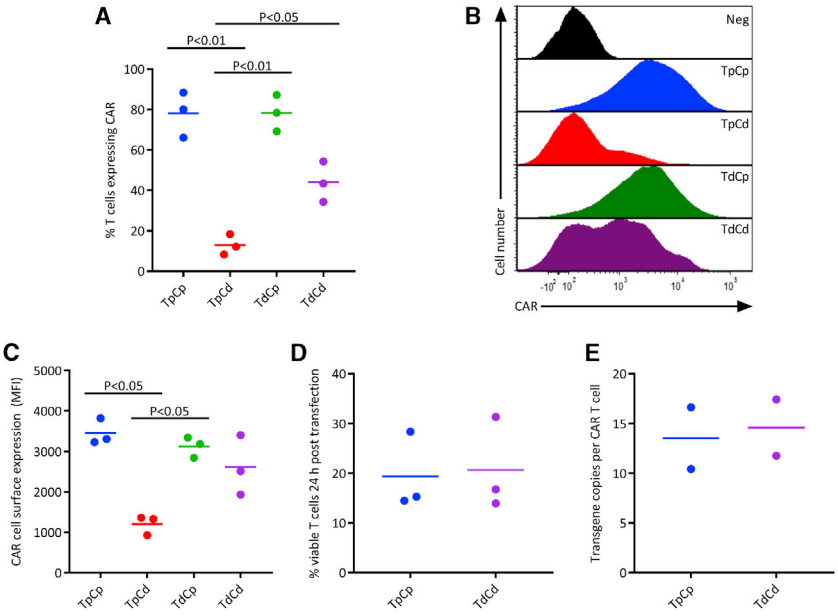

Figure 7. CAR Is Expressed from Transposon dbDNAs with Longer Sequences Flanking the ITR

An additional 200 bp of random DNA was inserted between protelomerase binding sites and ITRs flanking the *piggyBac* transposon cassette. All plasmid and dbDNA combinations of *piggyBac* transposase and transposon-CAR19h28TM41BBz were used to generate CAR T cells (n = 3 per combination). Tp, transposase plasmid; Td, transposase dbDNA; Cp, transposon-CAR plasmid; Cd, transposon-CAR dbDNA; neg, nontransfected T cells. (A) Proportion of $CD3^+$ T cells expressing CAR. (B) Representative histograms showing CAR expression on $CD3^+$ T cells. (C) Relative cell-surface expression of CAR on $CD3^+$ T cells, all on day 15 post-transfection. (D) Proportion of viable $CD3^+$ T cells, 24 h post-transfection. (E) CAR transgene copy number per CAR T cell using entirely plasmid or dbDNA components on day 15 post-transfection (n = 2 per condition). Dots represent individual data points, and solid lines represent the mean.

CAR primers/probe (5(6)-carboxyfluorescein [FAM]), 3 IU HindIII (New England BioLabs), and 3 ng gDNA. After droplet formation with the QX200 Droplet Generator (Bio-Rad), samples were transferred to a semi-skirted twin-tec 96-well PCR plate (Eppendorf, Hamburg, Germany), sealed with the PX1 PCR Plate Sealer (Bio-Rad). Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad) using the following conditions: enzyme activation (95°C for 10 min), followed by 40 PCR cycles (94°C for 30 s and 62°C for 1 min) and enzyme deactivation (98°C for 10 min), with ramp rate 2°C/s. Postamplification analysis was performed using the QX200 Droplet Reader (Bio-Rad) and QuantaSoft software (Bio-Rad).

Statistical Analysis

GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. The significance level used was $p < 0.05$. Repeated-measures one-way analysis of variance (ANOVA) with the Greenhouse-Geisser correction was performed to test for systematic within-subjects differences. Where a possible association was identified, Tukey's multiple comparisons test was performed, with individual variances calculated for each comparison. A paired t test was performed to identify any difference in integration copy number using plasmid-only or dbDNA-only components. Where replicates have been performed, the mean is presented in addition to individual data points.

AUTHOR CONTRIBUTIONS

Conceptualization, D.C.B., K.P.M., K.G., L.C., and J.T.; Methodology, D.C.B., K.P.M., L.C., and J.T.; Investigation, D.C.B., K.G., M.L., and K.K.; Formal Analysis, D.C.B.; Writing – Original Draft, D.C.B.; Writing – Review & Editing, D.C.B., K.P.M., L.C., J.T., and D.J.G.; Funding Acquisition, K.P.M., K.G., D.J.G., and D.C.B.; Supervision, K.P.M., L.C., J.T., and D.J.G.

CONFLICTS OF INTEREST

L.C., J.T., K.K., and M.L. are employees of Touchlight Genetics Ltd.

ACKNOWLEDGMENTS

This research was funded by Cancer Council NSW Project (grant RG17-09). D.C.B. is a doctoral candidate at The University of Sydney and has received funding from the Leukaemia Foundation of Australia, Haematology Society of Australia and New Zealand, Cancer Institute of New South Wales, and Sydney West Translational Cancer Research Centre. Flow cytometry was performed in the Flow Cytometry Core Facility that is supported by the Westmead Research Hub, Cancer Institute of New South Wales, and National Health and Medical Research Council.

REFERENCES

1. Sadelain, M., Brentjens, R., and Rivière, I. (2013). The basic principles of chimeric antigen receptor design. Cancer Discov. *3*, 388–398.

2. Cruz, C.R., Micklethwaite, K.P., Savoldo, B., Ramos, C.A., Lam, S., Ku, S., Diouf, O., Liu, E., Barrett, A.J., Ito, S., et al. (2013). Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. Blood *122*, 2965–2973.

3. Kochenderfer, J.N., Dudley, M.E., Carpenter, R.O., Kassim, S.H., Rose, J.J., Telford, W.G., Hakim, F.T., Halverson, D.C., Fowler, D.H., Hardy, N.M., et al. (2013). Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. Blood *122*, 4129–4139.

4. Kochenderfer, J.N., Dudley, M.E., Kassim, S.H., Somerville, R.P., Carpenter, R.O., Stetler-Stevenson, M., Yang, J.C., Phan, G.Q., Hughes, M.S., Sherry, R.M., et al. (2015). Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J. Clin. Oncol. *33*, 540–549.

5. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.K., Delbrook, C., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, M., Shah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet *385*, 517–528.

6. Fraietta, J.A., Beckwith, K.A., Patel, P.R., Ruella, M., Zheng, Z., Barrett, D.M., Lacey, S.F., Melenhorst, J.J., McGettigan, S.E., Cook, D.R., et al. (2016). Ibrutinib enhances chimeric antigen receptor T-cell engraftment and efficacy in leukemia. Blood *127*, 1117–1127.

7. Kebriaei, P., Singh, H., Huls, M.H., Figliola, M.J., Bassett, R., Olivares, S., Jena, B., Dawson, M.J., Kumaresan, P.R., Su, S., et al. (2016). Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. J. Clin. Invest. *126*, 3363–3376.

8. Turtle, C.J., Hanafi, L.A., Berger, C., Gooley, T.A., Cherian, S., Hudecek, M., Sommermeyer, D., Melville, K., Pender, B., Budiarto, T.M., et al. (2016). CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J. Clin. Invest. *126*, 2123–2138.

9. Turtle, C.J., Hanafi, L.A., Berger, C., Hudecek, M., Pender, B., Robinson, E., Hawkins, R., Chaney, C., Cherian, S., Chen, X., et al. (2016). Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. Sci. Transl. Med. *8*, 355ra116.

10. Gardner, R.A., Finney, O., Annesley, C., Brakke, H., Summers, C., Leger, K., Bleakley, M., Brown, C., Mgebroff, S., Kelly-Spratt, K.S., et al. (2017). Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. Blood *129*, 3322–3331.

11. Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., et al. (2017). Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. N. Engl. J. Med. *377*, 2531–2544.

12. Schuster, S.J., Svoboda, J., Chong, E.A., Nasta, S.D., Mato, A.R., Anak, Ö., Brogdon, J.L., Pruteanu-Malinici, I., Bhoj, V., Landsburg, D., et al. (2017). Chimeric Antigen Receptor T Cells in Refractory B-Cell Lymphomas. N. Engl. J. Med. *377*, 2545–2554.

13. Turtle, C.J., Hay, K.A., Hanafi, L.A., Li, D., Cherian, S., Chen, X., Wood, B., Lozanski, A., Byrd, J.C., Heimfeld, S., et al. (2017). Durable Molecular Remissions in Chronic Lymphocytic Leukemia Treated With CD19-Specific Chimeric Antigen Receptor-Modified T Cells After Failure of Ibrutinib. J. Clin. Oncol. *35*, 3010–3020.

14. Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., et al. (2018). Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. N. Engl. J. Med. *378*, 439–448.

15. Park, J.H., Rivière, I., Gonen, M., Wang, X., Sénéchal, B., Curran, K.J., Sauter, C., Wang, Y., Santomasso, B., Mead, E., et al. (2018). Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. N. Engl. J. Med. *378*, 449–459.

16. Lin, J.K., Lerman, B.J., Barnes, J.I., Boursiquot, B.C., Tan, Y.J., Robinson, A.Q.L., Davis, K.L., Owens, D.K., and Goldhaber-Fiebert, J.D. (2018). Cost Effectiveness of Chimeric Antigen Receptor T-Cell Therapy in Relapsed or Refractory Pediatric B-Cell Acute Lymphoblastic Leukemia. J. Clin. Oncol. *32*, 3192–3202.

17. Manuri, P.V., Wilson, M.H., Maiti, S.N., Mi, T., Singh, H., Olivares, S., Dawson, M.J., Huls, H., Lee, D.A., Rao, P.H., et al. (2010). piggyBac transposon/transposase system to generate CD19-specific T cells for the treatment of B-lineage malignancies. Hum. Gene Ther. *21*, 427–437.

18. Singh, H., Manuri, P.R., Olivares, S., Dara, N., Dawson, M.J., Huls, H., Hackett, P.B., Kohn, D.B., Shpall, E.J., Champlin, R.E., and Cooper, L.J. (2008). Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. Cancer Res. *68*, 2961–2971.

19. Galvan, D.L., Nakazawa, Y., Kaja, A., Kettlun, C., Cooper, L.J., Rooney, C.M., and Wilson, M.H. (2009). Genome-wide mapping of PiggyBac transposon integrations in primary human T cells. J. Immunother. *32*, 837–844.

20. Huang, X., Guo, H., Tammana, S., Jung, Y.C., Mellgren, E., Bassi, P., Cao, Q., Tu, Z.J., Kim, Y.C., Ekker, S.C., et al. (2010). Gene transfer efficiency and genome-wide integration profiling of Sleeping Beauty, Tol2, and piggyBac transposons in human primary T cells. Mol. Ther. *18*, 1803–1813.

21. Ramanayake, S., Bilmon, I., Bishop, D., Dubosq, M.C., Blyth, E., Clancy, L., Gottlieb, D., and Micklethwaite, K. (2015). Low-cost generation of Good Manufacturing Practice-grade CD19-specific chimeric antigen receptor-expressing T cells using piggyBac gene transfer and patient-derived materials. Cytotherapy *17*, 1251–1267.

22. Bishop, D.C., Xu, N., Tse, B., O'Brien, T.A., Gottlieb, D.J., Dolnikov, A., and Micklethwaite, K.P. (2018). PiggyBac-Engineered T Cells Expressing CD19-Specific CARs that Lack IgG1 Fc Spacers Have Potent Activity against B-ALL Xenografts. Mol. Ther. *26*, 1883–1895.

23. Bishop, D.C., Clancy, L.E., Burgess, J., Mathew, G., Atkins, E., Advic, S., Maddock, K., Street, J., Moezzi, L., Simms, R., et al. (2019). Matched sibling donor-derived piggyBac CAR19 T cells induce remission of relapsed/refractory CD19+ malignancy following haematopoietic stem cell transplant. Cytotherapy *21*, S9.

24. Vandermeulen, G., Marie, C., Scherman, D., and Préat, V. (2011). New generation of plasmid backbones devoid of antibiotic resistance marker for gene therapy trials. Mol. Ther. *19*, 1942–1949.

25. van der Heijden, I., Gomez-Eerland, R., van den Berg, J.H., Oosterhuis, K., Schumacher, T.N., Haanen, J.B., Beijnen, J.H., and Nuijen, B. (2013). Transposon leads to contamination of clinical pDNA vaccine. Vaccine *31*, 3274–3280.

26. Prather, K.J., Sagar, S., Murphy, J., and Chartrain, M. (2003). Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. Enzyme Microb. Technol. *33*, 865–883.

27. Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., and Klinman, D.M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature *374*, 546–549.

28. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740–745.

29. Bauer, S., Kirschning, C.J., Häcker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G.B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. USA *98*, 9237–9242.

30. Walters, A.A., Kinnear, E., Shattock, R.J., McDonald, J.U., Caproni, L.J., Porter, N., and Tregoning, J.S. (2014). Comparative analysis of enzymatically produced novel linear DNA constructs with plasmids for use as DNA vaccines. Gene Ther. *21*, 645–652.

31. Scott, V.L., Patel, A., Villarreal, D.O., Hensley, S.E., Ragwan, E., Yan, J., Sardesai, N.Y., Rothwell, P.J., Extance, J.P., Caproni, L.J., and Weiner, D.B. (2015). Novel synthetic plasmid and Doggybone DNA vaccines induce neutralizing antibodies and provide protection from lethal influenza challenge in mice. Hum. Vaccin. Immunother. *11*, 1972–1982.

32. Allen, A., Wang, C., Caproni, L.J., Sugiyarto, G., Harden, E., Douglas, L.R., Duriez, P.J., Karbowniczek, K., Extance, J., Rothwell, P.J., et al. (2018). Linear doggybone DNA vaccine induces similar immunological responses to conventional plasmid DNA independently of immune recognition by TLR9 in a pre-clinical model. Cancer Immunol. Immunother. *67*, 627–638.

33. Miller, D.G., Adam, M.A., and Miller, A.D. (1990). Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol. Cell. Biol. *10*, 4239–4242.

34. Lewis, P.F., and Emerman, M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J. Virol. *68*, 510–516.

35. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., and Wagner, E. (2000). Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther. *7*, 401–407.

36. Dean, D.A., Strong, D.D., and Zimmer, W.E. (2005). Nuclear entry of nonviral vectors. Gene Ther. *12*, 881–890.

37. Saito, S., Nakazawa, Y., Sueki, A., Matsuda, K., Tanaka, M., Yanagisawa, R., Maeda, Y., Sato, Y., Okabe, S., Inukai, T., et al. (2014). Anti-leukemic potency of piggyBac-mediated CD19-specific T cells against refractory Philadelphia chromosome-positive acute lymphoblastic leukemia. Cytotherapy *16*, 1257–1269.

38. Dolnikov, A., Shen, S., Klamer, G., Joshi, S., Xu, N., Yang, L., Micklethwaite, K., and O'Brien, T.A. (2015). Antileukemic potency of CD19-specific T cells against chemoresistant pediatric acute lymphoblastic leukemia. Exp. Hematol. *43*, 1001–1014.e5.

39. Morita, D., Nishio, N., Saito, S., Tanaka, M., Kawashima, N., Okuno, Y., Suzuki, S., Matsuda, K., Maeda, Y., Wilson, M.H., et al. (2017). Enhanced Expression of Anti-CD19 Chimeric Antigen Receptor in *piggyBac* Transposon-Engineered T Cells. Mol. Ther. Methods Clin. Dev. *8*, 131–140.

40. Nafissi, N., Alqawlaq, S., Lee, E.A., Foldvari, M., Spagnuolo, P.A., and Slavcev, R.A. (2014). DNA ministrings: highly safe and effective gene delivery vectors. Mol. Ther. Nucleic Acids *3*, e165.

41. Nafissi, N., and Slavcev, R. (2012). Construction and characterization of an in-vivo linear covalently closed DNA vector production system. Microb. Cell Fact. *11*, 154.

42. Izsvák, Z., Ivics, Z., and Plasterk, R.H. (2000). Sleeping Beauty, a wide host-range transposon vector for genetic transformation in vertebrates. J. Mol. Biol. *302*, 93–102.

43. Jin, Y., Chen, Y., Zhao, S., Guan, K.L., Zhuang, Y., Zhou, W., Wu, X., and Xu, T. (2017). DNA-PK facilitates *piggyBac* transposition by promoting paired-end complex formation. Proc. Natl. Acad. Sci. USA *114*, 7408–7413.

44. Rostovskaya, M., Fu, J., Obst, M., Baer, I., Weidlich, S., Wang, H., Smith, A.J., Anastassiadis, K., and Stewart, A.F. (2012). Transposon-mediated BAC transgenesis in human ES cells. Nucleic Acids Res. *40*, e150.

45. Nakanishi, H., Higuchi, Y., Kawakami, S., Yamashita, F., and Hashida, M. (2011). Comparison of piggyBac transposition efficiency between linear and circular donor vectors in mammalian cells. J. Biotechnol. *154*, 205–208.

46. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. *371*, 1507–1517.

47. Monjezi, R., Miskey, C., Gogishvili, T., Schleef, M., Schmeer, M., Einsele, H., Ivics, Z., and Hudecek, M. (2017). Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors. Leukemia *31*, 186–194.

48. Kay, M.A., He, C.Y., and Chen, Z.Y. (2010). A robust system for production of minicircle DNA vectors. Nat. Biotechnol. *28*, 1287–1289.

49. Rössig, C., Pscherer, S., Landmeier, S., Altvater, B., Jürgens, H., and Vormoor, J. (2005). Adoptive cellular immunotherapy with CD19-specific T cells. Klin. Padiatr. *217*, 351–356.

50. Micklethwaite, K.P., Savoldo, B., Hanley, P.J., Leen, A.M., Demmler-Harrison, G.J., Cooper, L.J., Liu, H., Gee, A.P., Shpall, E.J., Rooney, C.M., et al. (2010). Derivation of human T lymphocytes from cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. Blood *115*, 2695–2703.

51. Jena, B., Maiti, S., Huls, H., Singh, H., Lee, D.A., Champlin, R.E., and Cooper, L.J. (2013). Chimeric antigen receptor (CAR)-specific monoclonal antibody to detect CD19-specific T cells in clinical trials. PLoS ONE *8*, e57838.