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CAR-T CELL PRODUCTION

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ABSTRACT

The treatment of patients affected by hematologic neoplasms with autologous T cells expressing a chimeric antigen receptor (CAR) is one of the most promising adoptive cellular therapy approaches. Reproducible manufacturing of high-quality, clinical-grade CAR-T cell products is a prerequisite for the wide application of this technology. Product quality needs to be built-in within every step of the manufacturing process, including the choice of the vectors to modify T cells, such as viral vectors: lentiviral or gamma-retrovirus, and non-viral vectors: especially those based on transposons. Additionally, the CAR-T cell quality control must be in accordance to local Regulatory prior to infusion. Herein we summarize the state of art manufacturing platforms available. CAR-T cell therapy may be on the verge of becoming standard of care for a few clinical indications. Challenges in the manufacturing standardization and product characterization remain to be overcome in order to achieve broad usage and eventual commercialization of this therapeutic modality.

Keywords:

AIMS

Describe different techniques for the production of CAR-T cells, using viral vectors or non-viral techniques.

INTRODUCTION

Cell therapy with T cells expressing chimeric antigen receptor (CAR-T) is a type of immunotherapy that involves the manipulation and reprogramming of immune cells (T lymphocytes) in order to recognize and kill tumor cells. In its classic configuration, CAR represents a monoclonal antibody fragment, called a single-chain variable fragment (scFv), which resides in the extracellular portion of the T cell membrane and guides the cell to its target antigen. The scFv is linked to a loop, followed by a transmembrane portion and this portion, which to intracellular signaling domains[1,2] . Once the CAR-T cell connects with the antigen present in the target cell, the stimulatory molecules provide the necessary signals for the cell to become fully activated. T cells can effectively proliferate and attack cancer cells[3-5].

In most clinical studies and commercial CAR-T cell products, the cells are genetically modified using viral vectors. The viral vectors commonly used for the production of CAR-T cells are lentiviral or gamma-retrovirus derivatives. The viral vector is used to deliver a gene specifically, with high efficiency and, in this way, permanently integrate the transferred DNA into the genome allowing for long-term gene expression[6]. When a viral vector is used to modify the cellular genome for gene therapy, this vector must not induce allergic reactions or severe inflam-

matory processes, still fulfilling its therapeutic role, whether it exacerbates normal functions, corrects deficiencies, or inhibits deleterious activities (Table 1). Despite the profile of random integration and risk of mutagenesis, the use of viral vectors for the production of CAR-T lymphocytes has been shown to be safe[7,8]. These therapeutic vectors must be produced under Good Manufacturing Practices (GMP) conditions, purified in large quantities and high concentrations to be available on a large scale, which results in high production costs[9-11]. The vector must still be safe not only for the patient but also for the handler in the production process.

Despite the wide use of gamma-retroviral and lentiviral vectors for the manufacturing of CAR-T cells, there is a recent trend towards developing of non-viral vectors, especially those based on transposons[6]. Non-viral vectors generally consist of non-infectious DNA fragments that are carried into the target cell through physical methods, such as electroporations or microinjections, or chemically, through transfection reagents. Due to the low efficiency of lipofection protocols in T lymphocytes, the method of choice has been electroporation. The most used non-viral methods for the generation of CAR-T cells include Sleeping Beauty[12-14] and PiggyBac[15] transposons. Both have already had clinical use, in addition to the pre-clinical development carried out by different groups[16-18]. In general, gene delivery consists of transposons that carry the transgene of interest (in this case, CAR) in the form of DNA (either as plasmid[16] or minicircles[19]). The minicircles-based engineering CAR represents an advantage, since smaller masses of DNA are necessary for the delivery of the transgene, reducing the toxicity resulted from electroporation at high concentrations of DNA. The transposase can be delivered in the form of plasmid DNA, mini-circle, mRNA, or protein[6]. Following the same logic, decreased toxicities are found when using mini-circles and mRNA. The recent development of a transposase with greater solubility and potential for penetration into cells suggests that the efficiency of the system can be improved[20].

Although transposon-based systems present a genomic integration pattern considered to be less prone to insertional mutagenesis than that found in viral vectors, and Sleeping Beauty-based protocols have recently presented relevant clinical results with cells expressing anti-CD19 CARs, a clinical protocol based on PiggyBac carrying anti-CD19 CARs led to the transformation of CAR+ T cells causing the development of lymphomas in 2 of 10 treated patients[21]. This recent result was accompanied by antitumor response in 5 patients, demonstrating the potential for CAR-T cell response accompanied by a risk of T cell transformation not previously observed in any CAR-T cell generation protocol. The transformation mechanism and its potential association with the PiggyBac system are still under investigation[22]. No similar adverse events have been reported yet in protocols, including transposons Sleeping Beauty or PiggyBacs.

TABLE 1 - Viral vector used gene therapy and CAR-T cell manufacturing

	Retrovirus	**Lentivirus**	**Herpes Vírus**	**Adenovirus**	**Adeno associated**	Plasmid
Provírus	RNA	RNA	RNA	DNA	DNA	DNA
Capacity	~ 9 kB	~ 10 kB	> 30 kB	~ 30 kB	~ 4,6 kB	No limit
Integration into host genome	Yes	Yes	Yes	No	Rare	No
Time of transgene expression.	Long	Long	Transient	Transient	Long in post mitotic cells	Transient
Pre existing immunity	No	No	Yes	Yes	Yes	No
Adverse effects	Insertional Mutagenesis	Insertional Mutagenesis	Inflammatory response	Inflammatory response	Slighty Inflammatory response	No
Germline transmission	Possible	Yes	No	No	Possible	No
Applications	CAR-T, gene therapy as Gaucher, Fanconi anemia, B hemophilia	CAR-T	Neuronal diseases	Cystic fibrosis	Sickle cell disease	Cloning and protein isolation

Adapted from Misra S. Human gene therapy: a brief overview of the genetic revolution. J Assoc Physicians India. 2013;61(2):127-33 (9)

PROCEDURE DESCRIPTION

Indication: patients eligible for treatment who consented to participate in a clinical study case. In the production of CAR-T cells for clinical use, the service responsible for manufacturing must present official approval regarding technical standards. For clinical research situations, all steps involving ethical evaluation should be approved by CEP/CONEP and safety and quality should be approved by ANVISA and CTNBio agencies.

Contraindication: patients not eligible for treatment with CAR-T cells and lack of approval and regularization with the official norms and current techniques for the production of CAR-T cells.

HUMAN RESOURCES

It is recommended that the procedures should be carried out in a GMP environment or equivalent approved according to the legislation and with the leadership of multidisciplinary teams with proven ability in the manufacture of CAR-T cells.

MINIMAL REQUIREMENTS TO THE PROCEDURE

Lymphocytes collected by leukapheresis or blood collection are used as starting material. The collection from patients in the good clinical condition is recommended, with negative test for infectious agents such as C, C/II or B hepatitis, HIV, NAT, anti-HTLVI II, HBsAG, HBV NAT, anti-HCV, NAT for HCV, serological test for syphilis, antibody anti-*T. cruzi* for non-reactive Chagas disease and no signs of infection in the last 24 hours before collection.

Additionally, a checklist of all materials and reagents is suggested to perform the procedure.

CRITICAL POINTS AND RISKS

The manufacturing process of CAR-T cells must comply with good practices using human cells established by Resolution RDC number 508, of May 27, 2021, as described in the Regulation chapter of this Manual (ANVISA, RDC N° 508, 2021).

Reagents for the manufacturing process must be classified as GMP or for clinical use. Exceptions may be accepted by regulatory agencies for selected reagents under justification and risk minimization in reagent selection.

For manufacturing in closed and automated cellular processing systems, such as the CliniMACS Prodigy equipment, the critical points and risks are: inspection and proper handling of the equipment, identification and fixation of bubble and pressure sensors, fixation of the temperature control unit and the cell culture chamber.

STANDARD OPERATING PROCEDURE

Transduction efficiency, represented by the percentage of CAR positive T lymphocytes, generally identified by flow cytometry, can be used as a standard operating procedure. The median transduction of the product consisting of anti-CD19 CAR-T cells, such as tisagenlecleucel, when manufactured from cells from patients diagnosed with acute lymphocytic leukemia, is around 23% (5%-56%)[23]. When manufacturing in the automated Prodigy system is used, mean transduction of 30.6% ± 13.44 [24] and a median of 46.88% (29.02–61.09%) [25] is observed.

The manufacturing failure rate can also be considered a parameter to be evaluated in the standard operating procedure. Studies have shown manufacturing failure of CAR-T cells around 1 to 7% [26-28].

MATERIAL

Biological safety cabinet, human recombinant cytokines, culture medium, reagent for activating T lymphocytes, syringes, human AB serum, or defined chemical alternative. For manufacture on CliniMACS Prodigy: MACSQuant Flow Cytometer, CliniMACS Prodigy/TCT Software, CliniMACS Prodigy Tubing Set.

PROCEDURE

Autologous cells collected from peripheral blood or leukapheresis are the most used starting materials for manufacturing of CAR-T cells. Leukapheresis is the preferred technique in cases of lymphopenia or high tumor burden with low lymphocyte numbers[29,30]. Lymphocytes can be cryopreserved for shipment to the specialized manufacturing center or can be used as fresh starting material.

Some protocols establish the separation of lymphomononuclear cells by density gradient or elutriation, as well as enrichment of T lymphocytes or selection of subpopulations of T lymphocytes, such as CD4 and CD8, with the use of conjugated antibodies associated with magnetic beads for positive or negative selection[31,32]. These processes improve product purity and yield. T lymphocytes are then activated using reagents such as: anti-CD3 or anti-CD3/an-

ti-CD28 antibodies, antibodies linked to paramagnetic beads, reagents such as Transact, which uses anti-CD3 and anti-CD28 antibodies conjugated to a colloid of polymeric nanomatrix or via hydrogel "stimulation matrix" incorporating antibodies[33-35]. After the activation process, the T cells are genetically modified in a process known as transduction. In manufacturing protocols using viral vectors, the quality, the titer of the vectors, and their rapid use after thawing are determining factors for the efficiency of transduction, including high expression of CAR in T lymphocytes. Reagents such as protamine, retronectin, poly-L-lysine, and vectofusin can be used to facilitate this process[33,34,36].

CAR-T cells can be cultivated in culture medium containing recombinant human cytokines alone or in combinations (such as IL-2, IL-7 and/or IL-15) in an open system such as flasks known as G-REX bioreactors or by closed system cell expansion system such as Xuri, Wave or Quantum[33,37,38]. Currently, the production of CAR-T cells has become more automated with the development and implementation of new equipment that can perform an increasingly diverse set of functions, such as the CliniMACS Prodigy equipment, marketed by the company Miltenyi Biotec or the Cocoon device, provided by the company Lonza. Both systems operate in a closed and automated system, indicated for cell separation, T cell activation, transduction, expansion and cell collection. The equipment also records parameters at all stages of production, ensuring process traceability[24,39-41].

Although the generation of CAR-T cells with non-viral vectors generally follows the same standards of cell isolation, genetic modification, *in vitro* activation, and cell expansion in the laboratory, the use of non-viral systems allows T cells to be modified genetically without requiring prior activation. This feature allows ultra-fast CAR-T cell generation protocols to be generated through the infusion of freshly electroporated cells. Some initiatives in this direction describe CAR-T cells generated in less than 24h with antitumor potential demonstrated in preclinical models[42,43]. The automated systems mentioned in this chapter have the possibility of coupling electroporators, which allows the generation of T cells with non-viral platforms in these systems, as well as gene editing protocols with systems such as CRISPR/Cas9.

After the manufacture of CAR-T cells, the product is submitted to the quality control process, prior to infusion or cryopreservation, following the the National Health Surveillance Agency (ANVISA - RDC 506/2021 and 508/2021). The expected results are as follow and must be in the corresponding release document (Supplementary file):

1) High viability (above 70% is suggested - from flow cytometry analysis or trypan blue count of the transduced product)

2) Endotoxin: ≤ 5 EU/kg

3) Detection of microorganisms in the final product: no growth of bacteria or fungi after 14 days of incubation

4) Gram stain (for fresh infusion): absence of visualization of microorganisms

5) qPCR for mycoplasma: negative

6) VSVG and GAG qPCR for viral vector replication assessment: negative

7) Dose of CAR-T cells/kg: per cohort (indicated in processing and infusion orders)

8) Potency assay confirming tumor cell death induced by CAR-T cells

9) Absence of karyotypic changes

CAR-T cells can be infused fresh or after cryopreservation. The most used cryopreservation protocols include 5-10% dimethyl sulfoxide, human albumin, and plasmalyte followed by storage in vapor-phase liquid nitrogen (44), as described in the freezing of lymphocytes after leukapheresis, at the time of pre-manufacturing. Several studies demonstrate no significant differences in T cell percentage, transduction efficiency, and CD4:CD8 lymphocyte ratios pre- and post-cryopreservation and thawing[45,46].

FREQUENCY OF TRAINING OR COMPETENCE ASSESSMENT

Frequent training and competency assessment is suggested for the manufacturing process and product quality control.

QUALITY INDICATORS

The efficiency of T cell transduction by the vector of choice, potency test confirming CAR-T-induced cytotoxicity of target cells, and absence of contaminants for clinical infusion can be used as indicators.

SUPPLEMENTARY FILE
CAR-T CELLS MANUFACTURING

1.0 General Information	
Study:	Patient ID:
Patient Weight:	Dose: kg
Initial day of culture: / /	Time of culture: days
Performed by:	Checked by:

2.0 Product reception and verification		
2.1 Time and temperature details upon receiving the product		
Time:	Transportation temperature: °C	Overnight: ( ) Yes ( ) No
Temperature overnight: °C	Location overnight:	
Confirm if the product concentration is ≤ $2x10^8$/mL for overnight storage		
Show calculation to adjust the concentration (if necessary)		
Date:	Initials:	
Performed by:	Checked by:	

3.0 Preparation of reagents and samples	
3.1 Culture medium preparation (48h in advance)	
3.1.1	
Calculation:	
Date:	Initials:
:	
24 hours:	48 hours:
3.2 Buffer Preparation (24h to 48h in advance)	
3.2.1	
3.2.2	Name this buffer as: Processing buffer
Calculation:	

Date:	Initials:	
3.3	Getting the starting material samples	
3.3.1	Remove 0.5 mL for cell count	
3.3.2	Remove 2x10^6 cells for flow cytometry analysis	
Calculation (if necessary):		
Performed by:	Checked by:	

4.0 Pre-processing			
4.1	Leukapheresis volume: mL		
4.2	Calculation of starting material		
4.2.1.	Leucocyte count (WBC): x10^6/mL		
4.2.2	_____ x10^6/mL x _____ mL= WBC x10^6/mL (Item 4.2.1) Volume (Item 4.1)	_____x10^9 Total # WBC	
	Note: Starting material should not be more than 20x10^9 leucocytes (WBCs)		
Calculation (if necessary):			
4.3	Flow Cytometry analysis		
4.3.1	Markers: CD4+, CD8+, and CD4+/CD8+	%	
4.3.2	_____ _____ _x_____ _____ ____= Total WBC (Item 4.2.2) Stained cells (Item 4.3.1)	_____x10^9 Total stained cells	
	Note: Material should not exceed 3x10^9 CD4+ and CD8+ cells		
Checked by:		Initials:	
Comments (if necessary):			
Performed by:	Checked by:		

5.0 Fresh infusion			
5.1	**Only for fresh infusion:**		
5.1.1	Remove adequate volume for release tests (endotoxin, Gram, sterility)		
	_____mL x _____1_____ = _____mL mL from final product (Item 4.1) number of doses **Total volume**		
5.1.2	An additional amount of $20x10^6$ WBC must be added prior to final formulation for release testing.		
	$20x10^6$ WBC / _____10^6/mL = _____mL Correct WBC /mL (Item 4.2.2) Additional volume to be pipetted		
5.1.3	CAR-T cell total volume for final formulation		
	_____mL + _____/mL = _____mL (Item 5.1.1) (Item 5.1.2)		
5.1.4	Adjust WBC concentration to 4-$8x10^6$ WBC/mL		
	(_____x10^6/mL x _____/mL) / _____x10^6 = _____mL Corrected WBC /mL (Item 5.1.2) Final product total volume (Item 5.1.3) Concentration (4-$8x10^6$) Formulation total volume		
5.1.5	Calculate the final volume for distribution and infusion. Remove the volume needed for one dose, send the remainder release tests.		
	_____mL - _____mL = _____mL Formulation total volume (Item 5.1.4) Volume for release tests (Item 4.2.3) Final volume for infusion		
5.2	Take a note from date, time and final volume from formulated final product		
	Date: _____/_____/_____	Time:_____	Volume:_____mL
	Expiration date:	Expiration time:	
Date:		Initials:	
Performed by:		Checked by:	

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