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REVIEW

Manufacture of T cells using the *Sleeping Beauty* system to enforce expression of a CD19-specific chimeric antigen receptor

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T cells can be reprogrammed to redirect specificity to tumor-associated antigens (TAAs) through the enforced expression of chimeric antigen receptors (CARs). The prototypical CAR is a single-chain molecule that docks with TAA expressed on the cell surface and, in contrast to the T-cell receptor complex, recognizes target cells independent of human leukocyte antigen. The bioprocessing to generate CAR$^+$ T cells has been reduced to clinical practice based on two common steps that are accomplished in compliance with current good manufacturing practice. These are (1) gene transfer to stably integrate the CAR using viral and nonviral approaches and (2) activating the T cells for proliferation by crosslinking CD3 or antigen-driven numeric expansion using activating and propagating cells (AaPCs). Here, we outline our approach to nonviral gene transfer using the *Sleeping Beauty* system and the selective propagation of CD19-specific CAR$^+$ T cells on AaPCs.

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NONVIRAL GENE TRANSFER

T cell-based gene therapy for oncology is based on stably integrating a transgene into the host genome so that infused genetically modified progeny can provide long-term therapeutic effects. Viral vectors have been extensively and safely used to insert transgenes such as immunoreceptors into T cells, but immunogenicity,[1] production cost of clinical-grade recombinant viral vectors and genetic pay load restriction can limit clinical application. Nonviral gene transfer methods based on liposomal formulation, nanoparticles, advanced electroporation techniques (nucleofection) and cell-penetrating peptides offer attractive alternatives that can be inexpensive and can also deliver larger gene inserts.[2,3] However, these alternatives to transduction have less of a track record when used for human application chiefly because of low efficiency of gene transfer and subsequent integration. In order to improve long-term expression of transgenes, integration systems have been developed based on transposition systems such as phiC31,[4] Tol2,[5] *piggyBac*[6] and *Sleeping Beauty* (SB).[7] These transposon/transposase systems have merits as an approach to improving the efficiency of gene transfer, but only some, such as the SB system, can be reduced to clinical practice.[8,9]

T cells play an important role in immunity and as such have been candidates for gene therapy by redirecting specificity through the enforced expression of T-cell receptors (TCRs) and chimeric antigen receptors (CARs). The genetic introduction of defined αβ TCR chains provides for recognition of intracellular tumor-associated antigens (TAAs) in the context of human leukocyte antigen (HLA). However, the translation of this approach for therapy can be limited because of downregulation of HLA by targeted tumors,[10] the inappropriate recognition of normal cells expressing the TAA, restricted repertoire to target just a subset of HLA species and mispairing between endogenous and introduced αβ TCR chains. Nevertheless, there have been antitumor effects infusing T cells genetically modified to express αβ TCR such as targeting melanoma antigens and targeting tumors expressing NY-ESO-1.[11–14] The SB system can be used to express αβ TCR chains[15,16] and we are exploring the clinical applications of this approach. What is particularly appealing is that the low cost of the SB system renders feasible the expression of a single αβ TCR chain for the personalization of T-cell therapy to target an individual patient's unique tumor.

T-cell specificity can also be directed to TAA by the enforced expression of a single-chain CAR. This targeting molecule typically combines the binding properties of a monoclonal antibody (mAb) with signaling via the TCR complex. The extracellular portion of a CAR directly engages with the TAA and bypasses HLA for antigen recognition, resulting in T-cell activation as initiated by one or more motifs of the TCR complex embedded within the CAR endodomain. Such CARs can be expressed using the *piggyBac* and SB systems.[17–23]

The SB system, which was derived from fish, combines the advantages of cost and simplicity of naked DNA with the efficiency of gene transfer associated with recombinant retroviruses. The SB system adapted for human application comprises a two-component gene transfer vector system. The gene of interest designated the transposon (in this case the CAR) is cloned between the terminal inverted repeats that contain binding sites for the transposase (for example, SB11), the enzymatic factor of transposition.[24–26] Recent studies have shown that, in contrast to most retroviral-based vectors, SB integrates randomly in 2×10^8 TA sites in mammalian cells, without any discernable preference for

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actively transcribed genes, thereby apparently making SB a suitable candidate for a targeted integration system.[27,28] Our team at the University of Texas MD Anderson Cancer Center (MDACC) has adapted the SB system[8,9,29] to undertake a series of first in-human clinical protocols.[30] Four of these trials infuse SB-modified CD19-specific CAR$^+$ T cells manufactured in compliance with current good manufacturing practices (cGMP).[31]

ACTIVATING AND PROPAGATING CELLS (AAPCS) AND *EX VIVO* PRODUCTION OF CAR$^+$ T CELLS

Approaches to *ex vivo* numeric expansion of T cells include combining cytokines (typically interleukin (IL)-2) with crosslinking CD3, and increasingly CD28, using mAbs that are immobilized on beads, plates and cells. This may be accomplished using a 'rapid expansion protocol' requiring a double-feeder (irradiated) cell layer of peripheral blood mononuclear cells and lymphoblastoid cell line.[32–37] Activating T cells via the CD3 complex results in antigen-independent expansion of T cells and, depending on the time in culture, can result in a product with a terminally differentiated phenotype. AaPCs, previously referred to by us as artificial antigen-presenting cells, have been developed to undertake the propagation of T cells in an antigen-specific manner.[38,39] Indeed, we have adapted the AaPCs to present TAA in order to preferentially activate T cells in a CAR-dependent manner for sustained proliferation. To selectively propagate CAR$^+$ T cells from mononuclear cells (such as in peripheral and umbilical cord blood), we genetically modified K-562 cells to coexpress TAA and select costimulatory molecules to function as AaPCs. When lethally irradiated, these K562-derived AaPCs are a suitable platform for the numerical expansion of CAR$^+$ T cells because they (1) fail to express classical HLA A and B thereby likely avoiding stimulation of allo-specific T cells, (2) can be cultured in compliance with cGMP, (3) express desired endogenous T-cell costimulatory molecules and (4) can be readily modified to enforce the expression of TAA(s) and exogenous costimulatory molecules. For example, AaPCs have been modified to coexpress CD19, CD64 (high-affinity Fc receptor), costimulatory molecules (4-1BBL and CD86) and provide an IL-15-mediated activation signal via a membrane-bound form of this cytokine.[17] Some of these AaPCs have been produced as clinical-grade materials (Table 1) such as through Production Assistance for Cellular Therapies (PACT) under the auspices of the NIH Heart, Lung and Blood Institute (NHLBI).[31] Such master cell banks can be used to derive a working cell bank that in turn can be used to generate a reagent cell bank. Figure 1 describes the relationship between these banks, the time limits we permit our AaPCs to remain in continuous culture and the relationship between cryopreservation and subsequent use as thawed feeder cells to propagate CAR$^+$ T cells.

Although first-generation CAR signaling through only the CD3ζ chain has been demonstrated to provide a signal in initiating T-cell cytolysis,[40] these CARs are likely insufficient to sustain T-cell proliferation *in vivo*. Second-generation CARs containing costimulatory domains in addition to chimeric CD3ζ[41–44] increase activation, proliferative capacity and persistence of adoptively transferred cells.[45–47] CD19, a B-lineage antigen, has been targeted by us and others using CARs because it is expressed on the cell surface of most malignant B cells (as well as normal B cells). We developed a second-generation CD19-specific CAR (designated CD19RCD28) that signals through chimeric CD28 and CD3ζ and, as predicted, this demonstrated improved activation and persistence after adoptive transfer compared with first-generation technology.[45] To select for stable integrants expressing the CD19-specific CAR, T cells from peripheral blood (or umbilical cord blood) were nucleofected with two DNA plasmids coding for CD19RCD28 (SB transposon) and SB11 (SB transposase). After electroporation, the T cells were co-cultured with irradiated (100 Gy) K562-derived AaPCs capable of sustaining CAR-mediated propagation. This approach to electroporation and propagation resulted in 60-fold improved integration efficiency compared with illegitimate homologous recombination of naked DNA plasmid.[20] The manufacturing process, which has been adapted for compliance with cGMP, is based upon every 7-day stimulation with γ-irradiated AaPCs in the presence of soluble human recombinant cytokines (IL-2 and IL-21).[31,48] T cells retrieved at the end of the culturing period (typically 21–28 days) express CARs (90% expression), have memory/naive phenotype, maintain a diverse Vβ repertoire, have a normal karyotype and lyse tumor targets and produce cytokines in response to CD19[49] (Figures 2 and 3).

Table 1. Parameters for production of master cell bank of activating and propagating cells (AaPCs)

Parameter	*Specification*
Integrity	Viability, sterility, endotoxin, mycoplasma, viruses
Identity	Microscopy, fingerprinting
Immunophenotyping	Introduced transgenes

The high-affinity receptor (CD64) on AaPCs may also be used to load mAb on the cell surface that is then available for interaction/activation of T cells. We have successfully used K562-derived AaPCs loaded with mAb specific for the pattern recognition molecule Dectin-1 to selectively propagate T cells genetically modified to express a CAR to redirect specificity to carbohydrate. The introduced CAR derives its specificity for Dectin-1, enabling recognition and destruction of germinating *Aspergillus*.[23] Building on the principle that mAb bound to AaPCs can activate CAR$^+$ T cells, we developed an approach whereby AaPCs can sustain the proliferation of multiple CAR$^+$ T cells independent of specificity to TAA. These 'universal' AaPCs were genetically modified to express a single-chain variable fragment derived from the mAb clone 2D3 that is specific for the IgG4-based exodomain common to our CAR molecules.[20] The AaPC expressing this 2D3 ligand for CARs (designated CAR-L) binds to the stalk of multiple CARs, resulting in numeric expansion of genetically modified T cells with divergent specificities.[50]

CELL PROCESSING AND TESTING

Ex vivo processing of cellular products can be divided into two categories that define minimally manipulated and manipulated cellular products that are regulated by the Food and Drug Administration (FDA) under current good tissue practice or cGMP, respectively. Minimal manipulation procedures do not alter the basic characteristics or function of the cells and include cryopreservation, thawing, density-gradient separation, washing before reinfusion and simple depletion or positive selection. Manipulation procedures are those that likely alter the biological characteristics of the cellular products, including *ex vivo* numeric expansion, gene modification and complex selection such as by high-speed cell sorting. FDA oversight over facilities involved with cellular and tissue processing requires that both routine and complex processing procedures be performed in a facility that can handle the regulatory requirements. CAR$^+$ T cells that have been generated using the SB and AaPC systems for use in clinical trials are categorized as manipulated cellular products and are manufactured in MDACC's Cell Therapy Laboratory.

Peripheral blood, obtained from apheresis or venipuncture, is typically processed by cell fractionation methods such as ficoll or closed cell-processing systems such as Sepax II (Biosafe, Eysins, Switzerland) to provide T cells. The Sepax II device combines a

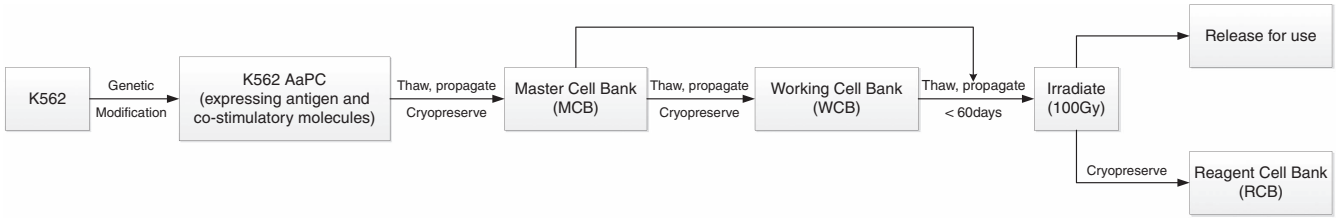

Figure 1. Production of K562-derived AaPC cell banks. K562 is a human erythroleukemic cell line derived from a patient in blast crisis and was genetically modified to coexpress antigen (CD19) and costimulatory molecules (CD86, CD137L and mIL-15). These activating and propagating cells (AaPCs) were propagated and cryopreserved as a master cell bank (MCB) that was used to derive a working cell bank (WCB), all of which meet release specifications.[31] It is our practice that an aliquot from MCB and WCB can be continuously propagated in culture (< 60 days to avoid differentiation). Cells in culture are irradiated (100 Gy) for direct use to propagate T cells, or vialed and cryopreserved as reagent cell bank (RCB) to be used for coculture with genetically modified T cells.

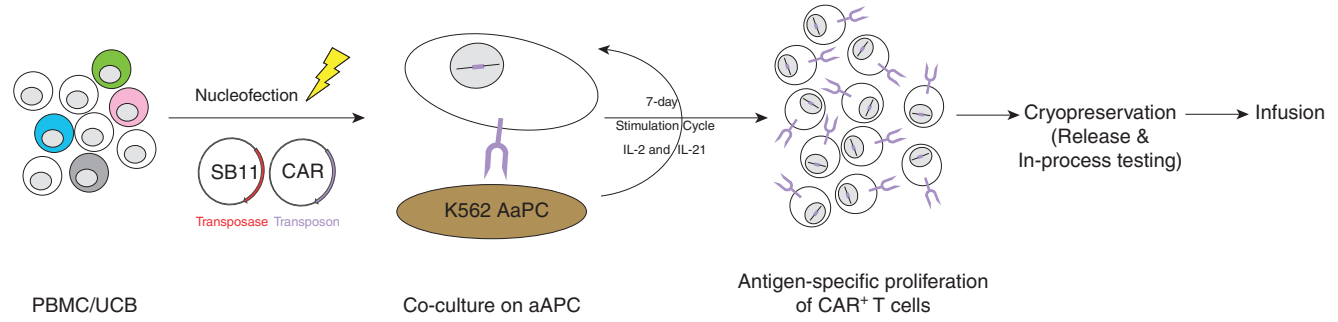

Figure 2. Schematic describing the generation of genetically modified CD19-specific CAR$^+$ T cells using the *Sleeping Beauty* (SB) system and activating and propagating cells (AaPCs). CAR, chimeric antigen receptor; IL, interleukin; PBMC, peripheral blood mononuclear cell; UCB, umbilical cord blood.

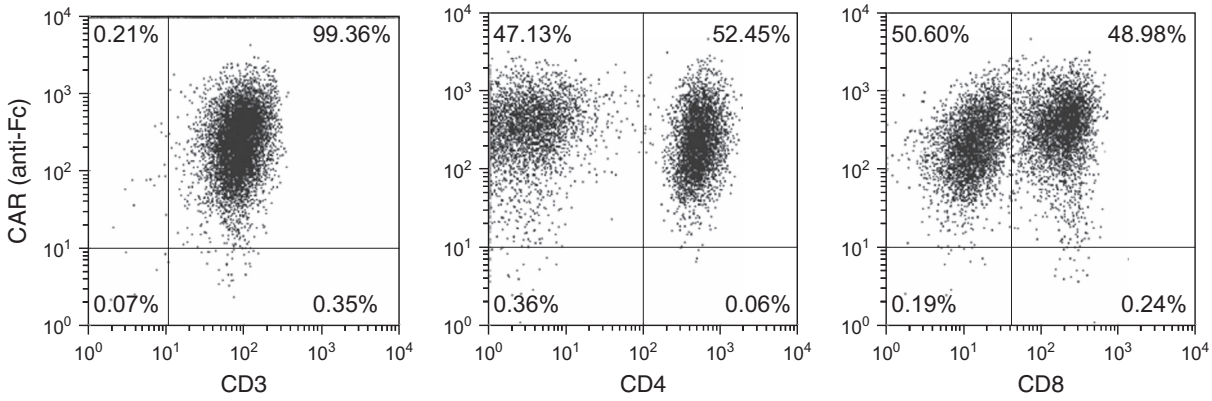

Figure 3. Expression of chimeric antigen receptor (CAR) on genetically modified CD19-specific T cells at the end of 28 days of co-culture with K562-derived activating and propagating cells (AaPCs) and cytokines. CAR expression is determined using a Fc-specific antibody to detect the extracellular stalk.

centrifuge and a pneumatic system capable of automated cell separation and volume reduction procedures in a closed system. Sepax II is being currently used by our group to isolate peripheral blood mononuclear cells in addition to harvesting the AaPCs that are grown in bags.[31] Our current approach for generating CD19-specific CAR$^+$ T cells for clinical trials involves: (1) Nucleofector II (Lonza Group, Basel, Switzerland), (2) two DNA plasmids, SB transposon (encoding CAR) and SB11 transposase, and (3) K562-derived AaPCs to select stable transfectants of CAR. Plasmid DNA encoding for CAR/SB11 was commercially produced and K562-derived AaPCS were manufactured as a master cell bank. AaPCs are cultured in VueLife bags and/or the WAVE Bioreactor, irradiated at 100 Gy (to inhibit proliferation), phenotyped to validate expression of introduced transgenes/costimulatory molecules and frozen in aliquots. We recently started using an automated vialer (Fill-It, TAP Biosystems, Sartorius Stedim, Hertfordshire, UK) to dispense the AaPCs for subsequent cryopreservation. Some of the equipment that we use during manufacture and analysis of antigen-specific T cells is shown in Figure 4. The manufacturing process involves electrotransfer of CD19RCD28 (second-generation CD19-specific CAR, transposon) and SB11 (transposase) in mononuclear cells and co-culture with γ-irradiated

Figure 4. Equipment used during manufacture and analysis of antigen-specific T cells. (**a**) Amaxa Nucleofector II (AAB-1001, Lonza Group Ltd) to electrotransfer DNA plasmids into quiescent cells. (**b**) Biosafe Sepax II to isolate mononuclear cells from blood as well as for volume reduction procedures for large cultures. (**c**) Cellometer (Cellometer Vision, Nexcelom Bioscience, Lawrence, MA, USA) to automate the enumeration of viable cells. (**d**) WAVE Bioreactor (System 2/10 EH, GE Healthcare Bio-Sciences) to culture T cells and activating and propagating cells (AaPCs) at higher densities and volume using gas/media perfusion. (**e**) Vialer (Fill-It, Model 0632L10, TAP Biosystems, Sartorius Stedim Biotech Group) to automate the dispensing of cells. (**f**) MACSQuant Analyzer 10 (Miltenyi BiotecGmbH, Bergisch Gladbach, Germany), a benchtop flow cytometer for multiparameter cell analysis.

K562 AaPCs along with cytokines (IL-2 and IL-21) to generate clinically meaningful numbers of CAR^+ T cells (Figure 1).[31]

Once the genetically modified T cells have been produced in compliance with cGMP, they are analyzed to ensure they meet predetermined release criteria established by FDA. These criteria, codified in the certificate of analysis, validate identity, purity, number viability and safety of the T-cell infusion product.[29,31] These release tests are performed by a quality control group that functions independently of the manufacturing group to avoid conflict of interest. The release criteria for SB-modified CD19-specific CAR^+ T cells (Table 2) include standard sterility parameters such as absence of endotoxin (<5 endotoxin units) and no mycoplasma or bacterial/fungal contamination. Integrity is determined using Trypan blue/7-aminoactinomycin D exclusion to assess the viability ($\geq$70%) and phenotype of $CD3^+$ T cells ($\geq$80%), introduced transgene (CAR, $\geq$10%) and absence of AaPC (CD32 and CD19; $<$5%). These release criteria also includes a measurement of safety of the engineered product, based on absence of aberrant T-cell growth. This assay is accomplished over 18 days to reveal the lack of autonomous T-cell growth cultured in the absence of AaPCs and cytokines. In-process testing (Table 2), which is not revealed in the certificate of analysis, is undertaken at defined times during the manufacturing process as well as on the final product. These results are examined to verify they meet established parameters. Some of the in-process testing is to help establish safety, and other assays reveal the therapeutic potential. To help maintain safety we validate a normal karyotype, a polyclonal TCR repertoire and lack of SB11 transposase. Potency is currently evaluated based on specific lysis of tumor cells using a 4-h chromium release assay. The presence of transgene (CAR) is not only assessed using flow cytometry (for the certificate of analysis), but also by western blot analysis and PCR.

Table 2. Release and in-process testing of CAR^+ T cells

Testing	*Criteria*	*Specification*
Release	Sterility	Mycoplasma, endotoxin, bacteria/fungi
	Viability	Trypan blue/7-AAD
	Immunophenotyping	T cells (CD3, CAR), AaPCs (CD19, CD32)
	Lack of autonomous growth	In the absence of external stimulation ($<$10%)
In-process	CAR expression	Western blot analysis
	CD19 specificity	CRA
	CAR integration	qPCR
	SB11 integration	PCR
	Polyclonality	TCR-Vβ repertoire
	Karyotype	G-banding

Abbreviations: 7-AAD, 7-aminoactinomycin D; AaPC, activating and propagating cell; CAR, chimeric antigen receptor; CRA, chromium release assay; qPCR, real-time quantitative PCR; TCR, T-cell receptor.

FUTURE

Recent advances in cell culture have helped overcome some of the limitations associated with scale-up to process and produce multiple products. For example, it is possible to culture cells at increased cell densities using bioreactors.[51] The WAVE Bioreactor (GE Healthcare Bio-Sciences, Uppsala, Sweden) uses automated feeding and maximizes the removal of waste products while keeping culture volumes to a minimum; the mechanical rocking induces waves in the culture providing for efficient mixing and oxygen transfer.[52,53] There is an increasing demand for an automated closed system to isolate and expand antigen-specific T cells to relieve the burden on technicians, improve processing time and lower the risk of contamination. The CliniMACS Prodigy system (Miltenyi Biotec, Bergisch Gladbach, Germany), which combines CliniMACS Separation Technology with cell processing capabilities to automate cytokine capture,[54] has been successfully used by our group to generate cytomegalovirus-specific T cells.[55]

There is an ongoing need to advance bioprocessing to translate immunology into immunotherapy. Our current approach to redirecting the specificity of T cells is based on the electrotransfer of DNA plasmids from the SB system to integrate CAR. The genetically modified T cells can be retrieved over culture time using AaPCs to generate large numbers that stably express high levels of CAR. As clinical experience is gained using SB and AaPC platforms, so we will be adapting this approach to shorten the manufacturing time and broaden the portfolio of genetically modified cellular products.

CONFLICT OF INTEREST

Dr Cooper founded and owns InCellerate. He has patents with Sangamo BioSciences with artificial nucleases. He consults with Targazyme (formerly American Stem Cells), GE Healthcare, Ferring Pharmaceuticals, Fate Therapeutics, Janssen Pharmaceuticals and Bristol-Myers Squibb. He is on the Scientific Advisory Board of Cellectis. He receives honoraria from Miltenyi Biotec.

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