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CAR-T Cells – Main Steps for Obtaining a Proper "Live Drug" Adoptive Therapy

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Abstract

Chimaeric antigen receptor (CAR) T lymphocytes (CAR-T) discovered in 1992 have been in the recent years in the spotlight of both researchers and clinicians. Immune therapy with engineered CAR-T cells has been approved by the FDA since 2017 for pediatric and young adult acute lymphoblastic leukemia. Since then, around ten therapies were approved for hematological cancers. In contrast to the other immune therapies CAR-T cell therapy is considered a "living drug" with the capacity of self-replicating. Having this particular characteristic, the manufacturing process has in general several steps that should be followed in the process of obtaining specific CAR-T cells. There are mandatory steps for obtaining a CAR-T cell culture: Leukapheresis, activation, gene delivery, expansion, and cryopreservation. All these seminal stages have various sub-stages that can differ from one procedure to other as detailed in the paper. Leukapheresis harvests peripheral blood mononuclear cells, followed by an enrichment of a certain cellular population or a depletion phase to remove contaminants as red blood cells and platelets. T cell population that is obtained needs to be activated using activating antibodies (Abs), magnetic beads coated with activating Abs or artificial antigen-presenting cells. After activation, T cells are subjected to genetic engineering that can be done using electroporation of naked DNA, plasmids, or viral vectors to insert the specific CAR. The last phases of CAR-T cell manufacturing are the expansion of these specific cells and, if not freshly inoculated to the patients, the final step is cryopreservation. The main drawback of CAR-T cell therapy is its high cost; therefore, new automated platforms would decrease their cost. The described stages are still variable within CAR-T manufacturing. Therefore, the future of this immune therapy will have to tackle standardization and automated technology, directions that will increase healthcare system availability.

Introduction

Chimaeric antigen receptor (CAR) T lymphocytes (CAR-T) have been in the recent years in the spotlight of both researchers and clinicians. CAR-T cells were first discovered by Zelig Eshhar in 1992 and starting from 2010, numerous clinical trials have begun using this novel, customized immunotherapy [1]. In contrast to the other immune therapies CAR-T cell therapy is considered a "living drug" with the capacity of self-replicating, therefore, particular phases in their production are to be followed.

As of 2021, there are several approved CAR-T cell therapies, denominated as second-generation constructs. These constructs include an antigen-binding domain, hinge and transmembrane domains, mandatory a costimulatory domain (from CD28 or 4-1BB), and a T cell activation domain from CD3ζ [1], [2]. The first generation of CAR-T cells had a T cell receptor (TCR) stimulatory domain linked to a single chain variable antibody fragment [3]. The second-generation of CAR-T had an addition of a costimulatory domain that significantly improved T-cell survival and proliferation on activation with antigen-expressing target cells [4]. The third-generation of CAR-T cells was the autologous T cells retrovirally transduced to have intracellular costimulatory domains derived from CD28 and 4-1BB [5]. Recently, the fourth-generation of CAR-T cells was designed and results of the clinical trial (ClinicalTrials.gov Identifier: NCT03778346) in Refractory/Recurrent (R/R) multiple myeloma were published [6]. This latest generation coexpresses interleukins and chemokines, or suicide genes that can enhance the efficacy of CAR-T therapy [7]. The general structure of a fourth generation of CAR-T cells is presented in Figure 1.

Various cellular designs differ in the specific domains that are included in the CAR-T cells. In the manufacturing of these cells, the viral vector that sustains the transgene delivery and several other details in the stages can differ [8].

In 2017, the FDA approved the first CAR-T cell therapy with tisagenlecleucel (Kymriah) for the treatment of pediatric and young adult acute lymphoblastic leukemia (ALL) [9]. In the same year, FDA approved three more CD19-specific CAR-T cells for the treatment of various B cell malignancies (e.g., axicabtagene ciloleucel – Yescarta, brexucabtagene autoleucel – Tecartus, lisocabtagene maraleucel – Breyanzi) [10], [11], [12]. CAR-T from

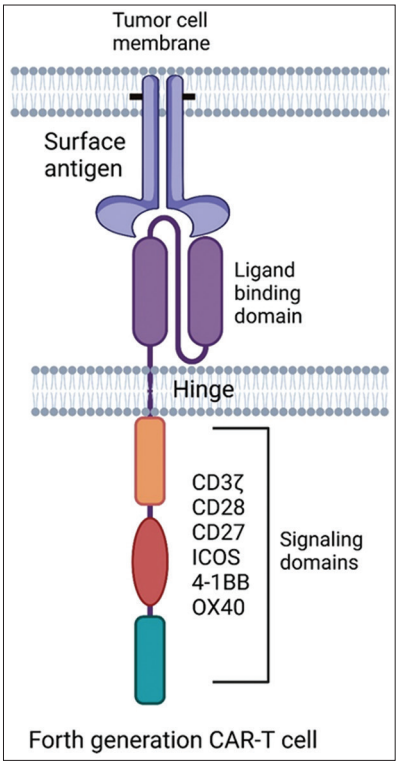

Figure 1: Structure of an engineered CAR-T cell. The ligand binding domain recognizes a specific antigen expressed by the tumor cell; the hinge region links the ligand binding domain to the intracellular domains that sustain the activation of T cells

Tecartus is similar to Yescarta, but Tecartus enriches T cells while Yescarta does not [13]. In 2021 and 2022, two B-cell maturation antigen (BCMA)-specific CAR-T cell therapies were approved for multiple myeloma (e.g., idecabtagene vicleucel – Abecma and ciltacabtagene autoleucel – Carvykti) [14], [15], and for relapsed or refractory follicular lymphoma tisagenlecleucel [16], [17]. Recently, reviewed by Cappell and Kochenderfer [18] CD19-targeted CAR-T cells are approved for R/R aggressive B cell lymphomas [12], mantle cell lymphomas [13], and indolent B cell lymphomas [17], [18]. The outstanding clinical results using CD19-targeted CAR-T cells with good complete response (CR) rates in B cell lymphomas were durable [18], [19]. For example, CR rates exceeded 70% in all clinical trials with R/R B cell acute lymphoblastic leukemia (B-ALL), in fact, B-ALL being a leukemia with limited treatment options [10], [20]. In R/R multiple myeloma BCMA-CAR-T cells, the overall response rates reached 98% [16].

The approved CAR-T cell therapies can trigger side-effects such as cytokine-release syndrome, immune neurotoxicity syndrome, hypogammaglobulinaemia, and cytopenias [21]. Therefore, alleviating the side effects implementing new stages of manufacturing the CAR-T cells can expand their clinical implementation beyond the hematological malignancies [22].

It is foreseen that by 2026, approved CAR-T cell therapies will be have the largest oncology drug sales in pediatric and young adult patients with ALL and in adults with diffuse large B-cell lymphoma [23]. Researchers and industry have agreed in the last years that for these therapies to expand and to be implemented in clinics, standard manufacturing platforms should be achieved, and more so, fully automated platforms that bypass the laboratory man-handling. In a real-life scenario, these platforms are expensive in terms of equipment (s), reagents, and consumable. Moreover, the work-flow for such an automated platform will manufacture only one type of product at a time [24]. In a paper published in October 2023, Albelda has shown that very soon the use of CAR-T cell therapy will reach the solid tumors domain [25] and when the commercial stages will be reached in this oncology domain the development of manufacturing platforms will be crucial. In a recent paper, Abou-el-Enein *et al*. have shown that robust and reproducible CAR-T cell production is associated with challenges and limitations [26].

Within this mini-review, we will touch on the main stages that are generally used to manufacture CAR-T cells to be further administered in patients [26].

Manufacturing Stages for CAR-T Cells Production

There are several mandatory stages for obtaining a CAR-T cell culture such as: Leukapheresis, activation, gene delivery, expansion, and cryopreservation. All these seminal stages have various sub-stages that can differ from one procedure to other as detailed further.

Leukapheresis

Leukapheresis and cell washing are the first procedures at the beginning of CAR-T production. From patient(s) white blood cells (WBCs) are obtained by leukapheresis and this is a phase where WBC is separating from the whole blood. This procedure is done by density gradient media with continuous or intermittent centrifugation of the harvested blood. The anticoagulants that were added during the process, contaminants such as red blood cells and platelets are removed in a washing step [27], [28], [29]. Used in research laboratories, but as well in clinical trials red blood cells and platelets, can be removed by Ficoll density gradient [30]. For example, in the clinical trial NCT01886976, this technique was used for obtaining CAR-T-cell for multiple myeloma patients [31]. The same method was used in clinical trial NCT01864902 for CAR-T therapy to be used in R/R acute myeloid leukemia patients [32]. Then again, clinical trials can use automated cell decontamination like in NCT02215967 [33]. After washing, the purified WBCs can be directly used or stored frozen in controlled freezers [34].

Leukapheresis can be followed by an enrichment and depletion stage. There are groups that try to enrich WBCs for CD4+ (T helper cells) and CD8+ (cytotoxic T cells) to infuse a cellular combination of CD8+ with CD4+ in a defined cell ratio [35]. Other groups enriched WBCs for central memory T cells (CD62L+) [36], [37]. Interestingly more than 10 years ago it was shown that a high concentration of natural killer (NK) cells would hinder T cell production so an NK depletion using CD56+ magnetic beads was used for WBCs if the NKs exceeded 10% in the process [38].

Activation

After leukapheresis and washing and enrichment/depletion of certain intended cellular population's stages, the following step is the activation of T cells. In normal physiological conditions, naive T cells enter in contact with antigen-presenting cells (APC) (e.g. dendritic cells [DCs]) and will mature, differentiate, and proliferate to initiate the specific immune response. The molecular interactions are done by the TCR and by the major histocompatibility complex on DCs through costimulatory molecules such as CD28, 4-1BB, and OX40 [39]. When the process was developing the activation stage was done with the co-cultivation of T cells with DC, but the process is difficult and lengthy so in the meantime several other methods mimicking this physiological process were put in place, developed, and implemented [40].

There are actually three major processes that mimic the activation of T cells *in vitro*: Using specific antibodies (Abs) and costimulatory cytokines, specific Abs-coated magnetic beads, and artificial APCs.

In terms of monoclonal Abs and cytokines, the most common method is to is use anti-CD3 monoclonal antibody (e.g., OKT3) and IL-2. IL-2 is used, depending on the group in various concentrations, but an excess concentration can exhaust T cells [41] displaying poor functionality and high apoptosis [42]. Two independent groups have shown that if IL-2 is replaced with IL-7 and IL-15 a higher percentage of memory subsets are obtained [43], [44]. Recently, various other methods were published such as membrane vesicles as target cell surrogates. Actually, antigenic micro-spheres were designed expressing surface-bound CAR antigens that can stimulate and expand CAR-T cells [45]. Another recent study showed that there is no need of additional T-cell activation. Namely, functional CAR-T cells are obtained within 24 h from T cells separation from peripheral blood without any additional activation, and the viral transduction process is influenced by the culture medium and the characteristics of the culture vessel [45].

Magnetic beads coated with anti-CD3/CD28 antibody were used as artificial antigen-presenting particles inducing the activation of cells and generating memory phenotype cells in an enhanced manner as compared to the simple OKT3/IL-2 treatment [35], [46]. During expansion, the beads continuously stimulate the cells. Cells were actively producing cytokine up to 100-fold higher as compared to other activation methods [47]. Besides several technical advantages, antibody-coated paramagnetic beads induce an activation that is less T cell exhausting and more persistent than activation with anti-CD3 mAb and IL-2 [44]. The first published study using magnetic beads to produce anti-gp120 CAR-T cells for HIV was in 2002 [48] and since then PubMed database we found tens of studies and several clinical trials.

Artificial APCs for activating T cells are another variant of this phase in the workflow of CAR-T production. CAR-T cells can be activated using non-viable APCs, such as K562 cell lines induced to coexpress stimulating molecules and tumor-associated antigens (TAA). After irradiation, these cells are dead, but they will express TAA and will selectively stimulate CAR-T cells [49]. For example, when producing CAR-T cells specific for EBV-specific TCRs an EBV-transformed lymphoblastoid B-cell line can be used for activation [50].

Gene delivery

The stage of gene delivery can be done using viral and non-viral methods. The main methods for accomplishing this phase in CAR-T cell therapy are electroporation of naked DNA, plasmid-based systems, and delivery using retro-viruses or lentiviruses vectors. Lentiviral vectors have sustained the success of CAR-T cell therapy because the process was based on lentivirus-mediated gene transfer [51]. The intended gene transfer induced from 4 to up to 70% efficacy [52] but the lentiviral technology is more expensive than plasmid transfection. The high-efficiency transduction when using retro-viruses or lentiviruses involves activation of the T cells because retroviruses will transduce in dividing cells, hence proliferation will be indispensable for gene delivery. In both retroviruses or lentiviruses vector methods, the risk of inserting an oncogenic gene exists, with a slightly lower probability in lentiviral integration [53]. In the clinical setting the introduction of an oncogene when using viruses vector method was not reported yet. However, insertional mutagenesis is an important risk with CAR-T cell therapy, and several years ago a new tagmentation-assisted PCR (tag-PCR) system with fewer steps compared to the other methods was reported. Tag-PCR was developed to identify the integration sites of transgenes and thus the integration sites of CD19-specific CAR genes in several final CAR-T cell cells were evaluated. Cells were generated using retrovirus-based, lentivirus-based gene transfer and the piggyBac transposon system (see below). The piggyBac transposon system was proven to have lower preference in comparison to the retroviral system for integration near transcriptional start sites and CpG islands, but a higher preference in

comparison to the lentiviral system for integration into genomic safe harbors. It was shown that the integration into or near proto-oncogenes was comparable in all three investigated systems [54].

In terms of economic issues, lentiviral vectors are more expensive than retroviral vectors because lentiviral vectors are produced using transient transfection and large amounts of plasmid DNA in comparison to retroviral vectors that can be produced using stable packaging cell lines [55], [56], [57], [58].

Plasmid-based gene delivery is another method that was used for CAR-T cell therapy. It was reported in the late ’90 using transposon/transposase systems. The transposon is a sequence of DNA that can to change position within the genome by the action of enzyme transposase excision and insertion [59]. There is a stable gene transfer through transposase, that and catalyzes the transposons movement by a replicative transposition mechanism [60]. The intended gene for CAR-T preparation can be inserted into a transposon sequence having the transposase within or separately. The plasmids containing the transposon and the gene are electroporated in T-cells, the transposase excises the transposon and inserts its sequence into the T-cell genome [61], [62]. The transposon system is more efficient in gene integration compared to the electroporation of naked DNA and matches viral transduction. The sleeping beauty (SB) transposon/transposase system is largely used in CAR-T-cell procedures. SB is the first synthetic DNA transposon used for two decades in various molecular applications [63]. The method is cheaper than viral vector generation [57]. One shortcoming for this method is that requires a longer cultivation period for the generation of the required cell numbers [63]. Viral-based gene delivery has the propensity to insert oncogenes or to disrupt other significant genes [64]. However, in 2016 a study was published investigating various gene delivery methods. It was found that SB gene integration displayed the lowest possibility of insertional oncogenesis in comparison to viral methods and the piggyBac transposon/transposase system [65].

In clinical trials, viral transduction is used in almost 94% of the studies. Lentiviral transduction is the predominant method while retroviral transduction is the second most used method, both methods being expensive [66]. Transposon/transposase systems like SB entered the clinical trials in 2015 with significant economic advantages in comparison to viral transduction [57].

Expansion

The final stage of the CAR-T production expansion phase will deliver an increased number of CAR-T cells. Briefly, the cultivation flasks will increase in volume and number, a step that requires trained operators and biosafety cabinets [67], [68]. This step and its procedures can be done at the research level with good efficiency, while at large-scale manufacturing, it becomes labor intensive. Therefore, for large-scale production of CAR-T cells, static culture bags are used for expansion, a procedure that can be semi-automated and can be connected by sterile tubing. In 2013, this procedure was published consisting of a work-flow that uses semi-closed system minimizing open-handling interaction [69].

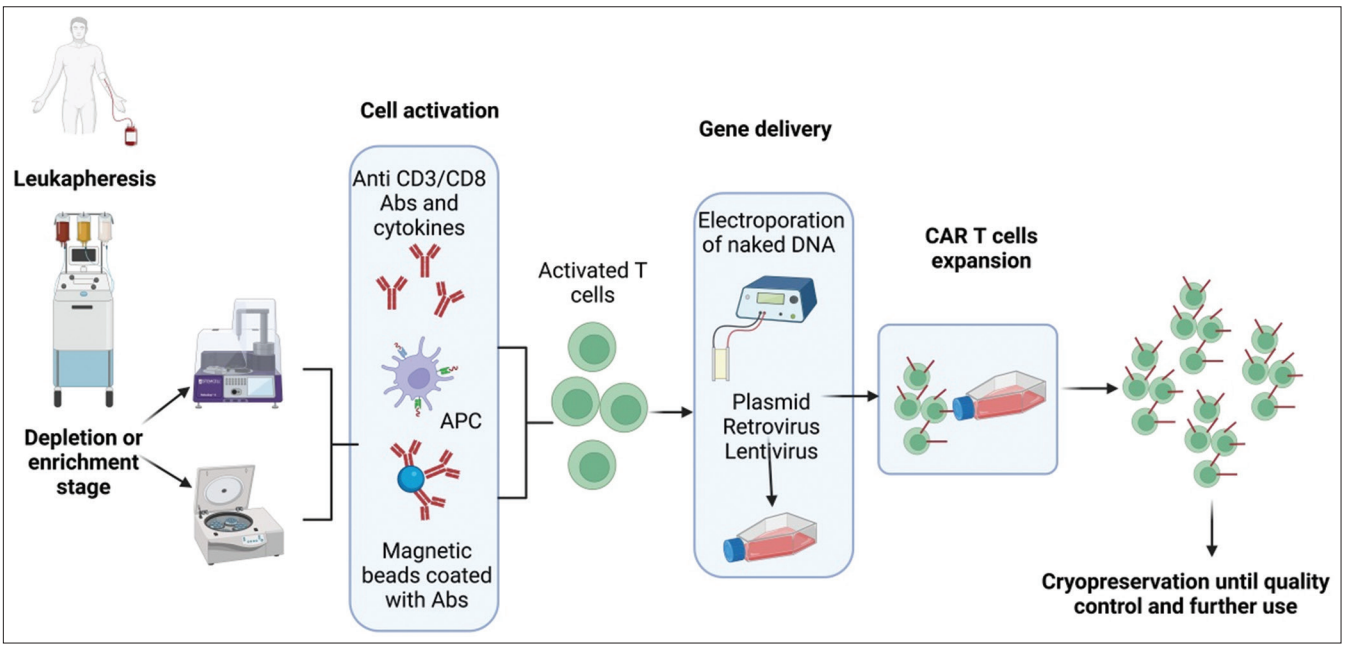

Figure 2: The steps of CAR-T cells manufacturing process. In the first stage, leukapheresis would isolate PBMC. The collected PBMCs can be cryopreserved at the site of harvest or enter immediately the further process. Density gradient centrifugation would deplete the cell suspension of granulocytes, red blood cells, and platelets. This stage can be replaced or followed by a selection of specific T-cell types (enrichment). After depletion or enrichment, T cells are activated by anti-CD3/CD8 Abs and cytokines, with artificial APCs, or with magnetic beads coated with activating Abs. After activation, T cells are genetically engineered by electroporation with naked DNA, or using plasmids, retroviruses, or lentiviruses. CAR-T cells are expanded and can be cryopreserved or freshly used depending on the protocol. PBMC: Peripheral blood mononuclear cells

Cryopreservation

If not used immediately, CAR-T cells can be cryopreserved. Several studies were done lately to evaluate the efficacy of CAR-T cells after cryopreservation [70]. Thus, evaluation of the fresh and frozen CAR-T cells has shown that although the viability of thawed cells is slightly lower than the fresh ones in the clinical trials where patients were receiving thawed CAR-T cells had the same clinical response as the ones receiving fresh cell cultures although the viability of thawed cells ranged from 73.7% to 98.4% [71-73].

In Figure 2 we present the main steps for CAR-T preparation.

Conclusion

Thoroughly described by Vormittag *et al*. [74], CAR-T cells have gained their place in the immune-therapy panel as "living drugs." There are already several FDA approved in hematological diseases and probably the following years several more will be approved. As they are offering a new therapy not only in oncology, probably we will assist in the coming years to their approval in other human diseases. A domain that will have in the very near future a CAR-T therapy will be in the solid tumors area. Nevertheless, research has still to tackle an array of issues. The main drawback of CAR-T cell therapy is the high cost of the manufacture; therefore, new platforms that are automated would decrease their cost. The steps that we have described within the paper: Leukapheresis, activation, gene delivery, expansion, and cryopreservation are still variable within CAR-T manufacturing. Therefore, the future of this immune therapy will have to tackle standardization and automated technology, directions that will increase health-care system availability.

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Authors' Contributions

Monica Neagu and Carolina Constantin designed and performed the bibliographic study. Monica Neagu and Carolina Constantin wrote the paper. Monica Neagu and Carolina Constantin wrote and revised the final form of the paper.

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