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### Development of Augmented Leukemia/Lymphoma-Specific T-Cell Immunotherapy for Deployment with Haploidentical Hematopoietic Progenitor-Cell Transplant

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This project develops novel immune-based therapies to target childhood leukemia. By combining gene therapy with T-cell therapy we have developed new biologic agents that specifically recognize CD19 molecule on the cell surface of B-cell acute lymphoblastic leukemia (B-ALL). To translate this approach to the clinic we have adapted the Sleeping Beauty (SB) transposon/transposase system to express a CD19-specific chimeric antigen receptor (CAR). T cells that have undergone transposition express the CAR and therefore redirected specificity for CD19. To selectively propagate CD19-specific T cells to clinically-meaningful numbers we have developed artificial antigen presenting cells (aAPC) by expressing CD19 molecule and desired co-stimulatory molecules on K562 cells. Thus, by combining SB transposition and aAPC, we can harness gene therapy and T-cell therapy to develop targeted treatment for B-ALL.
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INTRODUCTION

This annual report describes the second year of research accomplishments regarding the development of T cells that have potential to target B-cell malignancies. This is accomplished using genetic engineering to express a chimeric antigen receptor (CAR) to redirect the specificity of T cells for CD19 on malignant B cells. We report that successive generations of CD19-specific CARs have been built and validated and T cells have been modified to be specific for B-cell tumors. This grant's progress has been evaluated by the advisory board as described in the section on SUPPORTING DATA.

BODY

Task #1: Can CD10-specific-IL2 immunocytokine in combination with CD19-specific T cells improve persistence of infused T cells and to treat B-lineage malignancy?

Per last report in May 2008, the initial data for this aim has been published Cancer Res. 2007 Mar 15;67(6):2872-80 (as attached). These data demonstrate that a CD20-specific immunocytokine can augment the persistence and therapeutic effect of CD19-specific T cells. At this time we will not be generating a CD10-specific immunocytokine since addition approaches to providing T-cell help and improving persistence appear more promising (tasks #2 and #3).

Task #2: Can CD19-specific CD4+ Th cells improve the anti-tumor response of CD19-specific CD8+ T cells?

The initial data for this aim has been published Cancer Res. 2008 Apr 15;68(8):2961-71. These data demonstrate that both CD4+ and CD8+ T cells expressing CD19-specific chimeric antigen receptor (CAR) can be generated using a novel non-viral gene transfer approach employing the Sleeping Beauty (SB) transposon/transposase system. We have now adapted the transposon/transposase system to work using the piggyBac system (paper submitted to Leukemia and attached). Furthermore, we have developed IL-21 to improve the ex vivo preferential proliferation of CD8+CAR+ T cells (Figure 1). Since clinical-grade IL-21 is not widely available, we have further adapted K562 functioning as artificial antigen presenting cells (aAPC) to express a membrane-bound variant of IL-21 (Figure 2).

Task #3: Can CD28 co-stimulation can enhance the survival and potency of CD19-specific T cells?

Our published data indicates that second generation CAR (designated CD19RCD28) expressing CD28 endodomains provides a CD19-dependent fully-competent activation signal resulting in improved persistence (as measured by detection of T-cell fire fly luciferase, fFluc) after adoptive transfer and augmented elimination of an established B-lineage malignancy, compared with 1st generation technology (designated CD19, which signals only through chimeric CD3ζ). We have now expanded these observations to develop a new immunoreceptor that can function to provide T-cell costimulation.

To improve the survival of CAR+ T cells, we introduced membrane-bound cytokines (MBCs) into T cells as costimulatory molecules that will putatively enable these cells to obtain proliferative/pro-survival stimulatory signals that can act in concert with CAR-dependent signaling to improve T-cell persistence in the tumor microenvironment. While IL-2 is a cytokine known to be important for T cell activation characteristics, IL-7 has been shown to be crucial for T cell homeostatic expansion in lymphopenic conditions and in memory T cell subset maintenance. The homeostatic role of IL-7 may be better suited to providing T cells with the necessary proliferation/survival signals. This cytokine is promising for immunotherapy work as in vitro and
murine models both appear to up-regulate anti-apoptotic molecules in T cells, and cells expressing these molecules are resistant to apoptosis following IL-2 withdrawal in vitro. What is desired, but is currently not widely available, is IL-7 as a clinical-grade reagent. This MBC will act as a loco-regional replacement for the limited physiological “pools” of soluble endogenous IL-7 during immune reconstitution. It is anticipated that stimulation of CAR+ T cells through this MBC will preferentially expand CAR+ T cells after adoptive transfer while retaining the cytokine to regions of T cell activity lending to a decreased potential for systemic toxicity.

The specific aim was adapted to determine if costimulation through MBC IL-7 can improve in vitro CD19-dependent proliferation of CAR+ T cells. This MBC was expressed in primary T cells with and without co-expression of CD19-specific CAR. Artificial APC were used to select for genetically modified T cells which exhibit sustained proliferation. T cells will be evaluated for CAR-dependent redirected killing and cytokine-dependent (i) signaling, (ii) up-regulation of anti-apoptotic molecules, and (iii) cis/trans interactions. Generation of the MBC was achieved by fusion of the IL-7 cDNA sequence to the human Fc region and CD4 transmembrane region sequences that act as a stalk and anchor, respectively. The T cells were electroporated with multiple SB transposons to introduce CAR and the MBC transgenes followed by co-culture on a panel of irradiated aAPC. The panel of K562 aAPC will express +/-tCD19 (truncated CD19) and +/- MBIL-7 with or without the administration of exogenous IL-7. The CAR is expressed as a transposon from one SB transposon and the MBC is expressed from a second SB transposon plasmid. The improved kinetics of proliferation of CAR+ T cells co-expressing MBIL-7 is shown in Figure 3.
KEY RESEARCH ACCOMPLISHMENTS

- Developed piggyBac transposon/transposase system as an approach to genetically modifying human T cells to express CD19-specific CAR.
- Evaluated the use of soluble recombinant IL-21 to selectively propagate CD8+CAR+ T cells
- Generated membrane-bound IL-21 to provide a source of clinical-grade IL-21 as expressed on aAPC.
- Developed membrane-bound IL-7 as a novel co-stimulatory molecule to provide T-cell costimulation.

REPORTABLE OUTCOMES

- Manuscript submitted to Leukemia (reprinted as part of this progress report)
- Major funding applied for: Multiple stimulus grants through NIH April and May, 2009
- Major funding applied for: P01 NIH/NCI (Cooper – PI) 12/01/09– 11/30/2014; T-Cell Immunotherapy for Pediatric Solid Tumors, Major goal: Programmatic approach to generate cell-based immunotherapies for treatment of relapsed pediatric non-neural solid tumors.

CONCLUSION

We have made substantial progress in the development of T cells that can target CD19 on malignant cells. New technology has been developed with translational implications. These pre-clinical data not only provide a framework for the add-back of clinical-grade CAR+ tumor-specific T cells, but also the generation of T cells with capability for improved in vivo persistence and therefore therapeutic potential. The data are showing that T cells expressing the CD19RCD28 CAR is the preferred technology at this point to transition to clinical trials. Indeed, these data will be used to help support a clinical trial infusing donor-derived CAR+ T cells after haploidentical hematopoietic stem-cell transplantation.
REFERENCES

None

APPENDICES

- Paper attached has been submitted to Leukemia.
- Two movies that accompany the paper.
SUPPORTING DATA:

Attached is summary information from the Advisory committee who has evaluated my progress (page 50 supporting documentation from original grant):
Dr. Champlin, Professor, Chair, Blood & Marrow Transplantation
Dr. Gelovani, Professor and Chair, Experimental Diagnostic Imaging,
Dr. Hwu, Professor, Chair, Melanoma Medical Oncology
Dr. Kleinerman, Professor, Division Head
Dr. Liu, Professor, Chair, Immunology

The average score for each member are provided in Tables A and B below. From the original application: “…the committee will score the research since the last meeting for progress according to the Table A below using the following scale: 0 = no progress, 1 = adequate progress, 2 = progress beyond expectation, and 3 = exemplary progress. The committee will also assign an impact score according to Table B below using the following scale: 0 = no expected impact, 1 = impact expected to be adequate, 2 = impact likely to be beyond expectation, and 3 = impacted expected to be highly significant. These scores will be tabulated at each of the interim analyses and yearly meetings. To avoid bias, the PI, Dr. Cooper will not score the research, and the scoring will be anonymous. These scores as well as written comments from the committee members will be used to measure progress. Additional meetings may be requested by the PI, committee members and the DOD to discuss new data and plans, or if progress and impact has been less than expected as reflected by a downward trend in the scoring from Tables A and B.”

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<tr>
<th>Table A: Interim Progress Assessment</th>
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<tr>
<td>Blood-related cancer research</td>
<td>3</td>
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<td>Military’s needs</td>
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<td>Veterans’ needs</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
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</tbody>
</table>
A transition plan has been developed per Section 14 “Supporting Documentation” of the original grant application. An optimization score has been developed from this feedback and is provided in Table C. In this first year of the grant award the technology has been developed and in vitro testing has been performed. The in vivo modeling will be undertaken in the second year and this will allow the optimization score to be determined.

<table>
<thead>
<tr>
<th>Task/hypothesis</th>
<th>Product</th>
<th>In vivo persistence score</th>
<th>In vivo efficacy score</th>
<th>Synergy score</th>
<th>Manufacturing score</th>
<th>Regulatory score</th>
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<td>N/A</td>
<td>N/A</td>
<td>1</td>
<td>1</td>
<td>2</td>
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<td>2</td>
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<td>N/A</td>
<td>3</td>
<td>4</td>
<td>2</td>
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<tr>
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<tr>
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<td>CD19R+ T cells (1st-generation)</td>
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<td>1</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>9</td>
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</table>

3 Which of the three hypotheses/tasks resulted in improved in vivo T-cell persistence? Score 4 points for each new therapy which resulted in longer in vivo persistence compared with infusion of 1st-generation CD19R+ T cells. Score 1 point if the 2nd-generation technology is equivalent to the first, and no points if it is worse than the first.

4 Which of the three hypotheses/tasks resulted in improved in vivo T-cell anti-tumor effect? Score 4 points for each new therapy which resulted in an improved in vivo anti-tumor effect compared with infusion of 1st-generation CD19R+ T cells. Score 1 point if the 2nd-generation technology is equivalent to the first, and no points if it is worse than the first.

5 Score 3 points if a hypothesis/task resulted in at least equivalent T-cell persistence and at least equivalent anti-tumor effect, compared with adoptive transfer of CD19R+ T cells.

6 The relative ease of manufacturing the new reagents will be reflected in the scoring. The scoring reflects the following hierarchy: (i) CD19RCD28+ T cells (only one biologic product is needed), (ii) Combining CD19-specific CD4+ T cells with CD19-specific CD8+ T cells (both of these T-cell populations can be readily generated from a donor and genetic modification uses technology which is currently in clinical trials), (iii) Combining CD10-specific-IL2 ICK and CD19-specific T cells (requires generation of two products, one antibody-based reagent, and one cellular reagent). CD19R+ T cells are already in clinical trials.

7 Consideration will also be given to potential ease of obtaining regulatory approval. The a priori assumption is that some new technologies may be easier to obtain regulatory approval and this is reflected in the scoring in the table. CD19R+ T cells are already in clinical trials.
**Figure 1:** Addition of soluble recombinant IL-21 to the *ex vivo* culture results in improved outgrowth of CD8^+^ CAR^+^ T cells and improved redirected function. (A) Flow cytometry of CAR expression. (B) CAR-dependent activation measured by flow cytometry intracellular expression of γ-IFN using CD19$^+$ versus CD19$^{neg}$ stimulator cells. (C) Kinetics of expansion of CAR$^+$ T cells after electroporation on CD19$^+$ aAPC in presence of IL-2 and with/without IL-21. (D) Redirected killing of CD19$^+$ tumor cells (NALM-6) and transfected U251T glioma cells using T cells cultured with IL-2 and with/without IL-21.
Figure 2: Top: Expression of MB IL-21 on K562-aAPC (prepared as master cell bank, MCB) detected with mAb specific for IL-21 protein. Bottom: Expression of membrane-bound IL-7 (independent of other MB cytokines) on K562-aAPC. The IL-21, IL-15, and IL-7 MB cytokines are attached to K562 cell service via immunoglobulin Fc region.
Figure 3: Proliferation kinetics of CAR+ T cells with or without co-expression of MBIL-7. It is demonstrated that antigen-specific expansion is supported only in the presence of cytokine. CAR+MBIL-7+ T cells expand without the need for exogenous cytokines indicating that this membrane-bound cytokine is sufficient for antigen-specific T cell expansion.
Application of *piggyBac* transposon/transposase system to generate CD19-specific T cells for treatment of B-lineage malignancies

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Keywords: *piggyBac*, transposon/transposase, T cells, CD19, gene therapy

Running Title: *piggyBac* transposition for T-cell gene therapy

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ABSTRACT

Non-viral integrating vectors can be used for expression of therapeutic genes. To improve gene transfer efficiency, we and others have used transposon/transposase systems from Sleeping Beauty to integrate desired transgenes, such as chimeric antigen receptors (CARs), into primary T cells. To determine whether transposition of CARs is adaptable to other systems, we now show that the piggyBac (PB) can be adapted to express a CD19-specific CAR in human T cells. We demonstrate that T cells electroporated to introduce PB transposon and transposase stably express CD19-specific CAR upon propagation on CD19⁺ artificial antigen presenting cells and maintain a diverse repertoire of endogenous T-cell receptors consistent with efficient gene transfer. The numerically expanded T cells display a phenotype associated with both memory and effector T-cell populations and exhibit CD19-dependent killing of tumor targets. The electro-transfer of PB transposon to express the CAR was not associated with genotoxicity based upon fluorescent in situ hybridization and chromosome analysis. PB transposition of human T cells to redirect specificity to a desired target such as CD19 is a new approach with therapeutic implications for generating T-cell therapy for B-lineage malignancies.
INTRODUCTION

T cells can be genetically modified to redirect specificity through the introduction of full-length \( \alpha \beta \) T-cell receptors which recognize antigen in the context of major histocompatibility complex (MHC) or through the introduction of chimeric antigen receptors (CARs) to recognize cell-surface antigen independent of MHC (1, 2). Approaches to introduce CARs are viral (transduction with retrovirus/lentivirus) (3, 4) or non-viral using DNA plasmids (5-7) or mRNA (8-10). Electro-transfer of DNA plasmids has been adapted for clinical trials to introduce CAR transgenes into primary T cells (11-14). However, the integration efficiency of introduced naked DNA plasmids is low resulting in lengthy periods of ex vivo culturing under selection pressure to recover T cells expressing stable CAR integrants. Recently, we and others reported that the Sleeping Beauty (SB) transposon/transposase could be used to improve the efficiency of gene transfer to express a CAR in T cells and that this system may be adapted for clinical trials (15, 16), (17).

We now extend these observations to demonstrate that an alternate transposon/transposase system, namely piggyBac (PB) can also be used to introduce a CAR to redirect T cell specificity for CD19 expressed on malignant (and normal) B cells. The PB transposon, derived from the cabbage looper moth Trichoplusia ni, was originally identified in the genome of baculovirus infected insect cells giving rise to the name "piggyBac" (18-20). The original PB element was approximately 2.4 kb with identical 13 base pair (bp) terminal inverted repeats and additional asymmetric 19 bp internal repeats (21-23). PB is typically thought to mediate precise excision of transposon segments in mouse (24) and human cells through a cut and paste mechanism resulting in complementary TTAA overhangs on the ends of the donor DNA and ligation of these ends to restore the donor site to its pre-transposon sequence (19, 20, 24-27). Recently, PB has been used as a vector for reprogramming murine and human embryonic fibroblasts (28), and for introduction of reprogramming factor, Klf4 into murine epistem cells (29).

To evaluate the capability of PB as a vector for application in gene therapy we generated primary human T cells with redirected specificity for CD19, using the PB transposon/transposase system. We constructed a PB transposon expressing a 2\textsuperscript{nd} generation CD19-specific CAR designated CD19RCD28. We demonstrate that electroporation of primary human T cells with this PB transposon plasmid in the presence of codon optimized PB
transposase resulted in efficient integration of this CAR transgene, and numeric expansion of the CD19 CAR+ T cells to clinically-significant numbers could be readily achieved by recursive propagation on γ-irradiated K562-derived designer artificial antigen presenting cells (aAPC).

MATERIALS AND METHODS

Plasmids. The donor plasmid pXLBacIIPUbnlsEGFP(26), derived from pBSII-ITR1 (22) was a kind gift from Dr. Joseph Kaminski (Medical College of Georgia, GA) which is a minimal PB vector with terminal repeats of 308 bp and 238 bp at the 5' and 3' ends, respectively. The 2nd-generation CD19RCD28 CAR, was codon optimized (CoOp), to substitute codons with those optimally used in mammals (GENEART, Regensburg, Germany) without altering the anticipated amino acid sequence (16). This codon-optimized CD19RCD28 (CoOpCD19RCD28) CAR was subcloned into pXLBacIIPUbnlsEGFP vector by replacing the EGFP sequence with the CAR to create CoOpCD19RCD28/pXLBacIIUbnls (pPB-CAR) (Figure 1A). The PB transposase was also codon optimized for expression in human cells (GenScript Corp, Piscataway, NJ) and modified to include a 5' SacII restriction site immediately upstream of a strong Kozak initiation signal and a 3' PsiI restriction site after the stop codon. SacII/PsiI digested CoOp piggyBac transposase (hpB) was then subcloned into SacII/PsiI digested pCMV-piggyBac as described elsewhere (25) to create pCMV-hpB (Figure 1B). The SB plasmid ΔCD19/pSBSO was used to express truncated CD19 (30).

Cell lines and primary human T cells. Daudi (Burkitt lymphoma, ATCC cat # CCL-213) and K562 (erythroleukemia, ATCC cat # CCL-243) cells were obtained from American Type Culture Collection (Manassas, VA). Daudi co-expressing β2-microglobulin and GFP (Daudi-β2m-GFP) were a kind gift (31) from Dr. Brian Rabinovich (University of Texas M.D. Anderson Cancer Center, TX). These cell lines were cultured in HyQ RPMI 1640 (HyClone, Logan, UT) supplemented with 2mM Glutamax-1 (Gibco-Invitrogen, Carlsbad, CA), and 10% heat-inactivated defined FCS (HyClone, Waltham, MA), referred to as culture media (CM)(11). GFP+ U251T glioblastoma cell line (a kind gift from Dr. Waldemar Debinski, Wake Forest University, NC) was transfected with the ΔCD19/pSBSO vector and stable transfectants expressing truncated CD19 were grown. Both GFP+ U251T
and CD19^GFP^U251T cells (transfected to express truncated CD19) cells, were cultured in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT), supplemented with 10% heat-inactivated fetal calf serum (FCS). Human T cells were isolated by density gradient centrifugation over Ficoll-Paque-Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), from peripheral blood obtained from Gulf Coast Regional Blood Center (Houston, TX) after consent.

**Artificial antigen presenting cells (aAPC).** K562 cells transduced with lentivirus to co-express CD19, CD64, CD86, CD137L, and membrane bound IL-15 (co-expressed with GFP) were kindly provided by Dr. Carl June (University of Pennsylvania) and used as aAPC for *in vitro* expansion of genetically modified T cells in CM. A subclone of these aAPC (clone #4) are being prepared as a clinical-grade master cell bank by the Production Assistance for Cellular Therapies (PACT, www.pactgroup.net) under the auspices of the NHLBI.

**Electroporation of T cells and selective outgrowth of CAR^+ T cells.** On day 0 of a culture cycle, 10^7^ mononuclear cells from peripheral blood were resuspended in 100 μL of Amaxa Nucleofector solution (CD34 kit, Cat# VPA-1003), mixed with 15 μg of supercoiled plasmids pPB-CAR and pCMV-hpB (7.5 μg each), transferred to a cuvette, electroporated, and cultured overnight as (Program U-14) as described earlier (16). The next day (day 1) the cells were stimulated with γ-irradiated (100 Gy) K562-aAPC (clone #4) at a 1:1 T cell/aAPC ratio. The γ-irradiated aAPC were re-added every 7 days at 1:1 T cell/aAPC ratio. Recombinant human IL-2 (rhIL-2; Chiron, CA) was added to the cultures at 50 U/mL on a Monday-Wednesday-Friday schedule beginning on day 1 of each 7-day T-cell expansion cycle. T cells were enumerated every 7 days and viable cells counted based on Trypan blue exclusion.

**Cellometer.** Automated cell counting was accomplished using a Cellometer (Nexcelom Bioscience, MA). T-cell suspension (20 μL) and 0.2 % Trypan blue were mixed in a 1:1 ratio and 20 μL was loaded onto a disposable counting chamber and inserted into the Cellometer to automatically obtain concentration, live and dead cell counts. Data and images were saved and analysed.

**Western Blot.** Expression of the chimeric 79-kDa CD3-ζ from CD19RCD28 was assessed using a primary mouse anti–human CD3-ζ monoclonal antibody (mAb, 1 μg/mL) (BD Biosciences, CA) and secondary
horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:75,000; Pierce, IL) under reducing conditions, based on methods previously described(32). Protein lysates were transferred onto nitrocellulose membrane using iBlot Dry Blotting System (Invitrogen Corporation, CA) and developed with SuperSignal West Femto Maximum Sensitivity substrate (Pierce, IL) per the manufacturer’s instructions and chemiluminescence was captured after 1-minute exposure using VersaDoc™ 4000 gel documentation system (BioRad, CA).

**Flow cytometry.** Fluorochrome-conjugated reagents were obtained from BD Biosciences (San Jose, CA): anti-human CD4 PerCP Cy5.5 (cat # 341654), anti-human CD8 APC (cat # 555369), anti-human CD27 PE (cat # 555441), anti-human CD28 PerCP Cy5.5 (cat # 337181), anti-human CD62L APC (cat # 559772), anti-human CCR7 PE (cat # FAB197P, R&D systems, Minneapolis). Goat F(ab’)2 anti-human IgG (gamma) R-phycoerythrin conjugate (cat # H10104, Caltag Laboratories) or goat F(ab’)2 anti-human IgG (gamma) FITC conjugate (cat # 109-096-170, Jackson ImmunoResearch Laboratories Inc., PA) was used at 1/20 dilution to detect cell-surface expression of the CD19-specific CAR, CD19RCD28. Blocking of non-specific antibody binding was achieved using FACS wash buffer (2% FBS in PBS). Data acquisition was on a FACSCalibur (BD Biosciences, CA) using CellQuest version 3.3 (BD Biosciences, CA). Analyses and calculation of median fluorescence intensity (MFI) was undertaken using FlowJo version 7.2.2. T-cell receptor (TCR)-Vβ expression was determined with a panel of 24 TCR-Vβ specific mAbs (IO TEST Beta Mark TCR-Vβ repertoire kit, Cat # PN IM3497, Beckman Coulter Inc., CA) used in association with CD3 and appropriate isotype-matched control mAbs according to the manufacturer’s instructions.

**Chromium Release Assay.** The cytolytic activity of T cells was determined by 4 hour chromium release assay (CRA) (32). CD19-specific T cells were incubated with 5x10^3 ^{51}Cr-labeled target cells in a V-bottomed 96-well plate (Costar, Cambridge, MA). The percentage of specific cytolysis was calculated from the release of ^{51}Cr, as described earlier, using a TopCount NXT (Perkin-Elmer Life and Analytical Sciences, Inc., MA). Data are reported as mean ± standard deviation (SD).

**Fluorescent in situ hybridization (FISH).** Exponentially growing genetically modified T cells (5 x 10^8) were harvested after 21 days of co-culture on aAPC and incubated with 0.04 μg/mL demecolcine (Gibco, BRL) for
45 min at 37°C. The treated cells were centrifuged, and exposed to 75 mM KCl for 20 min followed by fixing in methanol/acetic acid mixture (3:1), washing three times with the fixative, and dropping on glass slides for air-drying. CD19RCD28-specific DNA probe was labeled by nick translation with Spectrum green (Vysis, Downers Grove, IL). Hybridization was performed according to the manufacturer's protocol. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and the images were captured using Quips PathVysion system (Applied Imaging Corp, Santa Clara, CA). To determine the number of integrants, 40 to 50 individual metaphase spreads were analyzed.

**Video time-lapse microscopy (VTLM).** To visualize killing of tumor targets by PB modified CD19-specific T cells, we undertook imaging by VTLM, using the BioStation IM Cell-S1/Cell-S1-P system (Nikon, NY). U251T cells were chosen as targets based on an ability to identify living, and dying/dead cells by phase contrast dynamic morphology(30). Parental GFP⁺ U251T cells (green) were used as CD19neg targets while CD19⁺GFP⁺U251T cells (transfected to express truncated CD19) stained with the PKH-26 red fluorescent dye (Cat # MINI26, Sigma, MO, according to the manufacturer’s protocol) which fluoresced orange (green + red) were used as CD19⁺ targets. The CD19neg and CD19⁺ U251T targets were mixed in a 1:1 ratio (0.25 x 10⁶ cell/target) and plated overnight on a T-35 mm glass bottom plate (Fisher scientific, PA) in CM. PB-modified CD19RCD28⁺ T cells (0.2 x 10⁶ in 200 µL CM) were added to the adherent U251T targets and were immediately imaged every 200 seconds at 37°C for up to 4 hours. Each image was recorded at 1,600x1,200 pixels using a 20X objective using a phase contrast channel, fluorescent channel 1 (orange, CD19⁺GFP⁺ U251T) and fluorescent channel 2 (green, CD19⁺ and CD19neg GFP U251T) with an exposure time at 1/125 and 1/5 seconds, respectively. Adherent live U251T cells appear flat and spread out while dying cells rounded up and imploded. Movies showing the killing events were made using the Microsoft® Windows Movie Maker software, Version 5.1.

**DNA PCR for PB transposase.** DNA was isolated from PBMC using the AllPrep DNA/RNA/Protein mini kit (Cat # 8004, Qiagen, CA). PCR was carried out with 50 ng of DNA/sample in a mix containing 10x PCR buffer, 2.5 mmol/L deoxynucleotide triphosphates, 3 µmol/L MgCl₂, and 0.5 units of DNA polymerase (AmpliTaq Gold, Applied Biosystems) in a final volume of 25 µL reaction volume and amplified in a thermal Cycler (PTC-200 DNA
Engine Cycler, Bio-Rad, CA), using piggyBac transposase-specific forward primer 5'-ACGAGCACATCTGTCTGCTCTGCTGCAG-3' (80 ng/µL) and reverse primer 5'-CACATATCGATGTTGTGCTCCCCGGCAGAT-3' (80 ng/µL). After an initial denaturation at 95°C for 5 min, the samples underwent 30 cycles of 95°C for 15 s, 58°C for 40 s, 72°C for 1 min followed by a prolonged extension step at 72°C for 7 min. The housekeeping gene GAPDH was also amplified in the same samples using the forward primer 5'-TCTCCAGAACATCATCCCTGCCAC-3' (80 ng/µL) and reverse primer 5'-TGGGCCATGAGGTCCACCACCCTG-3' (80 ng/µL). The pCMV-hpB transposase plasmid (1 ng/µL) was used a positive control. The PCR products were separated on a 0.8% agarose gel, using 4 µL of each sample per lane. The gel was stained with ethidium bromide (0.1 mg/ml), destained with distilled water and visualized using the VersaDoc™ 4000 gel documentation system (BioRad, CA).

**Chromosome banding analysis.** Exponentially growing PB-modified T-cells cultures were incubated for 2 hours at 37°C with colcemid (20 µL of 0.04 µg/mL) per 10 mL CM followed by 0.075 mol/L KCl at room temperature for 15 min, fixed with acetic acid/methanol (1:3), and thrice washed on a glass slide. For Giemsa (G) banding, slides were treated with trypsin were stained with Giemsa stain following standard techniques described previously(16). A total of 10 G-banded metaphases were photographed and 5 complete karyotypes were prepared using a karyotyping system from Applied Imaging Corporation.

**RESULTS**

**PB-mediated gene transfer and selected expansion of CAR+ T cells.** To evaluate whether the PB system can render primary human T cells specific for CD19, we cloned the 2nd generation CoOp CD19RCD28 CAR (16) into the PB backbone vector as a transposon (Figure 1A), creating the pPB-CAR plasmid. To achieve transposition a CoOp PB transposase was expressed from the plasmid designated pCMV-hpB (Figure 1B). Resting PBMCs were electroporated using the Nucleofector device with one or two PB plasmids: pB-CAR (to express CAR transposon) in the absence and presence (in trans) of pCMV-hpB (to express transposase). After electro-transfer the T cells expressing CAR were propagated on γ-irradiated K562-aAPC expressing CD19 antigen
and the desired co-stimulatory molecules CD86, CD137L, and membrane-bound IL-15 (Figure 1C). After 21 days of continuous co-culture on aAPC the percentage of CD3⁺ T cells expressing CAR was 50% (~70-fold improvement in CAR expression) in cells electroporated with PB transposon and transposase whereas the CAR expression remained at background levels found on T cells electroporated with no DNA (0.7%) or, transposon alone (~1%) (Figure 2A). Furthermore, by the end of 3 weeks an input number of 10⁶ of T cells modified with transposon and transposase increased by 1.5 logs on the K562-aAPC and these T cells continued to numerically expand thereafter. However, control T cells electroporated either with the single plasmid transposon or with no DNA could not be significantly numerically expanded, as shown in Figure 2B. With coculture there was outgrowth of CAR⁺ T cells, with flow cytometry revealing CAR expression peaking at 97% by day 49, and an increase in density as indicated by the MFI peaking at 70 units by day 49 (Figure 2C). To validate the flow cytometry data for the presence of CAR, Western blot analysis was undertaken and an expected 79-KDa band was detected corresponding to chimeric CD3-ζ expression in genetically modified T cells (Figure 2D).

**Redirected function of CAR⁺ T cells after electro-transfer of PB plasmids.** The genetically modified and numerically expanded T cells were evaluated for redirected killing of CD19neg (parental) and CD19⁺U251T (transfected) tumor targets (Figure 3A). They were able to lyse these CD19⁺ targets and specificity of killing was shown by a 3-fold increase in lysis of CD19⁺ U251T (51% specific lysis) over background lysis of CD19neg U251T cells at effector-to-target ratio of 25:1. The genetically modified T cells also demonstrated cytotoxicity against CD19⁺ Daudi cells (65% at 25:1 effector-to-target ratio, Figure 3C). Since, Daudi cells lack β2-microglobulin (β2m) and thus expression of MHC class I they may be sensitive to natural killer (NK) cell-mediated lysis. To determine the potential contribution of NK-cell mediated lysis in the propagated cells, Daudi cell targets were transfected to express β2m to restore MHC class I expression (Figure 3B). The genetically modified T cells were still capable of specific lysis of Daudi-β2m transfectants (43% at 25:1 effector-to-target ratio) as shown in Figure 3C which is consistent with continued CAR-mediated killing of HLA class I⁺ Daudi cells.

**Visualization of CD19⁺ tumor killing by genetically modified T cells.** We employed VTLM to directly visualize killing of CD19⁺ U251T tumor cells by genetically modified CAR⁺ T cells. The CD19neg parental and
CD19\(^+\) transfected U251T targets were admixed in a ratio of 1:1 prior to adding \(PB\)-modified CD19-specific T cells and killing was directly visualized over 4 hours to reveal the engagement/disengagement of T cells (small, irregular bodies shown moving across image frames) to adherent spindle-shaped green U251T tumor cells. After contact with the genetically modified T cells, the CD19\(^+\) U251T orange tumor cells rounded and imploded while the CD19\(^{\text{neg}}\) U251T tumor cells did not (Figure 3Di). Two movies (movie, 1 and 2), representing killing events each over 4 hours of visualization have been uploaded for online viewing (Figure 3Dii). These microscopy data validate the CRA experiments and show that the \(PB\) modified CD19\(^+\) T cells are redirected to specifically lyse CD19\(^+\) tumor cells.

**Memory and effector phenotype of \(PB\) modified CAR\(^+\) T cells.** It is recognized from human trials and experiments with non-human primates and mice that adoptive transfer of central memory T cells can lead to long-lived immune response (33-35). Therefore, flow cytometry was used to investigate the detection of cell-surface markers on T cells associated with central memory (CM) after \(PB\) transposition and propagation. We demonstrate that numerically-expanded \(PB\)-modified CAR\(^+\) T cells expressed both CM markers (34, 36, 37) as well as determinants of effector memory (EM) phenotype (Figure 4A). On further analysis, 62% of CAR\(^+\) T cells expressed CD62L and CD45RO, which is consistent with a T\(\text{CM}\) phenotype (Figure 4B). EM T cells can be distinguished from CM T cells by presence of both CD28 and Fas (33). Using these markers, we were able to identify that \(PB\)-modified and propagated CM (CD28\(^+\)CD95\(^+\)) T cells constituted \(\sim60\%\) of the total cell population, (25\% of which expressed CAR) and CD28\(^{\text{neg}}\)CD95\(^+\) EM T cells represented the remainder of the propagated T cells (25\% of which expressed CAR) (Figure 4C). These data demonstrate that the combination of electro-transfer of PB system and aAPC can be used to propagate populations of CAR\(^+\) T cells with a phenotype predictive for long term human engraftment.

**Electroporation and propagation does not lead to oligoclonality.** One potential deleterious problem after gene transfer in T cells is outgrowth of a clonal or oligoclonal sub-group of T cells. This could represent emergence of a transformed phenotype or indicate that only a subset of input T cells stably express the CAR which might bias against sustained persistence after their adoptive transfer. Since T cells express a broad diversity of endogenous \(\alpha\beta\) T-cell receptors (TCRs) before gene transfer, we examined the repertoire of V\(\beta\) to
determine whether there was an undesired loss of TCR family members indicating a bias in the propagation of just a subset of T cells. Therefore, the TCR repertoire was determined before and after gene transfer and based on a flow cytometry analysis of 24 Vβ TCR chains, we observed no major shifts in the expression/usage of TCR expression before and after electroporation/propagation (Figure 5A). Therefore, PB-mediated genetic modification of human primary T cells does not appear to significantly alter the TCR Vβ repertoire and is indicative that the CAR transposon is efficiently integrated into many T cells at the time of electro-transfer. The lack of emergence of a clonal or oligoclonal subpopulation of CAR+ T cells helps relieve concerns that the gene transfer event using the PB system may lead to aberrant T-cell growth. Furthermore, infusion of a broad repertoire of T cells increases the probability that a subpopulation of T cells can survive after adoptive transfer. Maintenance of a broad endogenous αβ TCR diversity also has implications for design of adoptive immunotherapy trials as this is desired to restore immune reconstitution after lymphodepleting or myeloablative preparative regimens.

**Lack of autonomous proliferation of T cells.** Gene transfer with PB system may cause genotoxicity and the potential for aberrant T-cell growth. Therefore we cultured the T cells in the absence/presence of K562-aAPC and cytokine (IL-2, 50 U/mL) and demonstrated that the PB-modified CD19-specific T cells survive and sustain proliferation only in the presence of K562-aAPC and IL-2 (Figure 5B). These data may be used in support of release criteria for T cells genetically modified by PB system.

**Lack of long-term expression of PB transposase.** Continued presence of transposase in PB modified T cells may lead to genotoxicity. Therefore, we carried out a genomic PCR analysis to evaluate for the continued presence of the codon optimized PB transposase. Using T cells which were electroporated with PB transposon and transposase and had undergone 5 weeks of co-culture with K562-aAPC, we could not detect the PB transposase gene (size ~1,750 bp) (Figure 6A). These results indicate that the PB transposase was not appreciably integrated into the genome of T cells expressing the CD19RCD28 CAR.

**Number of copies of integrated transposon by FISH.** FISH was performed to assess the copy number of the integrated CAR transgene after electro-transfer of PB system and numeric expansion of T cells for 4 weeks on K562-aAPC. The PB-modified CAR+ T cells were only observed to carry only one copy per cell of the
CD19RCD28 transgene (Figure 6B). These results are comparable to those observed with the CAR\(^+\) T cells modified with the SB transposon/transposase system (unpublished data).

**Karyotype of genetically modified T cells.** The overall integrity of the chromosome structure was evaluated as a measure of global genotoxicity associated with undesired and continued transposition. G-banding analysis of the \(PB\)-transfected T cells showed a normal male karyotype, 46 XY with no apparent significant numerical or structural chromosome alterations (Figure 6C). These data support the premise that \(PB\) transposition in human T cells is not associated with major translocations and chromosomal aberrations, although the possibility of chromosomal damage below the limit of detection of this technique cannot be excluded.

**DISCUSSION**

To obtain pre-clinical data for non-viral gene transfer by \(PB\) transposon/transposase system in gene therapy trials we genetically modified primary human T cells with the codon optimized CD19-specific 2\(^{\text{nd}}\) generation CAR, CD19RCD28. Our data demonstrate for the first time that the \(PB\) system can be electro-transferred into human T cells to express a desired CAR. The efficient integration efficiency of \(PB\) was confirmed by the stable expression of CD19-specific CAR within 3 weeks of coculture on K562-aAPC of human T cells co-electroporated with pPB-CAR (transposon) and pCMV-hpB (transposase) when compared to cells electroporated with transposon alone.

Electro-transfer with \(PB\) plasmids and subsequent CAR-mediated propagation on aAPC supported proliferation of memory T cells, in particular \(T_{CM}\) and \(T_{EM}\) with associated desired phenotypes, as subsets of the CAR\(^+\) T cells maintained expression of CD27, CD28, CD45RO, CD62L, and CCR7. These data have implications for improved *in vivo* efficacy as antigen-specific \(T_{CM}\) cell subsets are associated with long-term persistence after adoptive transfer in macaques (33). It is not currently known whether adoptive transfer of CAR\(^+\) T cells enriched for \(T_{CM}\) will provide superior protective immunity against cancer.

There are several potential advantages for electro-transfer of the \(PB\) transposon system for therapeutic genetic modification of T cells for clinical application. Non-viral therapies are less expensive to produce and thus may be more widely applicable for gene transfer compared with use of clinical-grade recombinant viruses. However, there are trade-offs to electro-transfer of DNA plasmids compared with viral-mediated transduction, such
as potentially reduced integration frequency. Nevertheless, in an initial direct comparison to other transposon systems, *PB* system showed the highest gene transfer efficiency in mammalian cells using a native transposase (i.e. not an hyperactive variant) (24-26, 38, 39). Creation of hyperactive *piggyBac* elements may further increase integration efficiency. The ability of *PB* to transpose large cassettes efficiently could be exploited in gene therapy trials where expression of large transgene, or co-expression of more than one therapeutic transgene, is necessary. Further, the *PB* transposase may be modified to achieve targeted integration such as by addition of zinc-finger DNA binding domain, resulting in site-directed integration (25, 26),(38),(40). Regulation of *PB* activity by an inducible *PB* system may provide further safety in clinical trials (38). Importantly, *PB* exhibits precise excision in human and mammalian cells (25, 39). Other systems, such as *SB*, leave behind a 5 bp footprint upon excision which might lead to mutagenesis within the human genome outside of the site of final integration (41). *PB* system’s lack of footprint upon excision may limit mutagenesis to the site of final integration increasing the chances that the rest of the cellular genome remains intact.

There are also potential obstacles in using the *PB* transposon/transposase system for therapeutic gene transfer. Any integrating element carries with it the potential risk of genotoxicity. However, site-directed transposable elements are one proposed strategy to limit genotoxicity(40). Additionally, like other transposable elements, there are domesticated *PB*-like elements within the human genome (42, 43). Although the protein and DNA sequences of these elements are different than the *PB* system used in this study, how these elements may affect *PB* activity or how *PB* may alter these elements within the human genome will need to be addressed in regards to clinical utility. Our initial results to address safety of using *PB* for genetic modification of T cells for cancer therapy showed, as had hoped, no significant oligoclonality or evidence of autonomous proliferation after gene transfer (16). As with the *SB* (16), we observed no expression of the *PB* transposase over the long-term which will limit potential ongoing transposition due to continued presence of *PB* transposase. Karyotype analysis revealed a normal karyotype in cells having undergone *PB* transposition. Ultimately, a suicide gene could be used in combination with the therapeutic gene(s) of interest in *piggyBac* applications for removal of cells which underwent gene transfer *in vivo.*
The simplicity of the approach, namely electroporating T cells with two DNA plasmids and selectively expanding CAR⁺ T cells, including T_{CM}, on γ-irradiated K562 aAPCs, lends impetus to the development of clinical-grade CAR⁺ T cells using the PB system for application in human immunotherapy trials.
Figure 1
Schematic of the two PB DNA plasmids to be electro-transferred and ex vivo culturing platform to generate CAR⁺ T cells. **A,** CoOpCD19RCD28/pXLBacIIUbns (pPB-CAR, Transposon). Polyubiquitin promoter; CoOpCD19RCD28, codon-optimized CD19RCD28 CAR; pBac3’ and pBac5’, PB-inverted/direct repeats; bGh polyA, polyadenylation signal from bovine growth hormone; AmpR, ampicillin resistance gene. **B,** pCMV-hpB (Transposase). hpB, codon optimize PB-transposase d; CMV promoter, CMV enhancer/promoter; pUC ori, minimal E.coli origin of replication. **C,** Scheme for electroporation with PB plasmids and propagation on CD19-specific K562-derived aAPC. Electroporation with transposon (blue) provides only transient expression unless incorporated into a transposon vector that can be cleaved from the plasmid and integrated into a host genome by a source of transposase (red). On the day after electroporation, T cells were co-cultured with γ-irradiated K562 genetically modified to co-express CD19, CD64, CD86, CD137L (4-1BBL) and cell-surface membrane-bound IL-15 (fusion of IL-15 cytokine peptide and human Fc), with addition of IL-2 every alternate day, resulting in expansion of stably transfected CAR⁺ T cells to clinically significant numbers.
Figure 2

CAR expression on PBMCs after electro-transfer of PB vectors and expansion of CAR⁺ T cells upon co-culture with aAPC.  

A, expression of CAR on CD3⁺ T cells by flow cytometry with anti-Fc antibody after electro-transfer of no DNA, PB transposon with or without hpB transposase at 24 hours and 3 weeks of co-culture on γ-irradiated K562-derived aAPC expressing tCD19, CD64, CD86, 4-1BBL and IL-15-Fc.  

B, Kinetics of propagation of T cells upon co-culture with aAPC. The average T-cell numerical expansion was ~70 fold after every 7d for up to 5 wk of continuous co-culture with aAPC.  

C, Kinetics of CAR expression. Median fluorescence intensity (MFI) and expression of CAR on T cells co-transfected with PB transposon and transposase upon co-culture with K562-aAPC over a period of time.  

D, Western blot analysis of CAR expression, detected by mAb specific for CD3-ζ. Whole-cell protein (20 μg) lysates from primary unmodified T cells (lane 1), primary T cells genetically modified with pPB-CAR and hpB and propagated for 3 weeks on aAPC (lane 2) and Jurkat cells electroporated to express CD19RCD28 CAR (lane 3, positive control) were resolved by SDS-PAGE under reducing conditions.
Figure 3

Redirected specificity of PBMCs genetically modified with PB system. A, GFP\(^{+}\)U251T targets were transfected with truncated CD19 expressing plasmid and stable transfectants were analysed for CD19 expression by flow cytometry. B, Expression of cell surface MHC class I and II on Daudi targets by flow cytometry. C, Killing of CD19\(^{+}\) target cells (HLA class I\(^{\text{neg}}\) Daudi; HLA class I\(^{+}\) Daudi β-2M; U251T CD19\(^{\text{neg}}\) glioblastoma cells; U251T cells transfected to express truncated CD19) in a standard 4-hour CRA. Points represent mean specific lysis of triplicate wells at effector to target (E:T) cell ratios; bars SD. D, VTLM to evaluate tumor killing by PB-modified CAR\(^{+}\) T cells. (i), To explain generation of tumor targets we created a paradigm. To distinguish the GFP\(^{+}\)CD19\(^{+}\) from the GFP\(^{+}\)CD19\(^{\text{neg}}\) U251T, the red fluorescent dye PHK-26 red fluorescent was pre-loaded onto the CD19\(^{+}\) target cells, which resulted in cells appearing orange (a merging of GFP-green with PHK-red). The CD19\(^{\text{neg}}\) and positive targets mixed in a 1:1 ratio were plated overnight. PB modified CAR\(^{+}\) T cells were added to these targets after overnight plating at an E:T ratio of 10:1. Cells were incubated for 4h and simultaneously imaged by VTLM as described in the materials and methods. CD19\(^{+}\) tumor targets which were engaged, disengaged and killed by the T cells, imploded and lysed and are shown as (greenish-yellow irregular cells), while live CD19\(^{\text{neg}}\) tumor targets remained flat and spread out (green). (ii), Two movies at low (movie #1) and high (movie #2) power showing tumor cell killing by PB modified CAR\(^{+}\) T cells. In each case the killing event was condensed to 12-14 seconds for visualization.
Figure 4
Characterization of CAR⁺ T cells on PBMC after electro-transfer of PB vectors. A, immunophenotype of memory cell markers (CD27, CD28, CD62L) on PB modified T cells generated after 4 weeks of co-culture on aAPC. The black solid histograms reveal the percentage of T cells expressing CD27, CD28 and CD62L in the lymphocyte-gated population. T cells expressing the memory cell markers were analyzed for co-expression of CAR and CD4 or CD8. B, expression of CD45RO and CD62L on T cells generated after co-culture. CD45RO and CD62L double-positive T cells were analyzed for the expression of CAR. C, T_{CM} defined as CD28⁺CD95⁺ and T_{EM} defined as CD28⁻⁰⁻CD95⁺ were analyzed for co-expression of CD62L and CAR.
Figure 5

Lack of oligoclonality and autonomous proliferation after electro-transfer with PB vectors.

A, TCR Vβ analysis by flow cytometry 4 weeks after electro-transfer of PB plasmids (filled columns) or matched no plasmid, unmodified T cells (open columns). B, T-cell proliferation analyses directly imaged using a Cellometer in the absence/presence of K562-aAPC and IL-2. Data show primarily dead T cells (shrivelled) when K562-aAPC and IL-2 are removed compared with healthy (refractile, rounded) T cells when K562-aAPC and IL-2 are present.
Figure 6
Safety issues regarding PB transposase and chromosomal aberrations. A, lack of integration of PB transposase by genomic PCR from genetically modified and propagated peripheral blood–derived T cells. DNA was isolated from T cells after mock electroporation (lanes 2 and 4 no DNA, 50 and 100 ng genomic DNA, respectively), from T cells 28 days after electroporation with the two-plasmid PB system (lanes 3 and 5, 50 and 100 ng genomic DNA, respectively). Lane 1, pCMV-hpB plasmid DNA (1 ng) loaded as a positive control. PCR was carried out using transposase-specific primers and GAPDH-specific primers in the same reaction. B, Idiogram of a G-banded karyotype of PB-transfected peripheral blood–derived T cells showing no apparent numerical or structural chromosome alterations. C, Fluorescence in situ hybridization (FISH) analysis of PB modified CAR+ T cells. Number of copies of CD19RCD28 transgene integrated upon electroporation with PB vectors and propagation on CD19-specific K562-derived aAPC was determined by FISH analysis as described in the materials and methods. A CD19-specific probe was prepared by labeling by nick translation method with Spectrum green (Vysis, Downers Grove, IL). Hybridization was performed with exponentially growing PB modified T cells expanded on CD19-specific K562 derived aAPC for 28 days according to the manufacturer's protocols. The slides were counterstained with DAPI and the images were captured using Quips PathVysion system (Applied Imaging). Data shown is a representation after analyzing 40-50 individual metaphase spreads. 23 pairs of chromosomes are shown in the picture and the arrow indicates the integration sites.
Acknowledgements

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FIGURE 1

(A) pPB-CAR (Transposon) 
- pBac 3' 
- pBac 5' 
- BGH-polyA 
- AmpR 

polyubiquitin promoter

CD19RCD28

(B) pCMV-hpB Transposase 
- CMV IE 
- SV40 
- AmpR 
- pUC 
- Ori 
- hpB

(C) Stably-transfected T cells
- Co-culture with K562-derived aAPC
- Antigen-specific proliferation of CAR+ T cells
- Numeric expansion of CAR+ T cells with integrated transposon on γ-irradiated K562-aAPC
- Transduction with lentivirus

Peripheral blood mononuclear cells

Electroporation with piggyBac DNA plasmids

Key
- CD86
- CD64
- CD19
- 4-1BBL
- IL-15-Fc
- EGFP
- Transposon
- Transposase (pCMV-hpB)

Masterbank of K562-aAPC (Not administered, used only ex vivo)

Infusion

Cryopreservation

K562 aAPC

T cell

K562
FIGURE 2

(A) No DNA

Day 1

No DNA
Transposon
Transposon + Transposase

Day 21

(B)

(C)

(D)

Viable T-cell number

Days of co-culture

% CAR expression

Days

Median Fluorescence Intensity (MFI)

Endogenous CD3-

Chimeric CD3-

88 kDa
42 kDa

88 kDa
42 kDa
FIGURE 3

(A) CD13+ U251T

(B) Qued

(C) Effector:Target ratio

(D) (i) CD13+ GFP+ U251T cell

(ii) MOVIE 1 START END MOVIE 2 START END
(A) % Vβ usage in lymphocyte subsets

- Pre-electroporation
- Transposon+Transposase

(B) TCR Vβ family

<table>
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<td>(1.3 x 10^6 cells/mL)</td>
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<tr>
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FIGURE 6

(A) Transposase

(B) GAPDH

(C) Chromosomes