Lentiviral Vectors in CAR-T Cell Manufacturing: Biological Principles, Manufacturing Process, and Frontier Development

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Abstract. Chimeric antigen receptor T cell (CAR-T) therapy, as the preeminent strategy in immunotherapy, has conferred benefits upon numerous cancer patients. Currently, there are six CAR-T therapies approved by the Food and Drug Administration (FDA), while the China National Medical Products Administration (NMPA) has approved three. The manufacturing process for CAR-T cells (CAR-Ts) entails using lentiviral vectors (LVVs), which are efficacious in transducing functional genes to both dividing and nondividing cells. Also, LVVs exert their capacity to integrate relatively large segments of DNA. Despite this fact, LVV-based CAR-Ts production can prove prohibitively expensive, and the yield of LVV can be further enhanced. In recent years, in-vivo generation of CAR-Ts has attracted significant attention since it stands to eliminate the ex-vivo isolation and activation process for T cells, thereby decreasing the cost and time involved in production. In view of this, this review summarizes the biological principles of LVVs and their manufacturing process, which encompasses lentiviral vector production, T cell isolation and activation, and CAR-Ts production. Additionally, the cutting-edge developments associated with LVVs are outlined herein, offering direction for the production of the next generation of CAR-Ts.

Keywords: CAR-T, Lentiviral vector, Serum-free suspension, In-vivo generation of CAR-T cells

1. Introduction

CAR-T therapies, first discussed in the late 20th century, have emerged as promising treatments for multiple cancers in the past few decades [1]. In 2017, the first two CAR-T therapy were approved by the FDA, to treat diffuse large B-cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL), respectively. There are now nine CAR-T therapies available for clinical use, targeting CD19 or BCMA on cancer cells [2]. The CAR structure is the main functional element of CAR-Ts, which can be divided into four domains, namely ligand-binding domain, spacer element, transmembrane domain and cytoplasmic domain [3]. Once the ligand-binding domain recognizes and binds to the antigen on the cancer cell's surface, the signal is transmitted downstream and the CAR-Ts are activated, stimulated to proliferate, release cytokines, and alternate metabolism. Furthermore, granzyme and perforin are released to destroy and digest cancer cells.

The procedure of manufacturing CAR-Ts ex vivo remains consistent despite the various genetic modifications of T cells. To begin with, peripheral blood is collected from the patient, and lymphocytes are isolated and washed for the depletion of monocytes and anti-coagulants. Then the T cells are separated and stimulated for the transduction of CAR genes. After gene modification, CAR-Ts are multiplied to the necessary cell numbers and prepared in a proper buffer for clinical utilization [4].

For gene modification of T cells, several methods can be utilized, which can be categorized into non-viral vectors and viral vectors. Electroporation via the transposon system is one of the novel non-viral methods. Still, it cannot be conducted in vivo, and exert inefficiency in stable CAR genes expression [5], although the produced CAR-Ts present faster cytotoxicity in vitro [6]. Viral vectors, which include γ-retroviral viruses, adenovirus-associated vectors (AAVs), and lentivirus, have superior transduction efficiency and are widely used for gene delivery in multiple applications. γ-retroviral vectors were the firstly-used viral vectors for CD19-targeted CAR-Ts production, and they have taken up approximately one-fifth of clinical trials involving gene delivery [7]. AAVs have a relatively lower risk of toxicity, but the limited package size (~50 kb) restricts their gene delivery

capability [8]. LVVs are the most versatile viral vectors for CAR expression. Besides their high gene transfer efficiency, they can transduce both non-dividing and dividing cells. Moreover, since the viral genome is passed to daughter cells, they can exert long-term transgene expression. Currently, several FDA-approved CAR-T therapies utilize lentiviral as the gene delivery strategy, namely Kymriah, Abecma, Breyanzi, Carvykti, Relmacabtagene Autoleucel, and Fucaso [9]. Although widely used, there remain shortages in LVV utilization of CAR-Ts establishment. This review presents the biological principles of LVVs, the process for manufacturing CAR-Ts using LVVs, and the frontier development of lentiviral transduction techniques that may improve their efficiency and function for clinical application.

2. Biological Principles of LVVs

The retroviridae viral family serves as the origin of lentiviral vectors. These vectors can be derived from various viral sources, including feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV), human immunodeficiency virus type 1 (HIV-1), which is the most commonly used. The HIV-1 genome and structure are shown in Fig. 1. They are engineered to transfer up to 10 kb of genes to the host cell, resulting in encoded protein expression.

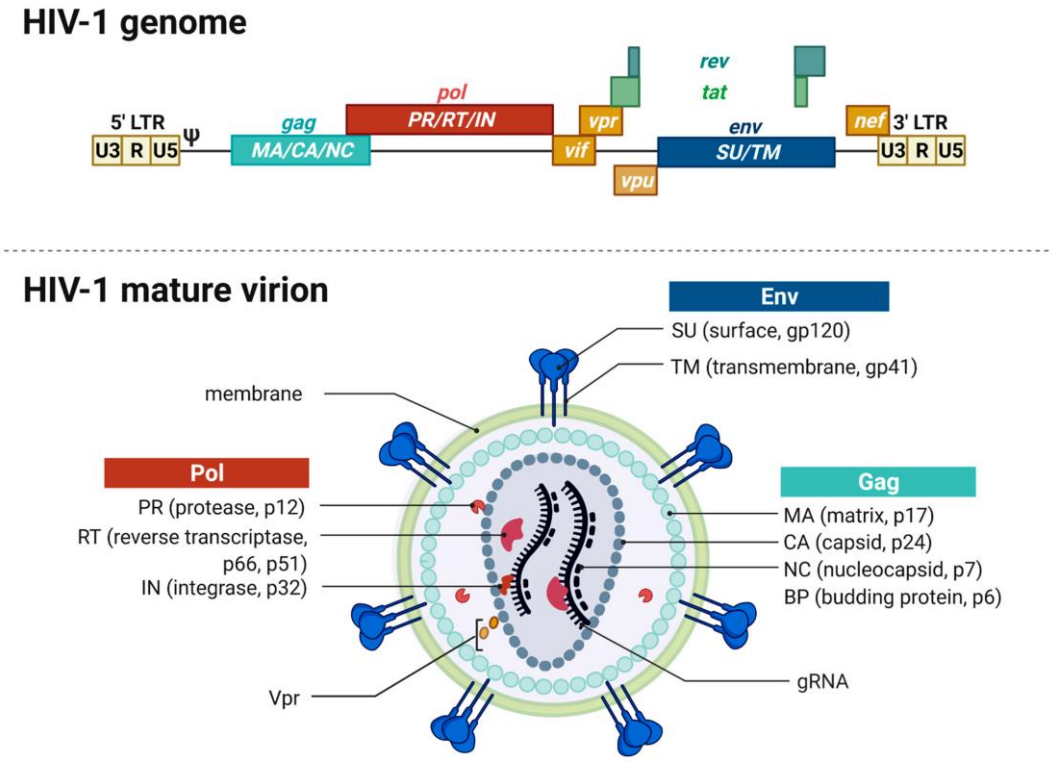

Fig. 1 HIV-1 genome and the structure of mature virion [10].

Gag, Pol, and Env are the three structural genes that exist in all lentivirus (Table 1). Moreover, Tat and Rev are crucial in regulation. Vif, Vpr, Vpu, and Nef are four additional accessory genes unique to HIV-1 that support virus replication and increase pathogenicity in vivo. The 3' terminus of the viral genome also has two untranslated regions, 5' R-U5 and U3-R [11].

The viral RNA functioned as a template for the reverse transcription of viral cDNA after the virus entered the host cells. The U3-U5 sequence is duplicated on both ends of the RNA, resulting in LTRs on the viral DNA. This DNA integrates into the host DNA, becoming a provirus. LVVs are essential for delivering genes because, in contrast to other retroviridae, they can infect nondividing cells. Following replication, the provirus spreads to daughter cells. Finally, transcription of the integrated DNA occurs, and the progeny viral genomes are then delivered to the cytoplasm. The virus forms a mature infectious virion by budding from the plasma membrane, simultaneously acquiring its lipid envelope [12].

To date, there have been three generations of the LVVs system derived from HIV-1, they are summarized in Fig. 2, and their genome components are shown in Table 1. The structural gag gene, the glycoproteins from the envelope plasmid, and the cassette flanked by two HIV LTRs were all present in the first generation of LVVs. The possibility of producing replication competent lentiviruses (RCLs) is reduced by using three different plasmids. The second generation eliminated accessory proteins (Nef, Vpr, Vif, and Vpu) associated with disease progression and pathogenic. In the third generation, a potent viral promoter took the role of the 5' HIV LTR sequence. Additionally, a new plasmid was created by moving the Rev element from the original one, increasing its resistance to RCL formulation [11].

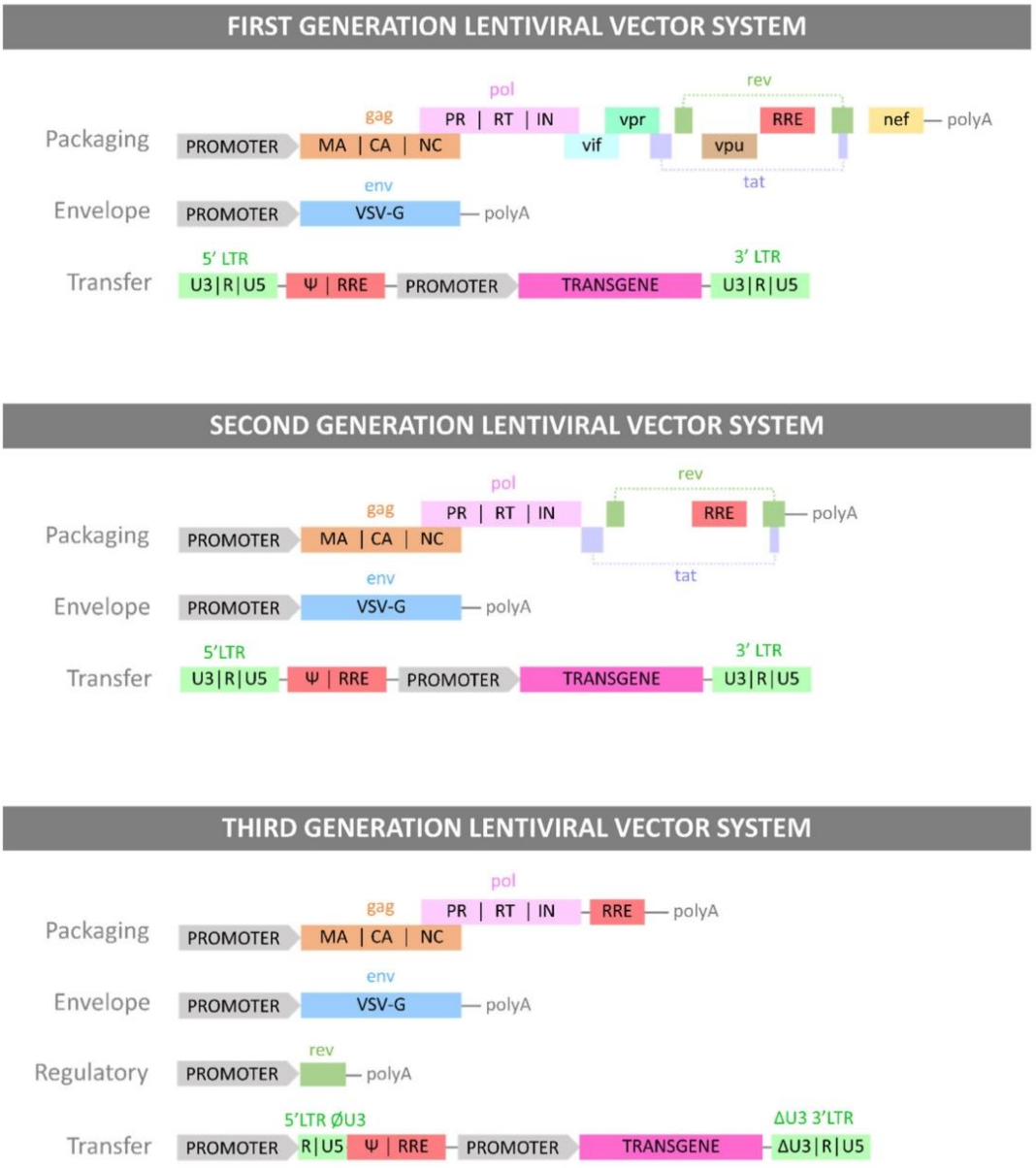

Fig. 2 The three generations of LVV plasmid systems [11].

Table 1. Components of lentiviral genome

Component	Abbreviation	Full name
Group Specific Antigen (Gag)	MA	Matrix
	CA	Capsid
	NC	Nucleocapsid
Polymerase (Pol)	PR	Protease
	RT	Reverse Transcriptase
	IN	Integrase
Envelope (Env)	VSV-G	Vesicular stomatitis virus G
Long Terminal Repeat (LTR)	ψ	Retroviral psi packaging element
	U3	Unique 3' region
	U5	Unique 5' region
	R	Repeat region
Other	Vif	Viral infectivity factor
	Vpr	Viral protein R
	Vpu	Viral protein U
	RRE	Rev response element
	Rev	Regulator of expression of viral protein
	Tat	Trans-activator of transcription
	Poly A	Poly-Adenine tail

3. Manufacturing Process of CAR-Ts using lentivirus vectors

The utilization of LVVs has attained a significant status in the propagation of CAR genes tailored for producing CAR-Ts. This is attributed to their established safety and efficacy, substantiated by various clinical studies [13]. To ensure the superiority of the maneuver, adherence to the Good Manufacturing Practice (GMP) criteria is fundamental in handling the relevant tools, technologies, and methods. The clinical production protocol is depicted in Fig. 3, where it can be reduced to three segments: lentiviral vector production, T-cell isolation and activation, and CAR-Ts production.

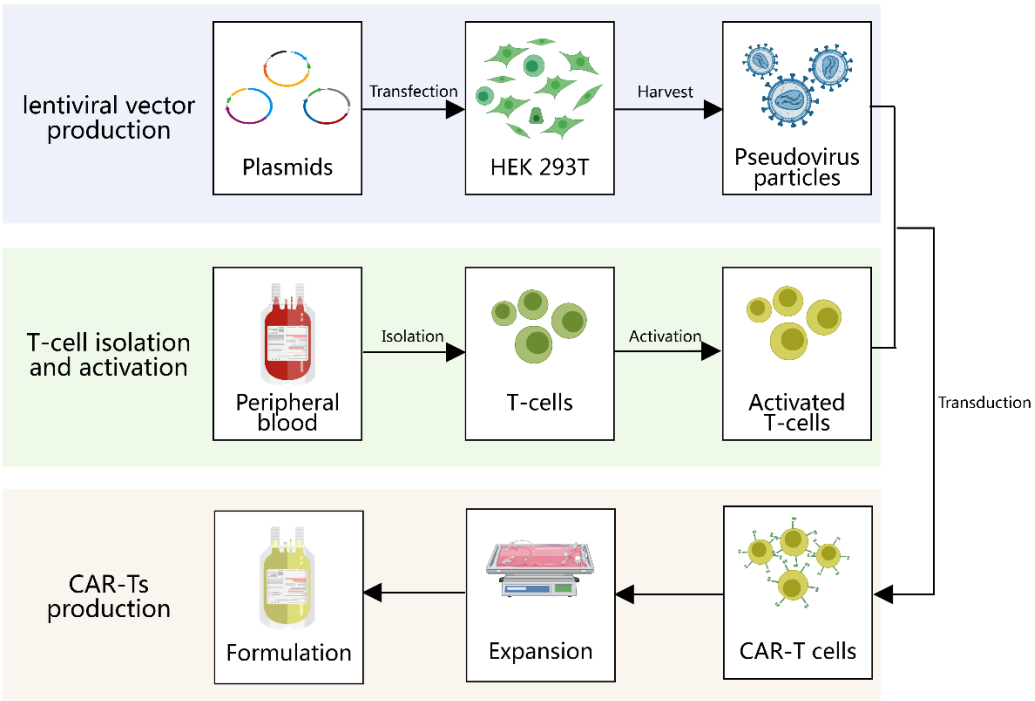

Fig. 3 Manufacturing process of CAR-Ts using LVVs
(Created with MedPeer: www.medpeer.cn)

3.1. Lentiviral Vector Production

To generate a lentiviral vector, it is necessary to design and produce plasmids that encode CAR structure and viral protein. Plasmids are usually expanded using a modified E.coli system, followed by extraction and purification processes, which effectively eliminate contaminants such as the host DNA, RNA, and endotoxin. Subsequently, mammalian Human Embryonic Kidney (HEK) 293T cells, which are the most popular immortalized cell lines to produce pseudovirus particles, are scaled for transfection and production of pseudovirus. Due to the adherent nature of HEK 293T, challenges in achieving high quantities are posed. Nonetheless, the problem is mitigated through the utilization of suspension cell culture techniques [14]. In addition, variant HEK 293T cell lines are being developed to address this issue [15]. The cells are then transfected with plasmid DNA (pDNA) by using polyethyleneimine or lipofectamine to generate pseudovirus. Then the lentiviral particles would release from the cell surface to the cell culture medium, obviating the need for cell lysis. Following the collection of the cell culture supernatant, several subsequent steps, including capturing, nuclease digestion, purification, and polishing are required to eliminate impurities from the complex heterogeneous system, ensuring the acquisition of a lentiviral vector buffer with high purity [11].

3.2. T-cell Isolation and Activation

Despite lentiviral vector production, T-cells are extracted from peripheral blood and activated for transduction. The initial step in this process involves the employment of density gradient centrifugation to excise red blood cells and platelets. In order to selectively enriched certain subsets of T lymphocytes, such as CD4, CD8, CD26, or CD62L T cells, antibody-conjugated magnetic beads are then added. Before plasmid transduction, isolated T cells must undergo activation. Activation can be achieved through several means, including the use of monoclonal antibodies and interleukins, magnetic beads coated with antibodies that prompt T-cell proliferation of memory, and artificial antigen-presenting cells [16].

3.3. CAR-Ts Production

After the activation of T cells, they become prepared for transduction with lentiviral vectors. Lentiviral vectors containing the CAR gene are introduced to the T cells, thereby facilitating the production of fully functional CAR-Ts. In order to obtain an adequate dose for patient treatment, the CAR-Ts undergo further cultivation and expansion. Subsequently, these cells are transferred from plates or flasks to bioreactors. As previously mentioned, antibody beads are used to activate T cells, but the beads tend to precipitate at the bottom of the system, resulting in diminished production of functional CAR-Ts. Therefore, the strategy of incorporating microbeads has been introduced to address this issue [17]. To regulate the ratio of CD4+ to CD8+ CAR-Ts, genetically engineered cells like K562 cells are employed. [18]. Once the desired quantity of CAR-Ts is achieved, they are formulated in an appropriate buffer for clinical application. Furthermore, the preservation of the viability and functionality of CAR-Ts is crucial during storage, and cryopreservation represents a viable method for achieving this objective [19].

4. Frontier Development of LVVs in CAR-Ts Production

4.1. Serum-free Suspension Lentiviral Vector Production

Currently, the high price of CAR-T therapy poses an essential obstacle to its widespread application, with prices ranging from US$320,000 to US$475,000 per dose, rendering it inaccessible to many patients [20]. The complicated nature of the manufacturing process and the production of LVVs for gene delivery to T cells contribute to this high cost. As a result, there has been a growing interest in strategies to reduce the expenses associated with CAR-T therapy and optimize the production procedure of CAR-Ts. One strategy is the production of serum-free suspension lentiviral vectors, which effectively reduces costs and mitigates the risk of contamination that could harm

patient health. Serum-free mediums typically contain essential supplements, including insulin, transferrin, selenium, glucose, etc. [21].

To compare the production of LVVs using serum-supplement monolayer and serum-free suspension culture, researchers conducted investigations using HEK293T cell line. The results showed that LVV production in serum-free suspension was significantly lower compared to serum-supplement monolayer. However, this issue was addressed and improved by modifying the transfection protocol. This involved using 1 mg of DNA per 10^6 cells, a cell concentration of 1×10^6 cells/mL, and a PEI: DNA ratio of 2.5:1. In addition, utilizing of 5mM sodium butyrate resulted in a notable enhancement in LVV production, with an average yield of 1.5×10^5 Tus/mL [22].

In another research, the efficiency of four commercially available serum-free cell culture media, namely BalanCD HEK293, Freestyle293, LV-MAX, and IVY, was assessed in terms of their ability to facilitate viral generation in suspension HEK293 cells. The transfection process involved the use of PEI 25K. It was observed that Freestyle293 exhibited the highest transfection efficiency at 50.1%, followed by 50% in IVY media [23].

Apart from HEK293 cells, SJ293TS cells were also utilized for LVV production in serum-free conditions. One novel approach reported in 2020 not only reduced the consumption of plasmid DNA but also resulted in an approximately 2-fold increase in virus titer and post-purification yields compared to serum-supplemented adherent HEK293T cell lines [24]. Furthermore, it was demonstrated that SJ293TS cells maintained high stability and could be continuously cultured for up to four months without a decline in LVV production ability.

4.2. Improving the Yield of LVVs

The production of lentiviral vectors is critical in manufacturing CAR-Ts, as the transduction step requires a sufficient quantity of LVVs. Therefore, it is imperative to enhance the titer of LVVs and identify suitable methods for large-scale production. Radhakrishnan et al. established a procedure that adheres to GMP to generate LVVs with high titers, enabling clinical presentation [25]. These LVVs were employed to create FRα-CAR-Ts. Remarkably, they achieved a physical titer of approximately 6×10^8 viral particles per cm^2 of cell culture surface. Considering a physical-to-functional titer ratio of 100:1, a total yield of around 10^9 infectious units (IFU) can be obtained from one T175 flask. Specifically, a cell density of 75000 cells/cm^2 and a DNA quantity of 2.5 μg/10^6 cells were found to produce the highest lentiviral titer. Furthermore, improvements in downstream processing techniques were made, such as optimizing the transfection agent, cell growth surface, clarity process for the elimination of cell debris, and the determination of the optimal timing for supernatant collection. In another study, a high-throughput microscale 96-deep well plate platform was developed for culturing HEK 293 cells and provided rapid condition screening to enhance the yield of LVVs [26]. Interestingly, it was observed that scaling down the culture volume did not significantly impact the concentration of produced LVVs, suggesting that the conditions identified using the microscale platform can be successfully applied to scale-up production. The final yields of LVVs achieved in this investigation reached 1.5×10^7 transducing units (TU) mL^{-1}.

4.3. In-vivo Generation of CAR-Ts Using Lentiviral Vectors

Despite the significant success of CAR-T therapy in numerous clinical trials, the manufacturing process typically requires several weeks and involves high costs. In addition, when T cells are extracted and expanded in vitro to obtain larger quantities, their lifespan and ability to proliferate in vivo are diminished [27]. Therefore, it is crucial to develop a strategy that can deliver LVVs in vivo, which may particularly benefit patients with aggressive diseases. To accomplish this, highly-specific LVVs are necessary. For one reason, the complex environment within the human body may impair transduction efficiency. For another, off-target transduction to single leukemic B cells can promote resistance to CAR-Ts [28]. Conventional pseudotyped LVVs with VSV-G, unfortunately, present significant broad tropisms and cannot efficiently target T cells in vivo [29].

To overcome this challenge, researchers have engineered novel envelope glycoprotein pseudotypes. For instance, the Nipah G glycoprotein has been introduced into LVV pseudotypes to specifically target CD8 T cells [30], while the measles virus (MV) hemagglutinin has been utilized to target CD4 T cells [31] for the production of human anti-CD19 CAR-Ts in humanized mouse models.

Furthermore, plasmids encoding anti-CD3 single-chain variable fragments (scFvs) like TR66 and HuM291 have been employed to confer targetability to CD3 T cells [32]. These engineered LVVs have demonstrated effective modification of T cells without prior activation in NSG mice [33].

Considering that LVV engineering for various targets is labor-intensive, adapters have been developed as a more rapid and flexible targeting strategy. A versatile adapter system for MV-LVs in vivo has been reported, wherein a mBio3-18E7-derived scFv is fused to the H protein, enabling binding to biotin through a specific linker. The biotinylated adapter can then target T cell through CD4 or CD8 [34]. Another study has designed a separate bispecific binder capable of binding to the mutant E2 glycoprotein on engineered Sindbis pseudotyped LVVs and CD3, thus facilitating the specific targeting of LVVs to T cells [27].

5. Conclusion

Lentiviral transduction is a prevalent technique employed in the manufacturing of CAR-Ts, given its robust and versatile gene delivery capability. At present, there exist three generations of LVVs, which offer reduced risks pertaining to the production of RCL. The process of CAR-Ts manufacturing using LVVs begins with the design and fabrication of plasmids, followed by the transfection of an appropriate cell line (usually HEK 293T) to generate pseudoviral particles. Subsequently, the LVVs infect activated T cells, thereby inducing the expression of CAR constructs. After expansion, sufficient quantities of CAR-Ts are generated for clinical use. To mitigate the high costs associated with manufacturing, several strategies have been developed to simplify the procedure, including the use of serum-free suspension lentiviral vectors to reduce expenses and contamination due to the utilization of fetal bovine serum (FBS). Furthermore, production conditions have been studied and improved to enhance LVV yields. Additionally, alternative candidates to viral vectors, such as virus-like particles, lipid nanoparticles, and synthetic polymer nanoparticles, have been demonstrated to possess diverse advantages. Nonetheless, further research and clinical trials are required to optimize the gene modification of T cells. In conclusion, this review provides an overview of the biological principles of LVVs and presents the manufacturing process for CAR-Ts while also highlighting the corresponding cutting-edge advancements, thereby offering prospects for future directions in LVV research.

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