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A new approach to gene therapy using *Sleeping Beauty* to genetically modify clinical-grade T cells to target CD19

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Summary

The advent of efficient approaches to the genetic modification of T cells has provided investigators with clinically appealing approaches to improve the potency of tumor-specific clinical grade T cells. For example, gene therapy has been successfully used to enforce expression of chimeric antigen receptors (CAR) that provide T cells with ability to directly recognize tumor-associated antigens without the need for presentation by human leukocyte antigen. Gene transfer of CARs can be undertaken using viral-based and non-viral approaches. We have advanced DNA vectors derived from the *Sleeping Beauty* (SB) system to avoid the expense and manufacturing difficulty associated with transducing T cells with recombinant viral vectors. After electroporation, the transposon/transposase system improves the efficiency of integration of plasmids used to express CAR and other transgenes in T cells. The SB system combined with artificial antigen-presenting cells (aAPC) can selectively propagate and thus retrieve CAR⁺ T cells suitable for human application. This review describes the translation of the SB system and aAPC for use in clinical trials and highlights how a nimble and cost-effective approach to developing genetically modified T cells can be used to implement clinical trials infusing next-generation T cells with improved therapeutic potential.

Keywords

gene therapy; T cells; Sleeping Beauty; transposon; transposase CD19; adoptive immunotherapy

Genetic modification *ex vivo* to enhance *in vivo* therapeutic potential

Gene therapy has been successfully combined with T-cell therapy to generate potent immune cells that upon administration can sustain proliferation, home to sites of malignant disease, and recycle effector functions in the tumor microenvironment. This bench-to-bedside-to-bench circle of innovation is driven by an understanding of translating immunology into immunotherapy and harnessing vector systems for safe gene transfer. The

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stable integration of transgenes into T cells can be accomplished using viral and non-viral systems. Among the latter, the electro-transfer of DNA plasmids is appealing as investigators can use commercial electroporation devices and readily produce, or contract to have made, DNA plasmids suitable for *ex vivo* genetic manipulation. Up to now, the widespread adoption of electroporation of T cells to express transgene from an introduced DNA plasmid has been limited due to low frequency of integration events from an approach that had relied on illegitimate homologous recombination events. We have advanced the transposon/transposase system from *Sleeping Beauty* (SB) as an approach to improve the rate of transgene integration upon synchronous electro-transfer of DNA plasmids coding for SB transposon and SB transposase. This review summarizes this advance to gene therapy in the context of redirecting T-cell specificity.

Redirecting T-cell specificity for tumor via CARs

Cancer typically arises in the host with a healthy immune system due, in part, to tolerance of the T-cell receptor (TCR) to tumor-associated antigens (TAA). Circumventing tolerance to engender a desired immune response can be achieved by using the mouse to generate antibody against TAA found on the cell surface of malignant cells. The antigen-binding region of a monoclonal antibody (mAb) can then be cloned and expressed as the scFv region imparting specificity to the prototypical chimeric antigen receptor (CAR). The complete CAR molecule consists of a scFv held in frame by an extracellular scaffold and fused via a transmembrane domain to one or more intracellular signaling domains. The CAR directly docks with TAA and can recognize tumor independent of human leukocyte antigen (HLA). Thus, this single-chain immunoreceptor can help broaden the application of adoptive immunotherapy as it avoids the need to pair TCR-mediated recognition of peptide antigen with the restricting HLA. The original first-generation CAR molecule was described by Zelig Eshhar in 1989 (1, 2) with a patent US 7,741,465 B1 filed in 1993 and issued in 2010 stemming from this work. It has required several decades of investigation in not-for-profit academic centers to develop and implement the gene therapy tools, refine the CAR design, and implement an approach to production in compliance with current good manufacturing practice (cGMP) to enable T cells to be genetically modified to stably express CAR in a manner suitable for their human application.

The B-lineage antigens have been recognized as a 'safe harbor' for the development and implementation of novel clinical trials infusing T cells genetically modified to be specific for CD19. This TAA is present on the cell surface of most malignant B cells and thus a CAR designed to target CD19 has the potential to target a wide variety of hematologic malignancies. Similarly, as the distribution of CD19 is apparently confined to malignant and normal B cells, it is anticipated that there will be the potential for 'on target' adverse events due to the loss of the recipient's healthy CD19⁺ B cells and damage to humoral immune system. We and others have successfully shown the T cells derived from peripheral blood can be genetically modified to express CD19-specific CARs (3, 4). However, other sources of T cells and sub-populations of T cells may have clinical appeal as a cellular substrate for the stable expression of CARs. For example, the ability to undertake allogeneic hematopoietic stem-cell transplantation (HSCT) across traditional HLA barriers makes umbilical cord blood (UCB) an attractive source of T cells. In an effort to improve the graft-

versus-tumor effect, we have demonstrated that CD19-specific CAR⁺ T cells can be manufactured from small amounts of UCB to provide recipients of allogeneic UCB transplantation (UCBT) with donor-derived tumor-specific T cells (5, 6). Currently studies infuse ‘bulk’ population of cells, therefore we and others seek to define and characterize the best cellular substrate for genetic modification and expression of CARs. Circulating T cells can be characterized by immunophenotype as naive (Tn), central memory (Tcm), and effector memory (Tem) cells. Naive-derived effector cells (Teff) are an attractive population of $\alpha\beta$ TCR⁺ T cells for adoptive immunotherapy, as they have conferred improved anti-tumor activity (7). We determined the relative frequency of Tn (CD62L⁺CD45RA⁺), Tcm (CD62L⁺CD45RA⁻), Tem (CD62L^{neg}CD45RA⁻), and Temra (CD62L⁻CD45RA⁺) subsets in the peripheral blood (PB) of healthy donors and observed an abundance of circulating Tn cells compared to Tcm ($p < 0.001$), Tem ($p < 0.05$), or Temra ($p < 0.001$) cells, which supports circulating T cells as a rich source of naive T cells for expression of CARs (8). Current clinical trials chiefly administer CAR⁺ on T cells co-expressing $\alpha\beta$ TCRs, as this population dominates (95%) of T cells in PB. The remaining 1-5% includes T cells expressing $\gamma\delta$ TCRs. This circulating T-cell sub-population are also attractive for adoptive immunotherapy, as they inherently recognize a broad range of tumor cells and potentially can be infused across HLA barriers without causing graft-versus-host-disease (GVHD). Therefore, we and others have expressed CD19-specific CARs in $\zeta\delta$ TCR⁺ T cells (9, 10).

In addition to the constitution of the T cell, the design of the CAR impacts the therapeutic potential of the genetically modified product. Most CARs for oncology applications typically consist of a (i) scFv, comprising of V_H and V_L domains of a TAA-specific mAb, linked via (ii) flexible hinge sequence to the cell membrane by (iii) transmembrane sequence, fused to an (iv) intracellular signaling domain (CD3- ζ from TCR complex) or with costimulatory molecules. To provide a fully competent T-cell activation signal, minimally defined as TAA-dependent (i) proliferation, (ii) cytokine production, and (iii) killing, multiple investigators have advanced CAR structures to include one or more costimulatory signaling domains, such as derived from CD28, CD134, CD137, DAP10, DAP12, ICOS, Lck (11-15). The clinical impact of such varied CAR designs to impact the ability of T cells to eliminate bulky disease, maintain remission, and prevent recurrence will need to be elucidated. For example, the CAR molecule that synchronously activates T cells to target relapsed hematologic disease may be different from the immunoreceptor species to address minimal residual disease or solid tumors.

Significant progress has been made towards the clinical application of CAR⁺ T cells especially to control B-cell malignancies. However, questions remain regarding the source of T cells and composition of the CAR, which may need to be personalized to meet the need of individual patients with regards to tumor type, burden, and biodistribution. Thus, while the CAR may be ‘universal’ in terms of the potential to recognize a TAA on a tumor type from multiple recipients, it is probable that this universality may not extend to other parts of the CAR molecule (e.g. the signaling moiety within the endodomain) and the cellular template on which the CAR is expressed. This is one of the reasons we implemented a new approach to human gene therapy based on non-viral gene transfer of the SB system recognizing that the approach could be rendered nimble and cost effective. DNA plasmids can be readily modified and produced for human application and this will enable

investigators to have a clear line-of-sight between ideas tested in the laboratory and infusions of T cells that draw upon panels of CAR⁺ T cells.

Non-viral gene transfer

Non-viral gene transfer of CAR transgenes offers some advantages over viral-based systems to integrate transgenes into the T-cell genome, as DNA plasmids are comparatively inexpensive to produce, are non-immunogenic, and are not generally constrained on delivery of DNA size (since there is no packaging). However, the stable introduction of naked DNA plasmids has been a low-efficiency event subject to silencing of transgene expression and can be associated with integration of multiple copies (16-18). We have overcome these hurdles using SB-derived transposons which do not depend on illegitimate recombination to achieve stable integration. Originally described by Perry Hackett in 1996 (19), the SB transposon/transposase system (20) (Fig. 1) is derived from fish and has been adapted for gene therapy (21-23). The SB system consists of a transposase enzyme which recognizes the inverted repeat containing direct repeated sequences (IR/DR) flanking the transgene (e.g. CAR) in a transposon. Recently, our group has employed the SB system to introduce CAR to redirect specificity of clinical-grade T cells (24, 25), as we have reviewed (26-29). Concern of genotoxicity is mitigated as SB transposase mediated insertion of the cargo load at TA dinucleotide repeats and unlike other gene transfer systems, the SB transposition event does not have preference for integration either into or proximal to transcriptional units. Concerns for remobilization of transposon due to residual SB transposase activity are assuaged as the SB11 transposase, as derived from fish, is likely immunogenic and post-translational control of the transposase is subject to a negative feedback loop by its degradation products (30, 31). Furthermore, re-hopping of integrated SB transposon is unlikely as the human genome does not contain endogenous transposase capable of remobilizing introduced fish-derived SB genetic elements (29). Thus, the SB system appears to have attractive features for human gene therapy and meets the need for an approach for non-viral gene transfer that might lower the cost and improve the speed to translate pre-clinical data into clinical trials.

Human application of SB system

We have worked since 2006 at MD Anderson Cancer Center (MDACC) to adapt the SB system to genetically modify T cells to express CD19-specific CAR in compliance with cGMP. Our first human trials infusing SB-modified CAR⁺ T cells, beginning in 2012 signify the fastest that a new gene therapy has been successfully brought from the bench to the bedside (32). Our approach to manufacture was based on (i) the use of readily-available commercial device (e.g. Nucleofector from Lonza, <http://www.lonza.com/>) to synchronously electro-transfer two supercoiled DNA plasmids coding for SB transposon (the CAR) and SB transposase (SB11), (ii) commercial vendor to produce DNA plasmid (e.g. Waisman Biomanufacturing, <http://gmpbio.org>, and Aldevron, <http://www.aldevron.com>), (iii) and engineered artificial antigen presenting cells (aAPC) to retrieve T cells stably expressing CAR integrants over culture time, with T cells lacking CAR unable to sustain proliferation. The criteria we use to release DNA plasmids for the electroporation in compliance with cGMP are shown in Table 1. The ability to selectively propagate T cells based upon

activating CAR by TAA and costimulatory molecules co-expressed on designer aAPC (4, 33) allows us to test the hypothesis that those T cells genetically modified to numerically expand *ex vivo* may exhibit improved ability to proliferate and survive *in vivo*. Quiescent T cells (typically $1-2 \times 10^7$ mononuclear cells per cuvette) from PB and UCB are electroporated in the presence of DNA plasmids coding for second-generation CD19-specific CAR [designated CD19RCD28 (34), that co-activates T cells via chimeric CD3- ζ and CD28] and SB11 transposase (transposon to transposase ratio of 3:1, 15:5 μ g per cuvette) using the human T-cell kit and Nucleofector II electroporation device. The SB transposon is under control of human elongation factor-1 α (EF-1 α)/human T-cell leukemia virus (HTLV) hybrid composite promoter (35) and flanked by the pT (20) and pT2 (36) IR/DR, while SB11 (37) is under control of a CMV promoter (25). We demonstrated a 60-fold improved integration efficiency of CAR transgene in SB-modified aAPC-propagated T cells, compared to electro-transfer of DNA without transposition (3). Our typical clinical CAR⁺ T-cell products are characterized based on release criteria (Table 2) and in-process testing (Table 3) as based on the manufacturing and correlative data as published (24, 25, 38). To date, we have opened four clinical trials at MDACC administering patient- and donor-derived CD19-specific CAR⁺ T cells after autologous and allogeneic HSCT, including UCBT, and after lymphodepleting chemotherapy (Table 4).

Next generation SB systems

Efforts to improve the frequency of integration of SB system have been based upon systematically improving the enzymatic activity of the SB transposase with three muteins currently being employed and ranked here with increasing ability to mediate transposition: SB10 (20), SB11(37) and SB100X (39). We compared SB11 and SB100X to generate genetically modified T cells and observed increased enzymatic activity of SB100X, which resulted in a more efficient outgrowth of CD19-specific CAR⁺ T cells on aAPC, compared with SB11. However, the increased rate of transposition resulted in increased copy number of CAR integration events resulting in our need to reduce the amount of DNA plasmid used to electroporate the T cells (40). Thus, on balance there was little immediate advantage to alter our approach to manufacturing CD19-specific CAR⁺ T cells based on swapping SB11 for SB100X.

One potential limitation of the SB system is that transposition rate is inversely proportional to the size of the cargo (41). The efficiency of SB-mediated transposition appears to decrease when the expression cassette size is larger than 5 kb (41). Other transposon systems such as *piggyBac* and *Tol2* apparently tolerate larger transgenes than the SB system. We and others have successfully adapted the *piggyBac* system to generate CAR⁺ T cells (33, 42). However, concerns remain *piggyBac* system has a pronounced preference for integration into transcriptional units compared to the SB system and that *piggyBac* transposase may liberate endogenous human sequences. The SB system has been modified by us to co-express more than one transgene. This can be achieved using bicistronic/multi-functional fusion SB DNA plasmids (43) or by the synchronous electro-transfer of two SB plasmids coding for different transposons that undergo transposition in the same T cell (44, 45) as mediated by electro-transfer of DNA (or mRNA) coding for SB transposase. This has been employed by us to stably introduce not one, but two transgenes at once (double

transposition). The advantage of this approach is that the CAR can be combined with other molecules such as for non-invasive imaging, homing, or improving persistence.

Our approach to generating SB-modified T cells in compliance with cGMP is currently based on the electro-transfer of two DNA plasmids coding for the SB transposon and transposase *in trans*. We are adapting the electroporation conditions to eliminate the possibility that the introduced SB transposase can integrate into the T-cell genome. This is based on the co-electro-transfer of SB-derived DNA coding for the CAR and *in vitro*-transcribed mRNA coding for SB11. There is precedence for this approach (46), and our efforts are focused on bringing this to the GMP facility in support of next-generation and multi-center clinical trials. We are also developing an alternative based on a self-inactivating DNA construct, similar to piggyBac system (47) that eliminates expression of SB transposase placed *in cis* with SB transposon.

aAPCs to selectively propagate CAR⁺ T cells

Currently, our approach to manufacturing CD19-specific T cells is based on electro-transfer of DNA plasmids from the SB system and selective propagation of CAR⁺ T cells to clinically appealing numbers upon recursive (every 7 to 10-day) additions of γ -irradiated aAPCs in the presence of soluble recombinant IL-2 and IL-21 (4). The immortalized K562 cells, a human erythroleukemic cell line derived from a patient with blast crisis (48), was selected as the source of aAPC, as these cells have been directly administered to humans (49, 50), can be readily genetically modified with lentivirus and SB systems, and express endogenous molecules [such as ICAM-1 and LFA-1 (51)] that can activate T cells (and NK cells). Furthermore, they lack of expression of HLA class I A and B alleles [express HLA class C molecules (52)] and all HLA class II alleles (53, 54) and thus are unlikely to propagate T cells with alloreactive potential. The K562-derived aAPCs can be pre-irradiated (100 Gy) prior to thawing and used in co-culture with T cells and thus offers an attractive platform that avoids the need, inconvenience and lot-to-lot variation associated with the use allogeneic peripheral blood mononuclear cells (PBMC) as irradiated feeder cells which are expensive and time-consuming to procure from volunteer donors. We initially showed that K562-derived aAPC co-expressing 4-1BBL and MICA (55) were more effective at numerically expanding CD19-specific CAR⁺ T cells compared with a rapid expansion protocol based on cross-linking CD3 (with mAb clone OKT3) in the presence of LCL and PBMCs (56). K562-derived aAPCs have further been modified by us and others to express a wide array of costimulatory molecules, including CD40, CD40L, CD70, CD80, CD83, CD86, CD137L, ICOSL, GITRL, CD134L, to facilitate proliferation of T cells (57, 58). In collaboration with Carl June, we genetically modified CD32⁺ K562 to co-express CD64 (the high affinity Fc receptor, for loading mAb), CD86, CD137L (4-1BBL) and a membrane-bound IL-15 mutein (mIL15). A clone (number 4) of these K562-derived aAPCs selectively propagated CAR⁺ T cells while preserving naïve and memory immunophenotypes (3, 4). The aAPC clone #4 were produced for using in compliance with cGMP as a master cell bank (in collaboration with PACT under the auspices of NHLBI) and a derived working cell bank at MDACC in support of on-going clinical trials (Table 4). The testing to release the banks of clinical-grade aAPCs are described in Table 5.

Correlative studies to inform on therapeutic potential of infused CAR⁺ T cells

Clinical trials infusing SB-modified and aAPC-propagated T cells will reveal the feasibility and safety of this new approach to gene therapy. Since persistence of CAR⁺ T cells is likely proportional to the anti-tumor effect, we developed a mAb (clone 136.20.1) that binds the scFv region of the CD19-specific monoclonal antibody clone FMC63 (59, 60). We demonstrated that clone 136.20.1 can detect (1:1000) CD19-specific CAR⁺ T cells (61), and this reagent has been successfully used by investigators to measure the abundance and biodistribution of CD19-specific CAR⁺ T cells after infusion (62, 63). In some instances, particularly when the bioburden of circulating tumor cells is low, the persistence of infused T cells in peripheral blood may be below the limit of detection by flow cytometry and survival of the genetically modified T cells may be detected by quantitative PCR (Q-PCR) using CAR specific primers (38).

Measuring persistence provides one measure of success, but the infused T cells need to find and engage CAR molecules with TAA to eliminate tumor. Flow cytometry and Q-PCR on samples serially obtained from tissues is one approximation to the homing potential of infused CAR⁺ T cells. The SB system has been adapted to avoid the need for biopsy and sampling by co-expressing a reporter gene with CAR for non-invasive imaging. For example, a four-function reporter transgene can be introduced by 'double transposition' to achieve (i) *in vitro* selection under cytotoxic concentration of hygromycin B, (ii) *in vivo* ganciclovir-dependent conditional ablation, and (iii) *in vivo* bioluminescence imaging (BLI) simultaneous with (iv) positron emission tomography (PET) (43, 64). Similarly, a CD19-specific CAR⁺ T cells co-expressing a fusion gene of hygromycin phosphotransferase and thymidine kinase (TK) derived from herpes simplex virus-1 can be tracked *in vivo* in mice (64). Developing methodologies capable of providing quantitative, non-invasive, longitudinal, and spatial *in vivo* information about the dynamic process of infused T cells will be critical to our understanding of adoptive immunotherapy in humans.

Each infusion product is a heterogeneous composition of genetically modified T cells, only some of which will persist and recycle effector function after adoptive transfer. The challenge to increase potency may be achieved by the *a priori* identification of subsets of CAR⁺ T cells that have superior anti-tumor activity for a given patient, tumor type, burden, and biodistribution. Standard immunologic assays report the overall functioning of populations of manufactured T cells but cannot readily inform on the therapeutic potential of individual CAR⁺ T cells. To personalize CAR⁺ T-cell therapy, we have employed an integrated technology (65) that combines single-cell functional screening with systems-level molecular profiling to describe individual T cells linking their cytotoxicity, cytokine production, and molecular profile (66, 67). It is expected that these data, compared before and after infusion, will help define desired T-cell populations that can be manufactured to meet the needs of a given patient.

Broadening the human application of CAR⁺ T cells

The SB and aAPC platforms can be readily used to genetically modify and propagate CAR⁺ T cells. This approach to manufacturing clinical grade T cells avoids the need, complexity, and cost of generating and releasing recombinant virus. Current practice is based on generating one population of CAR⁺ T cells to be infused into a given patient. This approach to manufacturing is costly, relies on both sufficient quality of quantity of host immune system from which to harvest T cells, and takes time, during which the intended recipient's medical condition may deteriorate precluding infusion of the genetically modified CAR⁺ T cells. Therefore, we have developed approaches whereby one (healthy) donor's T cells can be pre-prepared to express CAR and then infused on demand when needed, rather than when the T cells are available. The major impediment to the implementation of this embodiment of off-the-shelf therapy is the potential for serious adverse events stemming from the $\alpha\beta$ TCR on the infused CAR⁺ T cells recognizing major and minor histocompatibility antigens on normal cells in the recipient. This can be alleviated by eliminating alloreactive CAR⁺ T cells prior to infusion. One approach was based on allo-anergization of donor-derived T cells by *in vitro* induction of a hyporesponsive state which does not degrade signaling though introduced CAR, achieved by allo-stimulation with concomitant blockade of CD28-mediated costimulation (68). As an alternative, we have recently used engineered zinc finger nucleases (ZFNs) to target constant regions of the TCR α and β loci and thereby eliminate their expression. We combined the electro-transfer of SB-derived DNA plasmids to stably express CAR with electro-transfer of *in vitro*-transcribed mRNA coding for ZFNs which act in a hit-and-run fashion to introduce a desired double-strand break in the T-cell genome. The resultant CAR⁺TCR $\alpha\beta$ ^{neg} cells could not respond to TCR stimulation while maintained CD19-dependent effector function (69). An application is under review by NIH-OBA (#1307-1236) to undertake a clinical trial infusing CD19-specific T cells as an off-the-shelf therapy. Similarly, we have successfully used ZFNs to eliminate HLA-A expression in CAR⁺ T cells as an approach to prolonging the survival of infused off-the-shelf T cells by preventing their recognition by the recipient's immune system (70). The ability to stably introduce transgenes using the SB system and eliminate undesired endogenous genes with artificial nucleases provides an experimental path to generating T cells with improved potency and broad application.

Summary

The SB system provides investigators with a new approach to gene therapy. The initial clinical data are being obtained with CAR⁺ T cells targeting CD19 in patients with advanced B-cell malignancies. However, as approach to gene therapy is nimble and cost-effective it enables us to initiate next-generation clinical trials targeting ROR1 on CLL cells in attempt to spare normal B cells from CAR-mediated destruction (NIH-OBA #1210-1192) and test alternative CAR designs that activate T cells upon docking with CD19 via chimeric CD3- ζ and CD137 (NIH-OBA #1301-1203 and #1304-1225).

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Fig. 1. Schematic of *Sleeping Beauty* (SB) system used to genetically modify a T cell to express a chimeric antigen receptor (CAR)

The two DNA plasmid SB system consists of a SB11 transposase (red) and CAR transposon (blue) electro-transferred using a nucleofector device and solution. After translation, the SB11 enzyme (red circle) binds the inverted and direct repeats sequences (IR/DR, shown as grey arrows) flanking the CAR transposon. Transposition occurs via a cut-and-paste mechanism to insert the CAR into TA sites in the host T-cell genome (orange helix) resulting in stable CAR expression on the cell surface.

Table 1
Release criteria for manufacture of clinical grade DNA plasmids coding for SB system
(transposon and transposase) used *ex vivo* to genetically modify T cells

Test	Specification
Appearance	Clear colorless liquid
Identity	Restriction mapping and agarose gel electrophoresis agrees with predicted banding pattern
	Sequencing
Plasmid Concentration	2.0 ± 0.2 mg/mL
	1.8 - 2.0 (A260/A280)
	>90% supercoiled form
Bacterial host-cell macromolecules	Endotoxin <50 EU/mg
	Protein <0.3%
	RNA <10%
Sterility	Negative

Table 2
Criteria to release clinical grade CAR⁺ T cells after electroporation with SB-derived DNA plasmids and propagation on aAPC^{*}/IL-2/IL-21

Tests ^{**} performed to generate the certificate of analysis	Specification
Sterility	Visual inspection
	No growth at 14 days (BD BACTEC)
	PCR negative for Mycoplasma
	Endotoxin LAL < 5 EU/kg
Viability (Trypan Blue Exclusion)	70%
Immunophenotyping (ungated) ^{***}	CD32 ⁺ 7-AAD ⁻ < 5%
	CD19 ⁺ 7-AAD ⁻ < 5%
Immunophenotyping (gated on lymphocytes)	CD3 ⁺ 80%
	CAR ⁺ 10%
Autonomous Growth	10% cells viable at day 18 after withdrawal of cytokines and aAPCs

* K562-derived aAPC (e.g., clone #4 that endogenously expresses CD32 and genetically modified to co-express CD19, CD64, CD86, CD137L, mIL15)

** To establish chain-of-custody low resolution HLA class I may be confirmed with donor of T cells

*** To assess presence of aAPC

Table 3
In-process testing undertaken on CD19-specific CAR⁺ T cells cryopreserved for human administration

Parameter	Test	Method	Reference to specialized methodology	Notes
Phenotype	Cell surface expression of CAR [*]	Flow Cytometry	(25, 61)	Typically >80% T cells express CAR
	Total CAR expression	Western Blotting	(25)	A 73 kDa chimeric CD3 ζ band is seen for 2 nd generation CAR CD19RCD28
Potency ^{**}	Specific lysis	Chromium Release Assay	(25)	CAR-dependent lysis of CD19 ⁺ target cells
Potential for persistence	Memory/Naïve Phenotype	Flow Cytometry	(25)	Includes expression of CD28, CD62L, CCR7, CD45R0
	Markers of exhaustion	Flow Cytometry	(25)	Includes expression of PD1 and, CD57
	Telomere Length	Flow-FISH	(25)	Not degraded compared to unmanipulated control T cells
Safety	CAR copy number	Q-PCR	(25, 38)	Typically one to two integrants per T-cell genome
	Stable integration of DNA plasmid coding for SB11 by PCR	PCR	(25)	Absence of PCR band by agarose gel electrophoresis
	TCR Repertoire	Flow Cytometry for TCR V β and/or Direct TCR expression assay for TCR V α & V β	(25, 71)	Absence of skewing (emergence of oligoclonality or monoclonality in pattern of TCR abundance)
	Karyotype	G-banding	(3)	Normal

* This assay is also undertaken as part of release testing

** An assay is being developed to be compliant with guidance document from U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research¹

Table 4

Ongoing clinical trials at U.T. MD Anderson Cancer Center in fusing CD19-specific CAR* T cells electroporated with SB system and propagated on aAPC/IL-2/IL-21

Protocol title	IRB No.	IND No.	NIH-OBA	Clinicaltrials.gov
CD19-specific T-cell Infusion in Patients with B-Lineage Lymphoid Malignancies After Autologous Hematopoietic Stem Cell Transplantation	2007-0635	IND 14193	0804-922	NCT00968760
CD19-specific T-cell Infusion in Patients with B-Lineage Lymphoid Malignancies After Allogeneic Hematopoietic Stem Cell Transplantation	2009-0525	IND 14577	0910-1003	NCT01497184
Donor-Derived, CD19-specific T-cell Infusion in Patients with B-Lineage Lymphoid Malignancies After Umbilical Cord Blood Transplantation	2010-0835	IND 14739	1001-1022	NCT01362452
Autologous CD19 Specific T-cell Infusion in Patients with B-cell Chronic Lymphocytic Leukemia (B-CLL)	2011-1169	IND 15180	1201-1142	NCT01653717

Table 5

Criteria to produce a master cell bank of aAPC* suitable for use in compliance with cGMP

Parameter	Test**
Endotoxin LAL	<5 EU/mL
Sterility	Isolator sterility testing using a direct inoculation method (2-40 vials)
	Sterility isolator method suitable testing using a direct inoculation method
Viruses	Replication-competent lentivirus
	28-day in vitro virus assay GMP, 3 cell lines
	In vivo: suckling and adult mice, guinea pigs, embryo, hen eggs
	Bovine 9CFR in vitro assay for 7 viruses
	Bovine polyoma virus (BPV) detection by PCR (Fluorescent Endpoint)
	Quantitative product enhanced reverse transcriptase (Q-PERT) assay for the detection of retrovirus in biological samples (1 sample)-GMP
	Certificate of analysis detection of 14 viruses by real time polymerase chain reaction assay (Human panel I)
Mycoplasma	Qualification of the test article for the detection of agarcultivable mycoplasma in accordance with USP/EP/PTC/JP requirements (without avian controls)
	Test for presence of agar-cultivable and non-agar cultivable mycoplasma without avian controls (USP, EP, 1993 PTC)
Identity	Transmission electron microscopic examination of cell cultures (200 cell profiles)
	Cell Culture Identification and Characterization using Isoenzyme Analysis-GMP
	DNA fingerprinting per MDACC guidelines
Immunophenotyping (gated flow cytometry)	Viability 70%
	Introduced cell surface antigen(s) 40% CD32 75%

* K562-derived aAPC (e.g., clone #4 that endogenously expresses CD32 and genetically modified to co-express CD19, CD64, CD86, CD137L, mL15)

** Biologics safety testing are undertaken to ensure absence of contaminants. Some of these tests may be performed by contract services (e.g., BioReliance1)