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Improving CAR T cell therapy by optimizing critical quality attributes

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ABSTRACT

Whether as a cure or bridge to transplant, chimeric antigen receptor (CAR)-T cell therapies have shown dramatic outcomes for the treatment of hematologic malignancies, and particularly relapsed/refractory B cell leukemia and lymphoma. However, these therapies are not effective for all patients, and are not without toxicities. The challenge now is to optimize these products and their manufacture. The manufacturing process is complex and subject to numerous variabilities at each step. These variabilities can affect the critical quality attributes of the final product, and this can ultimately impact clinical outcomes. This review will focus on optimizing the manufacturing variables that can impact the safety, purity, potency, consistency and durability of CAR-T cells.

Introduction

The FDA approval of 2 chimeric antigen receptor (CAR)-T cell products in 2017 marked a historic moment for the field of cellular therapy as the first gene therapies for the treatment of cancer were made available in the United States. Since then, CAR-T cell clinical trials have rapidly expanded with targets for both solid tumors and hematologic malignancies [1]. Initial findings of these clinical trials have been promising [2-4]. Optimizing these therapies, however, remains a challenge as seen with the variable efficacy, persistence, and adverse effects of these cells.

Manufacturing CAR-T cells commonly occurs over 7 to 12 days and begins with an apheresis collection, followed by target cell selection and enrichment, CAR gene transfer, and cell expansion. At each step in manufacturing, several seemingly trivial details can impact the critical quality attributes of the final product, including safety, purity, potency, consistency, and durability. *Safety* includes not only negative sterility testing, but also producing a product lacking oncogenic or viral replicative potential; *purity* describes a product with a high concentration of CAR-T cells and minimal contaminating cells; *consistency* includes producing products that meet lot release criteria time after time; *potency* is characterized by a product capable of eliminating tumor cells; and *durability* is indicated by a product that persists in circulation and maintains its anti-tumor effects. At most steps during manufacture, 1 or more of these parameters may be compromised, but careful selection of methods and procedures may minimize these vulnerabilities. This review intends to address some of the manufacturing variables that can affect the critical quality attributes of CAR-T cells and efforts to optimize these attributes.

Apheresis Cell Collection

The starting material for CAR-T cell products is typically obtained through a non-mobilized autologous mononuclear cell apheresis collection. Even before the apheresis procedure begins, however, there are a number of patient factors that may be able to predict compromised quantity or quality of CAR-T cells in the final product, including certain disease types, advanced age, prior therapy, and peripheral blood characteristics [5,6] (Table 1). In comparison with healthy donors, patients referred for CAR-T cell therapy have often been previously treated with chemo- or radiation therapy, or have peripheral blood cell parameters that may complicate collections, such as lymphopenia or high peripheral disease burden.

Tuazon et al. examined variables associated with low lymphocyte collection efficiencies for patients participating in anti-CD19 CAR-T cell trials, and reported reduced collection efficiencies (<40%) in patients with advanced age, higher pre-apheresis platelet counts, and B-lymphoblastic leukemia (B-ALL), as compared to non-Hodgkin lymphoma and chronic lymphocytic leukemia (CLL) [5]. While this study and others have expressed feasibility in obtaining adequate quantities of lymphocytes for CAR-T cell manufacture, insufficient collections or cell expansion after collection does occur [7,8]. In a study of children with neuroblastoma, manufacturing failures due to insufficient cell growth of CD8+ cells were reported for two children who had received metaiodobenzylguanidine therapy within 8 weeks of apheresis, prompting a mandatory 12-week washout period prior to collection for future patients who had undergone this therapy [6].

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Table 1
Sources of variation during CAR-T cell manufacturing

Manufacturing phase	Variable
Collection	Patient characteristics (autologous products)
	Peripheral blood cell composition
	Apheresis procedure and collection efficiency
Cell enrichment	Method of cell enrichment
	Quantity of contaminating cells
	Lymphocyte phenotypes
Cell stimulation	Method of cell stimulation
	Duration of cell stimulation
	Presence of insoluble reagents
Gene transfer	CAR construct design
	Method of gene delivery
	Timing of transduction
	Use of transduction enhancers
	Vector envelope (viral vectors)
Cell expansion	Cytokine selection
	Culture systems
	Duration of expansion
Cryopreservation	Cryoprotectant type and concentration
	Method and rate of freezing

Several others have also investigated the impact of the differential state of T cells on CAR-T cell products, with most agreeing that having a higher proportion of naïve T cells phenotypes in the peripheral blood or starting material is associated with greater expansion and more sustained remissions, and accordingly, a more potent and durable product [9]. Hoffman and colleagues evaluated apheresis products from untreated CLL patients compared to healthy donors and found that CLL patients had expectedly higher numbers of circulating malignant B cells that may have contributed to reduced expansion of naïve T cells and increased expression of exhaustion markers [10]. Another study retrospectively analyzed cellular material for patients who ultimately suffered CD19 CAR-T cell treatment failures, and identified that the associated apheresis material contained an increased frequency of LAG-3^+/TNF-α^{lo} $CD8^+$ T cells [11]. In addition, these products demonstrated rapid expression of functional exhaustion markers following adoptive transfer. Apheresis material containing higher frequencies of $CD27^+CD45RO^-CD8^+$ T cells were also found to be associated with more sustained remissions for patients with CLL treated with CD19 CAR-T cells [12]. Likewise, CD8+ T cells in CLL have been found to have impaired mitochondrial biogenesis, which may affect expansion and persistence of the infused product [13].

While apheresis procedures are being increasingly utilized to procure lymphocytes for CAR-T cell manufacture, the parameters needed to optimize these autologous collections have not been well established. Collection efficiencies for mononuclear cells may vary between collection devices, with one study in pediatric populations reporting improved efficiencies on the Spectra Optia than with the COBE Spectra [8]. As with hematopoietic progenitor cell collections, processing volumes needed to obtain sufficient cells may also vary between patients. However, if collection targets are known, the duration of procedures can be tailored to meet manufacturing needs, particularly for patients with low peripheral blood counts [14]. To avoid some of these disease-related collection and cell quality challenges, there are ongoing studies utilizing healthy donor allogeneic material to produce CAR-T cells, though risks associated with graft-vs-host disease and T-cell rejection first need to be addressed [15,16]. The use of allogeneic donor cells allows for the manufacture of CAR T cells using only apheresis products meeting predetermined criteria. Allogeneic donor CAR-T cells can be made in large quantities and cryopreserved allowing for the establishment of an inventory of CAR-T cells that meet all critical quality attributes.

Starting Material Cell Enrichment

Following the apheresis procedure, a cell selection or enrichment step is undertaken to further isolate target cells. Contaminating red blood cells, monocytes, and neutrophils in the starting material may adversely affect T cell expansion in culture as well as final CAR-T cell product characteristics. Notably, increased quantities of monocytes have been shown to be associated with reduced T cell expansion, while excess neutrophils may be associated with reduced transduction efficiency [17,18]. Hence, as there is limited technical flexibility during the apheresis procedure to fully avoid the collection of undesired cells, employing a cell selection or enrichment step is a critical part of the manufacturing process.

The methods used for cell enrichment can be variable, however, this variability can permit flexibility during manufacturing, as techniques can be tailored to meet clinical needs and cost constraints. The available methods can be broadly characterized as either a method to eliminate contaminating cells, or one to specifically select for T cells. GMP-compliant cell elimination techniques include density gradient separation, counter-flow elutriation, and flask adherence techniques. Density gradient separation utilizes a lymphocyte separation medium, such as Ficoll, during centrifugation to separate red blood cells and granulocytes from peripheral blood mononuclear cells (PBMCs), platelets, and plasma. Counter flow elutriation separates cells based on size, and to a lesser extent density, and has been successfully used to generate CAR-T cells [19]. Cell separation can also be achieved through flask adherence techniques, which exploit the adherent properties of myeloid cells to plastic to permit isolation of lymphocytes in T flasks [18].

In terms of cell selection techniques, investigators have used various antibody-fluorochrome conjugates, antibody-magnetic bead conjugates, as well as Streptamer-based isolation methods [20,21]. These methods separate cells based on cell surface antigen expression and are capable of not only selecting for T cells, but also T cell subsets. Through cell selection techniques, Turtle et al. produced anti-CD19 CAR-T cells at a fixed 1:1 ratio of CD4:CD8 T cells in order to provide a more consistent final product [22]. Even without altering ratios, the use of CD4/CD8 selection during CAR-T cell manufacture has been found to yield more consistent transduction efficiencies, though the clinical impact of this remains to be determined as this may be associated with greater toxicities [23].

Though cell elimination techniques are cost-effective and have been successfully utilized to manufacture CAR-T cells, they result in greater contamination with unwanted cells as compared to more expensive cell selection techniques. While no single cell enrichment method has been shown to be superior to others, certain populations and disease states may require purer cellular material be used for beginning the manufacturing process. Likely the most compelling case for ensuring a pure T cell population was made in 2018 when it was reported that the unintentional transduction of a single leukemic B cell during anti-CD19 CAR T cell manufacture led to not only resistance to CAR-T cell therapy by masking the CD19 epitope, but also the iatrogenic creation of a CAR-B cell form of leukemia [24].

Cell Stimulation/Activation

Following cell selection or enrichment, polyclonal, antigen-independent T cell stimulation is used to facilitate T cell expansion so that T cell doses sufficient to treat patients can be obtained. With the expanding field of adoptive T cell therapies, GMP-grade reagents are rapidly becoming available to support clinical trials. There are now a number of different types of stimulatory reagents, but further research is needed to determine the differences between them and the downstream effects that they may induce. For example, while some stimulatory reagents may result in greater

cell expansion than others, this could also result in more differentiated T cell phenotypes, and accordingly, a less durable product [25].

Cell-based and bead-based artificial antigen presenting cells have been tested for the manufacture of adoptive cell therapies, and can effectively simulate the T cell activation effects of innate antigen presenting cells [26]. Antibody-based methods of stimulation are also commonly used, and require activation through both CD3 as well as a costimulatory signal in order to generate substantial expansion. These reagents may be soluble or utilize magnetic beads that require removal prior to cell infusion. Some of the GMP-grade activation reagents available at this time include: soluble anti-CD3 monoclonal antibody (clone OKT3), CTS™ Dynabeads™ CD3/CD28 (ThermoFisher), MACS® GMP T Cell TransACT™ CD3/CD28 (Miltenyi Biotec), and Cloudz™ T Cell Activation CD3/CD28 (R&D Systems).

The number of days that T cells are activated also needs to be carefully considered. At our institution, we have found that CAR-T cells manufactured on the CliniMACS Prodigy® (Miltenyi Biotec) demonstrated reduced viability, fold expansion, CD3%, and transduction efficiency when cell stimulation was extended to 5 days from 3 days (unpublished results). In addition, these cells demonstrated reduced Polyfunctional Strength Indexes (PSI™) as measured by technology from IsoPlexis, suggesting that they may have reduced immune programs that may affect the potency of the product.

CAR Gene Transfer

Variability during CAR-T cell manufacturing is also introduced during the gene transfer phase, and the method used may impact the critical quality attributes of the final product. Gene delivery may be accomplished through viral or nonviral systems, of which the former is highly efficient, though carries a higher risk of insertional mutagenesis [27]. Clinical CAR-T cell trials have largely utilized viral transduction-based methods, which include the use of γ-retroviral and lentiviral vectors. γ-retroviral vectors were the first viral vectors used in manufacture CAR-T cells, and benefit from high transduction efficiencies. Lentiviral vectors, on the other hand, have the advantage that they are capable of transducing both dividing and nondividing cells. Lentiviral vectors may also carry a lower risk of insertional mutagenesis based on integration patterns [28].

GMP-compliant viral vector production, however, is expensive, and there is currently manufacturing under-capacity. More accessible alternatives to viral vector-based transduction are available, though still being tested in clinical trials. Some of the methods being explored include electroporation of mRNA or via transposase systems, as well as targeted insertion strategies such as zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR/Cas9 [29-31]. Studies have used the latter approach to concurrently and strategically disrupt the T cell receptor alpha constant gene encoding T cell receptors, which could support the manufacture of allogeneic CAR-T cells, as the risks of graft-vs-host disease are alleviated [16,32]. Ultimately, whether CAR-T products manufactured with these nonviral techniques will be equally safe and effective remains to be determined.

When viral vectors are used to produce CAR-T cells, transduction related variables include the timing of transduction and the use of transduction enhancers. Vector transduction can be performed during or a few days after cell stimulation, and the transduction step can be performed more than once. Methods to enhance vector interaction with T cells have also been used, and include spinoculation, also known as centrifugal inoculation, which has been shown to improve transduction efficiency [33]. Spinoculation, however, also carries the risk of bag breakage, and in some cases, can be circumvented as a step in manufacturing without compromising transduction efficiency [34]. Chemical enhancers include polybrene and recombinant fibronectin (RetroNectin, Takara Bio), and are used to promote the co-localization of viral particles and T cells [34,35].

There is also significant variability with the CAR construct, which is typically composed of an extracellular target-binding domain, a hinge region, a transmembrane domain, and one or more intracellular domains. As CARs have developed, the intracellular domains have distinguished CARs as first (CD3ζ only), second (1 costimulatory domain and CD3ζ), or third generation (more than 1 costimulatory domain and CD3ζ). Each of these domains are designed with a purpose, with variabilities conferring both advantages and disadvantages, and together, the design of the construct can heavily impact the potency and durability of the final product.

The target-binding domain is designed to recognize tumor cells, and can be derived from both human and nonhuman sources [36]. The use of humanized constructs reduces the risk of development of an immune response directed toward the CAR-T cells. The ideal CAR-T cell target is present on tumor cells and absent from normal tissue, so that on-target, off-tumor toxicities can be reduced or avoided. The hinge region provides stability and enables flexibility for the construct. The transmembrane domain anchors the CAR to the cell membrane, and enables proper CAR T cell signaling and expression. Lastly, variability is also introduced with the costimulatory domain, which provides a second signal necessary for durable CAR-T cell activation. The two types of costimulatory molecules most commonly used include CD28 and 4-1BB, where the former results in more rapid T cell activation but shorter T cell persistence than the later [37]. These characteristics can be exploited to maximally tailor the design of the CAR to the disease being treated [36].

Cell Expansion

The cell expansion phase is another step where optimization is necessary to meet targeted doses for treatment. Effective CAR-T cell products may be produced from a relatively small amount of starting leukocytes, as the therapeutic cells may be expanded in culture after the gene transfer phase. Variabilities in the expansion phase include reagents used in the culture media, duration of culture, and choice of bioreactor or culture platform.

GMP-grade T cell culture media are available to support clinical CAR-T cell trials, and are typically supplemented with cytokines to enhance *ex vivo* CAR-T cell proliferation. Type 1 cytokines, and particularly members of the γ-chain family of cytokines, play critical roles in the survival, proliferation, and differentiation of T cells [38]. IL-2 has been the most common cytokine used for CAR-T cell manufacture and can induce robust T cell expansion, though potentially at the expense of differentiation and loss of the naïve T cell phenotype [39]. As a product composed of terminally-differentiated lymphocytes may have compromised potency and durability, future clinical studies are needed to determine whether the use of other γ-chain cytokines can improve CAR-T cell efficacy.

In addition, the duration of expansion needs to be carefully considered, as while extended cultures may produce more T cells, these could likewise be more differentiated and have less tumor killing capacity. For example, Kaartinen et al. found that by limiting the length of expansion and by adjusting the concentration of IL-2 used, the number of early memory T cells could be increased, which could enhance in vivo performance [40]. In a murine xenograft model of B-ALL, Ghassemi et al. showed that anti-CD19 CAR-T cells harvested at earlier time points demonstrated robust leukemic control even at lower doses, as compared to cells harvested after extended culture [41]. Hence, cytokine

Table 2
Measures of critical quality attributes

Critical quality attribute	Measure	Method of measurement
Safety	Sterility testing	Bacterial and fungal culture, Gram stain
	Mycoplasma	PCR or culture
	RCL/RCR	PCR or culture
	Vector copy number	PCR
	Bead content (insoluble material)	Microscopy
Purity	T cell content	Flow cytometry
	CAR-T cell content	Flow cytometry
	Contaminating cell content*	Cell count flow cytometry
Potency	CAR-T cell content	Flow cytometry or PCR
	In vitro cytotoxicity	Cytotoxicity assays
	Cytokine secretion	Cytokine release assays
Durability	CAR-T cell persistence	Flow cytometry PCR
	In vivo CAR-T cell expansion	Flow cytometry PCR
Consistency	Measures of consistency includes all of the above for safety, purity, potency, and durability, but are applied across different lots of CAR-T cell products.	

PCR = polymerase chain reaction; RCL/RCR = replication competent lentivirus/replication competent retrovirus
* Contaminating cells include red blood cells, circulating malignant cells, and non-T cell leukocytes.

types and doses as well as culture duration should be adjusted based on protocol needs.

Further, while traditional cell culture relied on flask systems, closed-system bags are becoming increasingly used to manufacture CAR-T cells and other immunotherapies [42]. In addition, partially- and fully-automated systems such as the CliniMACS Prodigy and the Cocoon (Lonza) are now available or being tested, and are capable of performing all functions of CAR-T cell manufacturing from cell selection, activation, gene transduction, expansion, and final harvest in a scalable, GMP-grade fashion. The shift to closed system processing and the incorporation of single-use disposable materials can also effectively reduce the risks of microbial contamination to provide a safer product, while automation can potentially promote a more consistent product. In addition, the choice of bioreactor is in itself a variability during manufacturing, as are the reagents associated with a given culture platform, and this can ultimately affect the critical quality attributes of the final product.

Cryopreservation and Thaw

Cell cryopreservation is an optional step during CAR-T cell manufacturing that can permit flexibility in scheduling patient infusions as well as time to complete extended quality control tests required for product release. This step may be performed prior to culture initiation and/or after the final product has been manufactured. From a quality perspective, this step is critical, as suboptimal cryopreservation can result in cell loss, impaired viability and altered cell phenotype and function. Accordingly, a validated process for this step is essential for clinical CAR-T cell manufacture.

Variables during this step include the type and concentration of cryoprotectant and additives in the freezing medium, the method of freezing, and the storage conditions. While most cryopreservation techniques utilize 5% to 10% dimethyl sulfoxide (DMSO) as a cryoprotectant, DMSO-free cryoprotectants are also being tested [43,44]. Post thaw recovery of cells may also be influenced by the method of freezing, with controlled-rate freezing representing an essential process to cool cells and minimize cell damage from ice crystals or cytotoxic solutes [45]. Following controlled rate freezing, cells are typically stored at <-150°C under temperature-monitored conditions. Lastly, automated devices to aliquot cells and reagents in preparation for cryopreservation are also becoming available, and may be able to provide more consistent products.

Following cryopreservation, PBMCs may be thawed to initiate culture, or final CAR-T cell products may be thawed for infusion. A number of variables may also be introduced at this step, and include the method, duration, and rate of thaw [46]. While water baths set at 37°C have been most commonly used to thaw cell therapy products, dry thawing systems that reduce water-borne contamination risks are also available, and may aid in supplying a safer product.

Given the potential variabilities seen during cryopreservation and thaw, the techniques used require optimization as well in order to maximize cell recovery. At our facility, we systematically examined the clinical impact of cryopreserving the starting material of PBMCs as well as the final CAR-T cell products [47]. Cryopreserved starting material did lose viability when thawed and placed in culture, particularly around two days into the process. However, all cultured products eventually recovered and expanded to a sufficient degree to meet clinical doses, and final CAR-T cells demonstrated no differences in viable cell recovery, transduction efficiency, %CD3 positivity, and CD4:CD8 ratios before and after cryopreservation. In addition, gene expression profiling performed in this study identified distinct differences between products that had or had not been cryopreserved, where products that had been cryopreserved demonstrated greater expression of apoptotic pathways, and less expression of T cell receptor signaling pathways. Ultimately, however, cryopreservation was not found to impact clinical outcomes including peak CAR-T cell levels or CAR-T cell persistence as measured in vivo. However, additional studies are needed in this regard.

Release Testing and Product Characterization

During and following CAR-T cell manufacturing, various in-process and quality control tests are performed to characterize the product and ensure its safety. CAR-T cell products are released under a certificate of analysis which features the minimum specifications required for several critical quality attributes, including safety, purity, and potency (Table 2). Release testing is typically performed according to regulatory agency-approved assays, but where this is not possible, assays developed in-house may be used, provided that they are validated to prove assay integrity. As there are a number of different ways to test any given item, release testing represents another source of variation during the manufacturing process.

Measures of safety include testing for microbial contamination, including bacterial and fungal sterility as well as mycoplasma, which can contaminate cell cultures and be difficult to isolate from routine bacterial testing. When viral vectors are used, safety testing also includes testing for replication competent viruses and vector copy number. Flow cytometry can be utilized to assess the purity of a CAR-T cell product, including the proportion of cells positive

for T cell surface markers such as CD3, CD4, and CD8, as well as markers of the CAR. In diseases where tumor cells may circulate in the periphery, flow cytometry may also be used as a safety measure to ensure that these cells have been eliminated from the final product. Viability testing is also performed to assess the proportion of viable cells before or possibility after cryopreservation, however, minimum viability specifications for clinical efficacy remain to be determined [48].

Conclusion

CAR-T cells as immunotherapies herald an exciting era, evolving as the fourth pillar in cancer treatment along with chemotherapy, radiation, and surgery. Nevertheless, standardizing manufacture of these nuanced “living agents” is a challenge, as variables may be introduced during each step in the process. Critical quality attributes of CAR-T cells are currently being assessed by a set of parameters similar across various studies, however, continued research and evaluation is needed to fine-tune these standards and their requirements. Alongside the major strides in automation and closed-system manufacturing solutions, it is critical to develop standardized assays for more robust testing of CAR-T cell functionality, in order to deliver better-characterized products to patients.

Notably, clearly-defined measures of CAR-T cell potency capable of predicting clinical outcomes do not yet exist. Potency may be assessed in vivo after the cells have been infused, such as by CAR-T cell expansion or tumor burden reduction, or through in vitro bioassays before the cells are released – though the latter may not correlate with clinical efficacy [49]. Measurements of transgene expression may serve as a surrogate marker of CAR-T cell activity, and potency has also been assessed by testing interferon-γ secretion or through cytotoxicity assays. Newer potency assays are also being evaluated to assess T cell gene expression and exhaustion states, T cell metabolic activity, and T cell immune programs [50-52]. As more is understood on how these measures correlate with clinical outcomes, these findings may be useful to further optimize manufacturing processes.

Conflicts of Interest

None.

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