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Review

Lentivirus for CAR-T and Other Cell Therapy Products: A Microbiological and Biosafety Perspective

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Abstract

Chimeric antigen receptor T cell (CAR-T) therapy is a significant and costly immunotherapy strategy that has provided benefits to many patients with cancer and autoimmune diseases. As of January 2025, only a few FDA- and NMPA-approved CAR-T therapies were available for clinical use, targeting CD19 or B cell maturation antigen (BCMA) on cancer cells. The manufacturing process for these approved CAR-T cells mainly relies on lentiviral vectors (LVVs) due to their high efficiency in transducing functional genes in both dividing and nondividing cells, as they have the unique ability to integrate into the genomes of those types of cells stably, and post-mitotic mammalian cells, a capability that ɣ-retroviruses do not possess to the same extent. This review summarizes the main CAR-T therapies derived from LVVs, the basic biological principles of HIV derived LVVs, the LV structure, capacities, and functions in biotechnology, a comparison between different vectors, the CAR T structure, and a summary of manufacturing processes, with an emphasis on the microbiological perspective for human and environmental biosafety. Additionally, we outline the latest developments in LVV technology, providing insights into the production of next-generation CAR-T therapies by using *in vivo* approaches.

Keywords: CAR-T; lentiviral vector; Serum-free suspension; *ex vivo* generation of CAR-T cells

1. Introduction

Lentivirus (LV), γ-retrovirus, and adeno-associated viruses (AAVs) are the most used viral vectors in various applications. [1–3] Those viral vectors have superior transduction efficiency. γ-retroviral vectors were the first viral vectors used for CD19-targeted CAR-T production, accounting for approximately one-fifth of clinical trials involving gene delivery. [2] AAVs have a relatively lower risk of toxicity; however, their limited package size (~50 kb) restricts their gene delivery capability. [3] Lentiviral vectors (LVVs) are the most versatile viral vectors for the expression of CAR. [1] 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, it can exert long-term transgene expression.[1] It has been reported that ≈94% of evaluable CAR-T products are prepared by viral vectors, with >50% mediated by LVVs. [4]

CAR-T cells are genetically modified T cells that express synthetic receptors on the cell surface to detect and eradicate cancer cells by identifying specific tumor antigens. Unlike T cells, CAR-T cells can recognize antigens on the surface of cancer cells without human major histocompatibility complex (MHC) molecules. [5] Therefore, CAR-T cells can distinguish a wider range of targets than

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natural T cells. When CAR-T cells bind to the targeted antigen, they are activated and function as "active drugs" that target and attack the tumor [2]. After the initial approval of the US Food and Drugs Administration (FDA) of the first two CAR-T therapies, Kymriah™ (*tisagenlecleucel*) for diffuse large B-cell lymphoma (DLBCL),[6–8] and Yescarta™ (*axicabtagene ciloleucel*) to treat acute lymphoblastic leukemia (ALL) respectively in 2017,[9,10] Terakus™ (*brexucabtagene autoleucel*) was approved for the treatment of adult patients with relapsed or refractory mantle cell lymphoma (MCL) in 2020,[11,12] followed by the approval of Abecma® (*idecabtagene vicleucel*) to treat adult patients with relapsed or refractory multiple myeloma (MM) after four or more prior lines of therapy, [13,14] and Breyanzi® (*lisocabtagene maraleucel*) to treat adult patients with large B-cell lymphoma (LBCL) both in 2021.[15,16] Lately, FDA-approved Carvykti™ (*ciltacabtagene autoleucel*) was also released for relapses and refractory MM in February 2022. [17,18] Finally, the Chinese National Medical Products Administration (NMPA) approved Fucaso® (*equecabtagene autoleucel*) in 2023 [19] and Carteyva® (*relmacabtagene autoleucel*) in 2024.[20] As shown in Table 1, most of the eight CAR-T therapies approved by the US and Chinese regulatory agencies utilize LVV as the gene delivery strategy, highlighting their widespread use. [21,22]

Table 1. Summary of [1]FDA- and [2]NMPA-approved CAR T cell therapies globally.[21,22].

Item	Drug name	Targeted gene	Delivery Strategy	Targeted malignancy	Country	Data source
1	**Kymriah™** (*tisagenlecleucel*) (Approved by FDA in 2017)	CD19	Lentiviral	• Patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia (ALL) that is refractory or in second or later relapse. • Adult patients with relapsed or refractory (r/r) large B-cell lymphoma after two or more lines of systemic therapy, including diffuse large B-cell lymphoma (DLBCL) not otherwise specified, high-grade B-cell lymphoma, and DLBCL arising from follicular lymphoma. • Adult patients with relapsed or refractory follicular lymphoma (FL) after two or more lines of systemic therapy.	USA	[6–8,23–25]
2	**Yescarta™** (*axicabtagene ciloleucel*) (Approved by FDA in 2017)	CD19	ɣ-retroviral	• Adult patients with large B-cell lymphoma that is refractory to first-line chemoimmunotherapy or that relapses within 12 months of first-line chemoimmunotherapy. • Adult patients with relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy, including DLBCL not otherwise specified, primary mediastinal large B-cell lymphoma, high-grade B-cell lymphoma, and DLBCL arising from follicular lymphoma. • Adult patients with relapsed or refractory FL after two or more lines of systemic therapy.	USA China	[9,10,26–29]
3	**Tecartus™** (*brexucabtagene autoleucel*) (Approved by FDA in 2020)	CD19		• Adult patients with relapsed or refractory mantle cell lymphoma (MCL). • Adult patients with relapsed or refractory B-cell precursor ALL.	USA	[11,12,30–32]
4	**Abecma®** (*idecabtagene vicleucel*) (Approved by FDA in 2021)	[3]BCMA	Lentiviral vector	• Adult patients with relapsed or refractory multiple myeloma (MM) after four or more prior lines of therapy, including an immunomodulatory agent, a	USA	[13,14,33,34]

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				proteasome inhibitor, and an anti-CD38 monoclonal antibody.		
5	**Breyanzi®** (*lisocabtagene maraleucel*) Approved by the FDA in 2021)	CD19	Lentiviral vector	• Adult patients with large B-cell lymphoma (LBCL), including DLBCL not otherwise specified (including DLBCL arising from indolent lymphoma), high-grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, and follicular lymphoma grade 3B, who have: • Refractory disease to first-line chemoimmunotherapy or relapse within 12 months of first-line chemoimmunotherapy; or refractory disease to first-line chemoimmunotherapy or relapse after first-line chemoimmunotherapy and are not eligible for hematopoietic stem cell transplantation (HSCT) due to comorbidities or age; or relapsed or refractory disease after two or more lines of systemic therapy.	USA	[15,16,35–37]
6	**Carvykti™** (*ciltacabtagene autoleucel*) (Approved by FDA in 2022)	[3]BCMA	Lentiviral vector	• Adult patients with relapsed or refractory multiple myeloma after four or more prior lines of therapy, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 monoclonal antibody.	USA China	[17,18,38–41]
7	**Fucaso®** (*equecabtagene autoleucel*) (Approved by China NMPA in 2023)	BCMA	Lentiviral vector	• It is indicated for the treatment of adults with relapsed or refractory multiple myeloma (RRMM) after three or more prior lines of therapy and progression, including a proteasome inhibitor and an immunomodulatory agent. It is an autologous cellular immunotherapy that involves integrating the chimeric antigen receptor (CAR) gene targeting B-cell maturation antigen (BCMA) into the patient's peripheral blood CD3-positive T cells using a lentiviral vector. Once reinfused into the patient's body, these modified T cells recognize the BCMA target on the surface of multiple myeloma cells and kill them, as a novel treatment option for patients with relapsed or refractory multiple myeloma.	China	[19]
8	**Carteyva®** (*relmacabtagene autoleucel*) (Approved by China NMPA in 2024)	CD19		• Treatment of adult patients with relapsed or refractory Mantle Cell Lymphoma (r/r MCL). "We are delighted to have a product that can deliver meaningful efficacy in this disease; nearly 70% of patients with r/r MCL have achieved complete remission after treatment with Carteyva®, and the overall safety data demonstrated that the treatment was generally well-tolerated".	China	[20]

[1] FDA: US Food and Drug Administration; [2] NMPA: China National Medical Products Administration; [3]BCMA: B cell maturation antigen.

The CAR-T therapies, first described in the late 20th century, have emerged as promising treatments for multiple cancers. [1–3] The CAR structure is the primary functional element of CAR-

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Ts, comprising four distinct domains: the ligand-binding domain, spacer element, transmembrane domain, and cytoplasmic domain.[4] Once the ligand-binding domain recognizes and binds to the antigen on the surface of the cancer cell, the signal is transmitted downstream, and the CAR-Ts are activated and stimulated to proliferate, release cytokines, and alter their metabolism. Furthermore, granzyme and perforin are released to destroy and digest cancer cells. [7]

In recent years, CAR technology has emerged and developed rapidly. CAR-T immunotherapy has shown impressive clinical success for refractory and relapsed (r/r) hematopoietic malignancies, including CD19+ leukemia and lymphoma and BCMA+ multiple myeloma. Several CAR-T products have been commercially approved worldwide for treating the above blood tumors. Motivated by the achievements made, researchers have expanded CAR technology from CAR-T to CAR-NK, CAR-CIK, and CAR-MΦ applications, and have employed CAR-engineered cell therapy for broader indications to treat aggressive diseases. [3,22,42]

In Abu Dhabi, United Arab Emirates, the Abu Dhabi Stem Cells Center (ADSCC) GMP laboratory has been producing and applying autologous clinical-grade CAR-T cells for the treatment of hematological malignancies since 2024, utilizing the CliniMacs Prodigy® platform (Miltenyi Biotec). The cells were stimulated and transduced with a lentiviral vector (Lentigen Technology), provided by Miltenyi Biotec, encoding a CAR protein targeting CD19. [43] Miltenyi CliniMACs Prodigy® is one of the newest technologies for CAR-T cell expansion. This system is currently utilized for stem cell enrichment and the preparation of virus-reactive T cells. [44] It is a technology system that combines a cell washer, a magnetic cell separation system, and a cell culture device in a closed, sterilized system. [45] Equipped with a flexible programming suite, the CliniMACs Prodigy technology enables us to fully integrate and automate the complex, multi-step CAR T cell processing and manufacturing procedures within the GMP lab.

In this review article, we referred to the biological principles of LVVs, a brief description of the CAR-Ts manufacturing platform using LVVs, the regulatory framework for biosafety and biosecurity in LVVs production facilities, and finally, the frontier development of lentiviral transduction techniques that may improve their efficiency and function to meet the growing demand for clinical application.

2. Basic Biology of the Human Immunodeficiency Virus-1 (HIV-1) LVVs.

2.1. What are LVVs?

The *Retroviridae* viral family is the origin of LVVs. These vectors can be derived from various viral sources, including feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV), and human immunodeficiency virus type 1 (HIV-1), which is the most used. [46,47] Within the *Retroviridae* family, LV is a genus of enveloped RNA viruses. These viruses have a spherical structure with an outer envelope, a matrix protein, a capsid, and a core that contains two identical RNA strands and an enzyme. LV, including AIDS-causing HIV, induces chronic and often fatal diseases in humans and other mammals. They are globally distributed and infect hosts like apes, cows, goats, horses, cats, and sheep. Known for their ability to integrate viral DNA into host genomes, they are highly efficient for gene delivery and can be inherited by the host's descendants. The virion's morphology reveals enveloped viruses, 80-100 nm in diameter, that are spherical or pleomorphic, with capsid cores that mature into a cylindrical or conical shape. The virus's surface appears rough because the envelope projections, or tiny spikes (approximately 8 nm), are dispersed evenly over the surface. [48] The virus is engineered to transfer up to 10 kb of genes to the host cell, resulting in the expression of encoded proteins. [14] Figure 1 illustrates a wild HIV-1 morphology (**A**) a genomic structure (**B**).

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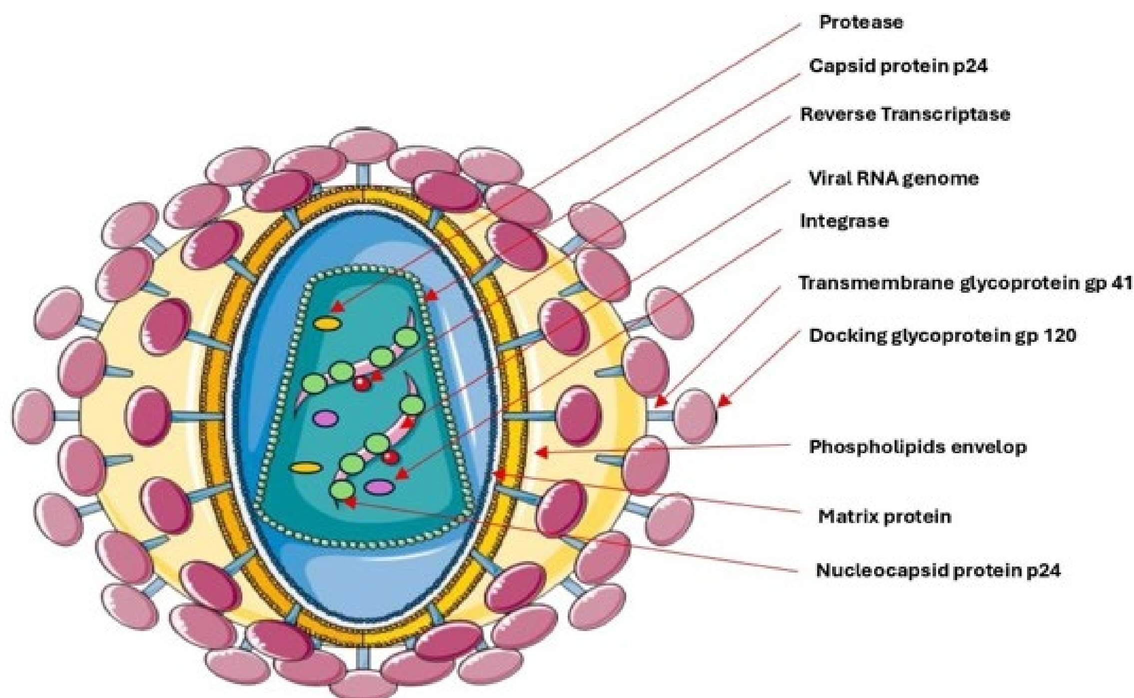
A)



B)

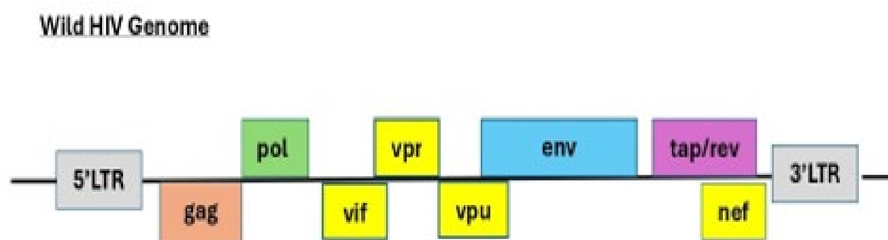


Figure 1. The human immunodeficiency virus type-1 (HIV-1) genome and the structure of the mature virion. (**A**) Particle structure of the virus. (**B**) Simplified schematic of the wild-type HIV-1 genome. [1][12].

A summary of the key genetic elements, their encoded proteins, and functional roles in the HIV-1 lentiviral vector system, as well as the design of modern self-inactivating (SIN) transfer vectors, along with the separation of functions across multiple plasmids for enhanced biosafety, is illustrated in Table 2 below.

Table 2. HIV genome and HIV-derived lentiviral vectors components: gene of origin, proteins encoded, functions, and abbreviations.

Component	Gene	Proteins Encoded	Functions in Wild Type HIV	Role in Recombinant Vector System	Abbreviation
Structural gene	gag	MA (matrix), CA (capsid), NC (nucleocapsid), p6	Structural core proteins: form the viral capsid and are essential for particle assembly and packaging.	Provided "*in trans*" by the "*packaging plasmid*". Forms the viral core	gag

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				that encapsulates the vector RNA	
Enzymatic gene	pol	RT (reverse transcriptase), IN (integrase), PR (protease)	Reverse transcription, integration, polyprotein cleavage	Provided "*in trans*" by the "*packaging plasmid*". Essential for creating an infectious vector particle	pol
Envelope gene	env	gp120, gp41 (envelope glycoproteins)	Mediates viral entry by binding to CD4 and CCR5/CXCR4 co-receptors on target cells	Deleted from the vector system. Replaced "*in trans*" by a heterologous envelope plasmid, most commonly "VSV-G", which confers broad tropism	env
		Vesicular Stomatitis Virus protein G		To support an extremely broad range of tropism	VSV-G
Regulatory element	RRE (in env RNA)	– (RNA motif, not protein)	Binding site for the Rev protein. Allows unspliced and partially spliced viral RNAs to be exported from the nucleus to the cytoplasm	Retained in the vector to enable nuclear export of the full-length vector RNA (especially important for genomes >4kb)	RRE
Regulatory gene	rev	Rev protein	RNA-binding protein that binds to RRE to shuttle unspliced RNAs from the nucleus to the cytoplasm	Provided "*in trans*" on a "*separate plasmid*" (in 3rd/4th gen systems) or with packaging genes. Essential for high-titer production of vectors with RRE.	rev
	tat	Tat protein	A key transcriptional activator that binds the TAR element to dramatically enhance transcription from the LTR.	Deleted. LTR in packaging plasmid is often replaced by a strong constitutive promoter (e.g., CMV).	tat
Accessory gene	nef	Nef protein	Immune evasion downmodulates CD4	Deleted	nef

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			and MHC-I, enhancing infectivity.		
	vif	Vif protein	Blocks APOBEC3G (antiviral restriction factor)	Deleted	vif
	vpr	Vpr protein	Cell cycle arrest, nuclear import of pre-integration complex	Deleted	vpr
	vpu	Vpu protein	CD4 degradation, virus release enhancement	Deleted	vpu
LTR (5′ and 3′)	U3	– (cis-DNA/RNA element)	Promoter/enhancer region (drives transcription)	In "*SIN vectors*", its U3 region is often deleted to be replaced by a specific promoter.	U3
	R	–	Repeat: transcription initiation and polyadenylation site	It drives transcription of the vector RNA.	R
	U5	–	Needed for reverse transcription and integration		U5
LTR (3′)	PolyA (in 3′ LTR)	–	Signals proper 3′ end processing and mRNA stability	In "*SIN vectors*", the U3 region is "deleted". This deletion is copied to the 5' LTR during reverse transcription, "*inactivating the viral promoter*" in the provirus	PolyA

Note: For LVV: Only **cis-acting sequences** are retained (LTRs, RRE, packaging signal Ψ, PolyA). Protein-coding regions (*gag, pol, env, tat, rev,* etc.) are supplied **in trans** from helper plasmids during vector production. The **U3 region is modified** in *self-inactivating (SIN)* vectors to eliminate unwanted promoter activity.

2.2. What Are the LV Structure, Capacities, and Functions in Biotechnology?

As a type of simple retrovirus, HIV-1-derived LV are capable of hijacking host-mediated machinery to sustain efficient nuclear import across the intact nuclear membrane. [49] This feature has allowed them to efficiently transduce nondividing and terminally differentiated cells (e.g., postmitotic neurons, hepatocytes, or macrophages) with superb efficiency.[50] The long-lasting effect of the viral transduction supports long-term production of the therapeutic gene-of-interest (thus providing permanent steady-state "dosing" after a single administration of the virus), which is essential for gene therapy applications. The LV genome comprises ~10.7 kb of single-stranded RNA (ssRNA) enclosed within a lipid-enriched spherical capsid measuring ~100 nm in diameter. The viral genome encodes both structural and enzymatic genes, including gag and pol. The polycistronic gag gene encodes three products, namely matrix (MA), capsid (CA), and nucleoproteins (NC). The polycistronic pol gene encodes three viral enzymes: reverse transcriptase (RT), protease (PR), and integrase (IN). HIV-derived LV is an enveloped virus that uses a glycoprotein envelope to attach and enter the host cell.

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Nevertheless, the creation of heterologous envelopes used for viral particle pseudo-typing was one of the main progresses in the field, allowing for the dramatic diversification and extension of transduction tropism. The host range of retroviral vectors including LVV can be expanded or altered by a process known as pseudotyping. Pseudotyped LVV consist of vector particles bearing glycoproteins (GPs) derived from other enveloped viruses. Such particles possess the tropism of the virus from which the GP was derived. For example, to exploit the natural neural tropism of rabies virus, vectors designed to target the central nervous system have been pseudotyped using rabies virus derived GPs. Among the first and still most widely used GPs for pseudotyping LVV is the vesicular stomatitis virus GP (VSV-G), due to the very broad tropism and stability of the resulting pseudotypes. Pseudotypes involving VSV-G have become effectively the standard for evaluating the efficiency of other pseudotypes. Additionally, supplementing viral particles with heterologous envelopes has positively impacted vector safety. [50][51] As mentioned above, LVs can be efficiently pseudotyped with a broad range of heterologous envelopes, thereby enabling broad viral tropism. For example, LV supplemented with Mokola virus (MV), Ross River virus (RRV), and Rabies virus (RV) demonstrated a strong preference for the transduction into neuronal cells.[51] However, the most common envelope used to pseudotype viral particles is that of vesicular stomatitis virus protein G (VSV-G). The envelope has been shown to support an extremely broad range of tropism, and as such, it is used for transduction into most cells and tissues. [50]

In addition to gag and pol, LV carry six supplementary genes: rev and tat, which are involved in viral transcription and export, respectively, and nef, vif, vpr, and vpu, which are involved in viral entry, assembly, replication, particle formation, and release. [52] 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. [16] 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, unlike 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.[18]

To date, there have been four generations of the LVV system derived from HIV; they are summarized in Figure 2. The core principle behind this generational classification is the progressive removal of accessory genes and the splitting of the remaining essential genes across multiple plasmids. This minimizes sequence overlap, making it increasingly improbable for a replication-competent lentivirus (RCL) to form through recombination. It is essential to note that the latter four accessory products are dispensable for vector production and can therefore be omitted from the packaging cassette of the vector (Figure 2A). The removal of these products has been shown to have a positive effect on the vector safety; importantly, their deletion also creates space for cloning larger inserts. [53–56] The first generation was released between 1996–1997 with 3 plasmids prove in-vivo, long-term expression: transfer + packaging (carrying many HIV genes) + envelope (often VSV-G). These papers established stable integration and expression in non-dividing cells and set the basic plasmid split [53]. Indeed, the second generation of the packaging system carried only the tat and rev genes (Figure 2B). [57] In 1998, the Self-inactivating (SIN) LTRs: 3′LTR ΔU3 copied to 5′LTR after reverse transcription provided LTRs transcriptionally inactive in the provirus; a major safety leap, and in the same year a third generation conditional packaging was created by rev moved to its own plasmid, tat no longer required when the transfer uses a heterologous promoter; overall system becomes 4 plasmids (transfer SIN + gag/pol + rev + env). This split greatly reduces RCL risk and becomes today’s clinical workhorse. [55]. The third generation of the packaging system lacks the tat gene, which is coincidental with the deletion of the endogenous promoter harbouring the tat-responsive element, TAR, at the U30 region of the LTRs (Figure 2C). Instead, full-length RNA of the

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virus is transcribed from the strong ubiquitous promoter derived from Rous sarcoma virus (RSV) or cytomegalovirus (CMV). It is a still the gold standard system for clinical applications. Further improvement in virus safety has been achieved with the development of the fourth-generation packaging plasmid, highlighted by the separation of the gag/pol and rev sequences into two distinct cassettes. The fourth generation of the packaging systems is the safest to date but still in experimental [55].

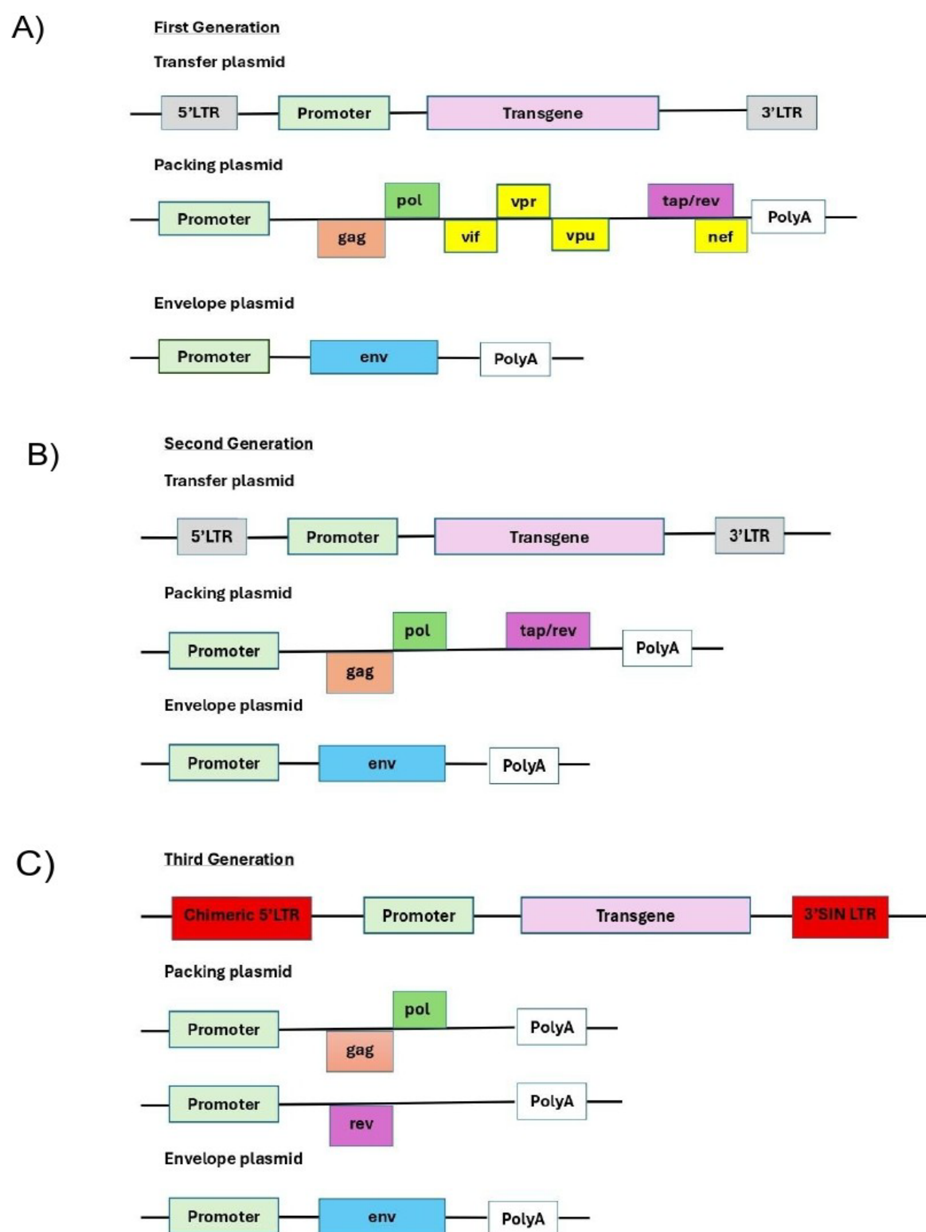

Figure 2. Schematic Structure of the First Three Generations of HIV-derived Lentiviral Systems.[12].

(A) *The first generation is composed by three plasmids: Transfer +* ***Packaging*** *+ Envelope. Full HIV-1 genome minus* ***env*** *in packaging; accessory genes present (****vif, vpr, vpu, nef****). Tat-dependent LTR; non-SIN; high homology to wild-type..*

(B) *The second generation is composed also by three plasmids: Transfer +* ***gag/pol + tat + rev*** *(packaging) + Envelope. The difference from the first is that accessory genes* are ***deleted*** (**Δvif**, **Δvpr**, **Δvpu**, **Δnef**); *often still non-SIN;* ***tat + rev*** *retained in packaging.*

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(C) *The third generation is composed by four plasmids: Transfer (SIN) + **gag/pol** + **rev** + Envelope. At a difference from previous generations **rev** is moved to a separate plasmid; **SIN LTRs (ΔU3)** standard; heterologous 5′ promoter; **cPPT/CTS** and **WPRE** commonly used.* [12]

It should be noted that the REV gene is present in all packaging systems, as its product, REV, plays a key role in exporting fully length and partially spliced viral RNA (vRNA) from the nucleus into the cytoplasm. [58] The advanced generations of the packaging plasmids also harbor a strong, heterologous poly-adenylation signal (poly-A), derived from the SV40 virus or bovine/human growth hormone (bGH/hGH). These potent poly-As enable high-level vRNA stability, and as such, their inclusion is advantageous for packaging and viral titers. [55][58] Moreover, the inclusion in the third generation of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the central polypurine tract (cPPT) in the viral transfer cassette has been shown to improve further vRNA stability, transcription efficiency, and overall viral titer. [59][60] Importantly, the above modifications significantly reduce the likelihood of recombination-competent retroviruses (RCR) appearing, which positively impacts viral safety characteristics.

Table 3 summarizes the comparison between the 4 generations of LVV and their key genomic features, plasmid system, safety profile, and primary use.

Table 3. Comparison of the Fourth Generation of HIV-derived Lentiviral Systems.

Generation	Key Genomic Feature	Plasmid System	Safety Profile	Primary Use
1st.	Full HIV-1 genome minus **env** in packaging; accessory genes present (**vif, vpr, vpu, nef**). Tat-dependent LTR; non-SIN; high homology to wild-type. [21]	**3-Plasmid:** Transfer + **Packaging** + Envelope.	**Low**	Basic research; historical.
2nd.	Accessory genes **deleted** (Δ**vif**, Δ**vpr**, Δ**vpu**, Δ**nef**); often still non-SIN; **tat + rev** retained in packaging.	**3-Plasmid:** Transfer + **gag/pol + tat + rev** (packaging) + Envelope.	**Medium**	In-vitro cell modification; preclinical.
3rd.	**rev** moved to a separate plasmid; **SIN LTRs (ΔU3)** standard; heterologous 5′ promoter; **cPPT/CTS** and **WPRE** commonly used.	**4-Plasmid:** Transfer (SIN) + **gag/pol + rev** + Envelope.	**High**	Gold standard for clinical applications (CAR-T, HSC).
4th.	Further HIV sequence minimization; **tat-independent** with heterologous promoters; possible insulators/extra safety modules; may **split gag from pol**.	**≥4-Plasmid** (extends 3rd-gen split; optional extra splits).	**Very High**	Cutting-edge trials/platforms where maximal safety is required.

2.3. The Comparison Between Viral Vectors

LVs have the unique ability to stably integrate into the genomes of dividing, nondividing, and post-mitotic mammalian cells, a capability that ɣ-retroviruses do not possess to the same extent. While adenoviruses can also transduce nondividing cells, they cannot integrate stably into the host genome and require significantly more time for design and preparation. Additionally, LVs are far less immunogenic than ɣ-retroviruses and adenoviruses, making them more suitable for various cell types and animal models.

In Table 4 the comparison highlights the differences between LVs, ɣ-retroviruses, and adenoviruses regarding genome integration ability, target cell types, immunogenicity, design and preparation time, application range, vector capacity, and stability.[1]

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Table 4. Comparison between lentivirus, ɣ retrovirus, and adenovirus vectors.[1].

Characteristic	Lentivirus (LV)	ɣ Retrovirus	Adenovirus (AAVs)
Genome Integration Ability	Stable integration into the host genome	Less stable integration compared to LV	Cannot integrate into the host genome
Cell Type Target	Dividing, non-dividing, and post-mitotic cells	Primarily dividing cells	Dividing and non-dividing cells
Immunogenicity	Low	Moderate	High
Design and Preparation Time	Short	Moderate	Long
Application Range	Broad, suitable for various cell types and animal models	Limited, mainly used in dividing cells	Generally used for short-term gene expression or vaccine development
Vector Capacity	Relatively small	Relatively small	Large
Stability	High	Moderate	High

2.4. LVs' Summary of Advantages: [48]

- High-efficiency gene delivery: LVs can efficiently deliver exogenous genes into target cells, including those that are difficult to transfect, such as primary and stem cells.
- Long-term gene expression: Once integrated into the host genome, LVs can achieve long-term, stable gene expression, a crucial aspect for treating chronic diseases.
- Broad host range: LVs can infect various cell types, including dividing and nondividing cells, expanding their range of applications.
- Low immunogenicity: Modified LVVs typically have low immunogenicity, reducing the risk of host immune responses.
- Safety: Modern LVVs have been genetically engineered to remove pathogenic genes, enhancing their safety.

2.5. Other Applications of LVs

LV packaging can also be crucial in the development of gene and cell therapies. [8] Based on the characteristics of the LV system, it may be used in the following applications:

- Basic research: In molecular and cell biology, LLVs are utilized for gene overexpression, gene knockout, and knock-in experiments. Gene knockout involves inactivating a gene by replacing it with an artificial piece of DNA. In contrast, knock-in experiments involve the insertion of a gene into a specific location in the genome. These techniques aid in the study of gene functions and disease mechanisms. Large-scale collaborative efforts are underway to use LVs to block the expression of specific genes using RNA interference technology in high-throughput formats. Conversely, LVVs are also employed to stably over-express certain genes, thus allowing researchers to examine the effects of increased gene expression in a model system. For example, gene editing technologies mediated by LV, such as CRISPR/Cas9, can repair or replace mutated genes.[12]
- Stable cell line construction: LV can be used to make stable cell lines in the same manner as standard retroviruses. The process involves infecting host cells with recombinant LLVs or pseudo-typed LVVs that carry selectable markers, such as the puromycin resistance gene. This gene confers antibiotic resistance to the infected host cells. When these antibiotics are added to the growth medium of the host cells, they kill any cells that have not incorporated the LV genome. [1]Those surviving cells can be expanded to create stable cell lines that include the lentiviral genome and harbor the genetic information encoded by it.[1] For instance, in vaccine development, LLVs can be vaccine carriers to express pathogen antigens, thereby inducing an immune response.[61] It is used in the development of HIV vaccines and other vaccines for infectious diseases. [14,16,62]

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3. Manufacturing CAR T Cells Ex Vivo

The procedure for manufacturing CAR-Ts *ex vivo* remains consistent despite various genetic modifications of T cells; however, it involves the patient's participation. Subjects approved for CAR T-cell therapy typically undergo the following treatment process, as illustrated in **Figure 3**.[46]

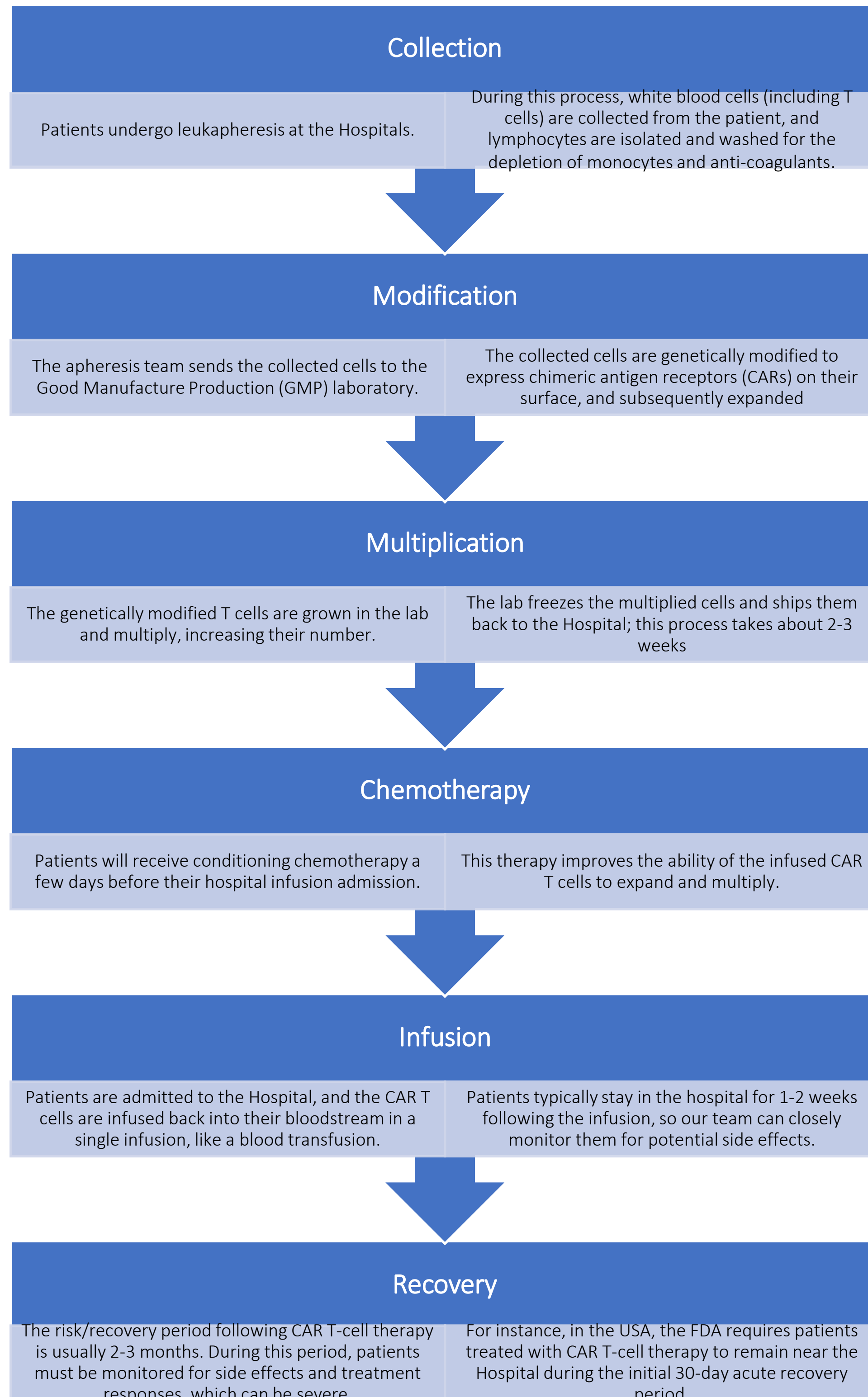

Figure 3. CAR T Patients process.[46] .

3.1. How Is the CAR-T Cell Structure?

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The CAR's primary structure comprises a tumor-antigen receptor and a signal transduction domain.[2] The tumor-antigen receptor recognizes specific tumor-associated antigens (TAAs), including proteins, glycoproteins, and other elements, while the signal transduction domain primarily enhances T-cell proliferation and differentiation. Diverse intracellular signal transduction domains characterize different generations of CAR structure. [3] CAR structures of four generations have been described.[4] The original CAR framework features an intracellular CD3ζ signaling module, which oversimplifies and conveys signals ineffectively.[7] Although the first-generation CARs can specifically recognize TTAs and enhance T cell anti-tumor activity, their therapeutic effect is unsatisfactory *in vivo* due to their decreased proliferation ability.[8] CAR's second generation, which integrates a costimulatory domain, such as CD28 or 4-1BB, with the CD3ζ molecule, has remarkably improved cell multiplication and reduced senescence. [10]

4. Risk Assessment While Working with LVs

HIV-derived LVV are powerful tools in genetic research and gene therapy applications. These vectors are derived from HIV but have been significantly modified to enhance safety while maintaining transduction efficiency. The evolution of vector systems has progressed through multiple generations as shown before. These advanced systems split viral components across multiple plasmids, eliminating essential pathogenic genes and incorporating self-inactivating (SIN) LTR configurations to minimize risks. Despite these safety improvements, working with LVV requires strict adherence to biosafety protocols to prevent laboratory-acquired infections and ensure patient safety in clinical applications. The fundamental biosafety concerns with HIV-derived LVV include their potential to generate replication-competent lentiviruses (RCL) through recombination events, the risk of insertional mutagenesis when integrating into the host genome, and the potential effects of the transgene products (e.g., oncogenes or toxins). Additionally, the common practice of pseudotyping with VSV-G expands cellular tropism, potentially increasing risks if exposure occurs. [52] These concerns necessitate rigorous containment measures, thorough risk assessment, and comprehensive personnel training to ensure safe handling practices across both laboratory and clinical settings.[63]

4.1. Modes of Transmission

LVs may be transmitted through skin penetration via puncture or absorption (from scratches, cuts, abrasions, dermatitis, or other lesions) and exposure to the eyes, nose, and mouth through mucous membranes.[18,63]

4.2. Containment Level

Working with LVV requires the use of Biological Safety Level 2+ (BSL-2+) practices and procedures. Biosafety Level 2+ (BSL-2+) is the commonly used term for laboratories where work with microorganisms is conducted, utilizing biosafety practices and procedures typically found in BSL-3 laboratories. Most research institutions still struggle to determine when to use this approach and which BSL-3 practices to employ, as BSL-2+ is not a recognized containment level. No standardized list of microorganisms, viral vectors, or research projects to be conducted within BSL-2+ environments. Each decision to use selected BSL-3 practices in a BSL-2 laboratory must be made via a risk assessment. [64] Examples of when BSL-2+ may be appropriate include viral vectors with gene inserts consisting of oncogenes or genes of unknown function. Second-generation LVVs have an increased risk of recombination, which can generate replication-competent LVs.

4.3. Project Review Process

The project review process is part of the risk assessment process.[65] This review includes several steps before work can be approved and commenced.[66] The laboratory supervisor or the Principal Investigator (PI) must complete and submit a project registration document to the Biosafety

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Officer (BSO). The document must clearly outline the project's purpose and detail the steps to be taken with the biohazardous material. Review and discuss the project registration document with the laboratory supervisor or PI, BSO, and, in some cases, selected Institutional Biosafety Committee (IBC) members with expertise in BSL-2+ research. For example, a virologist may be asked to review a project involving viral vectors. A suitable BSL-2 laboratory space should be proposed, and the BSL-3 practices should be outlined. IBC review and consensus must occur before the project's initiation. At the IBC meeting, the BSO outlines the proposed project and the BSL-3 practices for use in the BSL-2 laboratory space. The IBC members should agree on the appropriate BSL-3 practices for the proposed work and decide upon a suitable BSL-2 laboratory space. Risk communication and training must be conducted after IBC approval and before any work is performed in the laboratory. The BSO should review the required BSL-3 procedures with the laboratory supervisor or PI and his/her laboratory staff. Ideally, these should be written in the form of an SOP. Additionally, it is essential to review the laboratory space to ensure that the required BSL-2 elements are in place, including, but not limited to, biowaste containers, a sink with soap and paper towels, and certified biological safety cabinets (BSCs).[64]

4.4. Selection of a BSL-2+ Lab Space

BSL-2 laboratories are often large spaces occupied by many personnel working on diverse projects and sharing laboratory equipment. This scenario may not be conducive to adhering to BSL-3 practices. Dedicating a separate BSL-2 laboratory space to the project that requires BSL-3 practices allows other projects to maintain standard BSL-2 practices. Creating a BSL-2+ lab space means dedicating a smaller BSL-2 or "tissue culture" laboratory room to the project. It allows limited access to only those listed on the research protocol who have received the necessary training.[64]

5. Facility Considerations

The lab supervisor or the PI must designate a laboratory that fulfills the facility requirements outlined in the US CDC/NIH publications *Biosafety in Microbiological and Biomedical Laboratories*. [64] It should be an inner lab with two doors between the BSC and the hallway. Air must flow from the hallway to this lab (in the negative direction, to the lab). All air must be exhausted outside the building, not recirculated. An Environmental Health and Safety (EHS) officer [68] can evaluate the laboratory's negative pressure status.

5.1. Engineering Controls

The following safety equipment must be used when working with LVVs:[69]

- Certified Class II BSCs;
- Sealed centrifuge rotors and safety cups;
- Vacuum lines with an in-line HEPA filter and a primary and secondary vacuum flask containing a 10% bleach solution.

5.2. Examples of Modifications to BSL-3 Practices

One example of a potential modification to BSL-3 practices for use in a BSL-2+ space is the management of waste.[70] While decontamination within the immediate laboratory is preferable, removing materials from the decontamination facility is an option. All materials encountered with LVVs should be disinfected using a 1:10 bleach solution before disposal. Additionally, all work surfaces must be disinfected with a 1:10 bleach solution once work is completed and at the end of the workday. (Note: At least a 15-minute contact time is required for decontamination.)[69]

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5.3. Personal Protective Equipment (PPE)

When working with LVVs, the following personal protective equipment must be worn: gloves (consider double gloving depending on the procedures performed), a lab coat, goggles, and a face shield.[69]

5.4. Waste Disposal Procedures

Non-Sharp Waste—All cultures, stocks, and cell culture materials must be disinfected and autoclaved before being disposed of in a double-lined biohazard box. Sharps Waste, including needles, syringes, razors, scalpels, Pasteur pipettes, and tips, must be disposed of in an approved, puncture-resistant sharps container. Sharps containers must not be filled more than 2/3 of their capacity.[70]

5.5. Additional Considerations for Implementing BSL-2+

Consider whether it is appropriate for laboratory personnel to bring laboratory notebooks and portable electronic devices into and out of the BSL-2+ laboratory. The best practice is to prohibit this to avoid bringing contamination out of the laboratory, but provide provisions for information to be transmitted to the office through an electronic device dedicated for use within the BSL-2+ laboratory.[69]Alternatively, a procedure could be implemented to wipe down a laptop with a disinfectant wipe before leaving the lab. If the BSL-2 laboratory is to be renovated or built for a project that utilizes BSL-3 practices, it may be beneficial to incorporate some BSL-3 laboratory facilities for convenience. Examples include installing a hands-free or automatically operated sink for hand washing and locating an anteroom between the laboratory and external areas.[70]

As there is no "one size fits all" approach to implementing BSL-2+ practices, a risk assessment is crucial in determining whether BSL-3 practices are necessary in a BSL-2 laboratory facility and identifying the specific practices that will be required. Collaboration between the laboratory supervisor or PI, BSO, IBC, and laboratory personnel is crucial to a successful outcome. Modifying BSL-3 practices for work in a BSL-2 laboratory will depend on the work conducted, the risk assessment results, and your institution's IBC. [69]

5.6. Other Facility Infrastructure Requirements

The streamlining of workflows, enhancement of process robustness, and adoption of automated closed systems are encouraged to facilitate scalability and lower the cost of goods, while preserving the efficiency of CAR-T (CAR-NK) cell products. To achieve commercial-scale production of CAR-T (CAR-NK) cell therapies, end-to-end automation is desirable, including but not limited to fully automated closed systems and partially automated systems. Fully automated closed systems can be used in less stringent cleanrooms; however, the step of the final product should still be performed in an ISO 7 (Class 10,000, Clean C) cleanroom environment. (See Table 4).

Table 4. Cleanroom classification guidelines.

Cleanroom Standard	**Cleanroom Classification Guidelines**					
ISO 14644-1	Class 3	Class 4	Class 5	Class 6	Class 7	Class 8
EU GMP (at rest)	-	-	A/B	-	C	D
US Federal Standard 209F (replaced by ISO 14644 in 2011	1	-10	100	1,000	10,000	100,000

In case of cell isolation involving open manipulation, steps must be carried out in an ISO 5 (Class 100) BSC. The environment surrounding the BSCs must maintain aseptic processing operations for cellular therapies that are manipulated and manufactured under cGMPs; the environment surrounding the ISO 5 (Class 100) BSCs should be controlled and classified as an ISO 7 (Class 10,000,

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Clean C) clean room. ISO 5 (formerly Class 100) BSCs must comply with ISO 5 standards, which include a HEPA-filtered laminar airflow over the work area and a UV sterilization lamp as a minimum. [64]

6. Recommendations for Working with LVVs

6.1. Practices and Procedures while Working with LVVs

The BSO and the IBC specified the following additional practices and procedures. For instance, the laboratory must have limited access. According to signage posted on the door, only individuals listed in the project registration, the BSO, and select Environmental Health and Safety (EH&S) department members are permitted access. The laboratory supervisor, PI, BSO, or project personnel must escort the janitorial and maintenance staff to the laboratory.[70,71] Phone numbers for the laboratory supervisor, PI, and key laboratory staff are posted during an off-hour emergency. BSL-2 work is permitted in the BSL-2+ laboratory, provided that it is performed using the same BSL-3 practices as those required for the LV project.[68] The laboratory supervisor or PI developed an SOP detailing the work practices and procedures as required by the IBC, using a template provided by the BSO. The BSO reviewed the SOP, which is used for training additional project personnel. Signage for the laboratory door with the universal biohazard symbol includes the Laboratory supervisor or PI's name, a list of approved/trained project personnel, including others who may have approved access (BSO, EH&S personnel), materials in use (LV and human cells), emergency contacts, and phone numbers. The signage indicates that only approved/trained personnel may enter, and an approved/trained person must accompany visitors.[71]

Based on consultation with the institution's occupational health physician, no medical surveillance is required for laboratory members working on the project. Laboratory staff were instructed to contact the occupational health physician with any medical questions or concerns. Since human materials are used in the project, all project personnel were offered Hepatitis B vaccination in accordance with the *Occupational Safety and Health Administration's (OSHA) Bloodborne Pathogen and Hazard Communications Standards*. [68] Additionally, project personnel were provided with a review of the institution's procedure for immediately reporting all occupational injuries and illnesses.[69]

In addition to the two BSCs already in the BSL-2+ laboratory, a tabletop centrifuge, a microscope, two incubators, and a laptop computer were purchased and designated for the laboratory. The laptop transmits notes outside the laboratory, as notebooks cannot be taken in and out.[70]

The PPE consists of a disposable solid front gown with cuffed sleeves, safety glasses with side shields, and nitrile gloves. In the entry area of the laboratory, coat hooks are available, allowing gowns to be hung up for reuse if deemed non-contaminated. A set of hooks immediately outside the laboratory is available to hang cotton laboratory coats utilized for work in the main BSL-2 laboratory. Each researcher is required to bring a box of gloves into the BSL-2+ laboratory in the appropriate size. Sharps such as Pasteur pipettes and needles are prohibited in the BSL-2+ laboratory. Plasticware is substituted for glass, and plastic pipette tips are allowed. All work is conducted in the BSC, including loading and unloading centrifuge safety cups for the laboratory's tabletop centrifuge. [70]

Freshly prepared bleach solutions and 70% ethanol are available and utilized in the laboratory to disinfect surfaces and equipment. There is no autoclave in the BSL-2+ laboratory or the larger BSL-2 laboratory. While an autoclave located in another part of the building is used for media preparation, the institution utilizes a vendor's services to dispose of biomedical waste and sharps. [70]

The solid, non-sharp waste, including but not limited to plastic culture flasks and gloves, is collected within the BSC in a small red biohazard bag contained within a Nalgene container with a lid. When two-thirds full, the researcher removed the bag, tied it at the top with a rubber band, and placed it within a vendor-supplied large cardboard waste box lined with two red bags. When full, the box is taped, labeled, and placed immediately outside of the BSL-2+ laboratory for the vendor to remove from the facility and transport for off-site incineration.[70]

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The used pipette tips generated in the BSC are immediately put in a sharp plastic container located within the BSC. Liquid waste is treated with mercury-free bleach (1 to 9 parts liquid waste), allowed to sit for at least 30 minutes, and carefully disposed of via the sink. Materials in labeled secondary containers can be removed from the laboratory and moved to the main BSL-2 laboratory for storage in the -80°C freezer.[70] Additionally, fixed cells may be removed from the laboratory in a secondary container for cell sorting. A laboratory member must be appointed to serve as the BSL-2+ "manager" and oversee daily lab operations, including ensuring that adequate PPE and supplies, such as disinfectants, are available, monitoring conditions in the lab, including PPE usage, and reporting issues that may require retraining. The BSL-2+ manager coordinates with and accompanies the maintenance department and equipment vendors when access to the BSL-2+ laboratory is necessary.[70]

6.2. Special Handling Procedures

Cells exposed to LVVs may not be removed from the laboratory for experimental purposes unless inactivated by approved procedures. If culture needs to be aerated, it must be done slowly and in a manner that minimizes the potential for aerosol creation. This action must be carried out in class II BSCs. Pouring and pipetting samples must be done gently and slowly, and must be carried out in a Class II BSC.

Extra precautions must be taken when using sharps. Appropriate substitutes for sharp items must be used whenever they are available. Sharps (including needles and Pasteur pipettes) may not be used to work with LV-infected cell cultures or harvest virus pellets. Use plastic aspiration pipettes instead of glass Pasteur pipettes.[63] For aspiration, use a plastic vacuum flask with a second vacuum flask connected to it as a backup, using non-collapsible tubing that can withstand disinfection. Attach a hydrophobic filter and a HEPA filter (or a combination filter) to the second vacuum flask to prevent anything from being sucked into the house vacuum system. These three items must be connected to a series from the vacuum source in the hood or a vacuum pump.[63]

6.3. Impatient Design Rooms for CAR-T Application to Human Subjects

There must be a designated outpatient care area that protects the patient from contagious agents and allows for patient isolation, confidential examination, evaluation, and administration of intravenous fluids, medications, and blood products. For procedures performed in an ambulatory setting, a designated area with adequate space and design must be available to minimize the risk of microbial contamination. Provisions must be in place for prompt evaluation and treatment by an attending physician available 24/7.[72]

7. Emergency Procedures and Exposure Management

7.1. Exposure Response and First Aid Measures

Immediate response to potential exposures to LVV is crucial for minimizing risks. In the event of exposure, personnel should immediately:

(1) Flush mucous membranes (eyes, nose, mouth) with copious amounts of water for 15 minutes at an eyewash station;

(2) Wash exposed skin with soap and water for at least 15 minutes (5 minutes for intact skin); and

(3) for puncture wounds or cuts, encourage bleeding by gently squeezing the area while washing with soap and water.

After providing immediate first aid, the affected individual should seek medical evaluation promptly, even if the exposure seems minor. Medical providers should be informed about the specific nature of the LVV involved, including the method of generation, the transgene being expressed, and any other relevant details. [73]

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Post-exposure prophylaxis (PEP) with antiretroviral drugs may be considered for significant exposures to HIV-based LVV. Some guidelines recommend that physicians consider initiating a 7-day course of a nucleoside reverse transcriptase inhibitor (NRTI), such as tenofovir, and an integrase inhibitor, such as raltegravir, as soon as possible after exposure (within 72 hours). [73] The need for PEP should be determined through medical evaluation based on the type and extent of exposure. All exposure incidents must be reported to the supervisor and the institutional biosafety office immediately, with completion of appropriate incident report forms and follow-up as required by institutional policies.

7.2. Spill Response and Decontamination Procedures

Spill response procedures for LVV depend on the volume and location of the spill. For small spills (<1 liter) inside a biological safety cabinet, the sash should be closed and the cabinet allowed to operate for 15 minutes before beginning cleanup. For spills outside the BSC, personnel should immediately evacuate the area and allow 30 minutes for aerosols to settle before re-entering. Appropriate PPE should be done before cleanup, including double gloves, a lab coat, eye protection, and respiratory protection if significant aerosolization may have occurred. The spill cleanup process involves covering the area with absorbent material, applying an appropriate disinfectant (such as a 1:10 fresh bleach solution), and working from the perimeter toward the center to prevent the spread of contamination. After allowing 30 minutes of contact time, the materials can be carefully collected for disposal as biohazardous waste.[64]

Decontamination protocols for LVV require the use of appropriate disinfectants with demonstrated efficacy against enveloped viruses. For instance, the NIH guidelines recommend sodium hypochlorite (0.5%: use a 1:10 dilution of fresh bleach) with a minimum contact time of 20 minutes, although 5% phenol or a 70% ethanol/isopropanol solution is also effective. [73] All potentially contaminated materials must be decontaminated before being disposed of or reused. Work surfaces and equipment should be decontaminated after each use and at the end of each workday. Other guidelines emphasize the importance of using absorbent pads on work surfaces within biological safety cabinets to prevent spills from generating aerosols and to facilitate decontamination. [64,68,69]

8. Concluding Remarks

LLVs are widely used in cell gene therapy to treat genetic diseases, cancer, and other chronic diseases. These diseases include, but are not limited to, cystic fibrosis, sickle cell anemia, and various types of cancer. While LVs are primarily a research tool for introducing a gene product into in vitro systems or animal models, their applications in therapeutic development are significant, particularly in adoptive cell transfer therapeutics. These include CAR-T, CAR-NK, TCR-T, and TIL therapies, representing the primary use of LVs in this field.[74]

LLVs are opening new doors in regenerative medicine and tissue engineering. By effectively transducing stem cells, LLVs enable the genetic modification of these cells, offering a promising avenue for a better understanding and treatment of various diseases. [75]

Although widely used, shortages persist in LVV utilization in CAR-T establishment. However, several shortcomings exist that are hard to bypass for LVVs. First, in CAR gene integration into T cells, LVVs often undergo random integration into the cellular genome, which can potentially cause adverse effects on the host genome. It can lead to unintended gene silencing, overexpression, or genetic mutations, thereby increasing potential safety risks. [8]

Moreover, the limited transcriptional capacity of LVVs restricts the size and complexity of the CAR gene payloads, as well as the associated regulatory elements that can be accommodated. Lastly, the large-scale production of LVVs for clinical applications requires matching BSL2+ or BSL3 good manufacturing practice (GMP)-graded laboratory and manufacturing reagents, resulting in close-to-prohibitive manufacturing costs and regulatory hurdles. [67,68,74]

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Several methods can be utilized for gene modification of T cells, categorized into viral vectors and non-viral. However, these methods, including electroporation via the transposon system, are inefficient in achieving stable CAR gene expression, although the produced CAR-T cells exhibit faster cytotoxicity in vitro. This inefficiency highlights the need for safer and lower-cost virus-free gene delivery vectors, which are typically represented by transposon systems, CRISPR/Cas9 systems, and mRNA electroporation platforms.[59–61] Autologous CAR-T cells have demonstrated remarkable clinical outcomes, significantly altering the treatment of blood cancers. However, there are still issues that prevent patients from receiving CAR-T cell therapy. In addition to the efficacy and safety issues of CAR-T therapy mentioned in the previous sections, the high cost, complex process, and lengthy waiting time of approximately 3 weeks required for manufacturing personalized T cells are also factors that hinder patients' access to treatment [76]. Consequently, to overcome these obstacles, the development of universal allogeneic CAR-T cells (also known as "off-the-shelf" CAR-T cells) and other CARs using alternative effector cells is underway.[77]

Various alternative engineering approaches and sourcing strategies have recently been developed for generating CAR-engineered cells. Given the successful applications of CAR-T therapy in oncology, developing additional strategies with new technologies and improved ease of operation to reduce costs and increase accessibility is warranted. Continuous advances in broadening cell sources and engineering approaches have revealed new ways to improve the supply of CAR-immune cells and simplify the manufacturing of CAR products.[77] The diverse sourcing strategies, encompassing autologous, donor-derived, third-party, and off-the-shelf cellular products, have unveiled a spectrum of cellular reservoirs with distinct attributes and potential. These strategies have expanded the repertoire of therapeutic candidates and addressed limitations associated with cell availability and functionality. Moreover, ingenious engineering approaches have propelled the optimization of CAR-based immunotherapies. Techniques such as genome editing, synthetic biology, and multi-gene integration have enabled the tailoring of immune cells with enhanced persistence, specificity, and safety profiles. Concurrently, advancements in modular CAR designs, incorporation of costimulatory domains, and switchable CAR systems have fine-tuned the therapeutic response and mitigated adverse events, underscoring the remarkable progress achieved in refining CAR-engineered immune cells.[78]

The ability of LVVs to generate CAR-T cells *in vivo* has garnered considerable attention, as it could eliminate the need for *ex vivo* isolation and activation of T cells, thereby reducing both the cost and time required for in vitro production.[79]

Further investigations will be warranted to comprehensively elucidate the long-term safety and efficacy of these advanced therapies. The development of standardized protocols for sourcing, engineering, and characterizing CAR-modified immune cells will be instrumental in ensuring reproducibility and facilitating regulatory approval.[80]

Biosafety practices for HIV-derived LVVs continue to evolve as vector technology advances and our understanding of potential risks improves. The current standards emphasize evidence-based approaches that focus on actual risks rather than predetermined prescriptions, allowing for more flexible and effective safety protocols. The international harmonization of biosafety guidelines facilitates global collaboration in LVV research and therapy development. As these technologies increasingly move into clinical applications, the integration of GMP standards with traditional biosafety practices creates a comprehensive framework for ensuring safety, from the laboratory bench to the patient's bedside. [64]

Emerging challenges in LVVs biosafety include the growing use of CRISPR/Cas9 systems delivered via LVV, which necessitate enhanced containment due to their potential for genotoxicity. The scale-up of production for clinical applications also presents novel biosafety considerations that differ from research-scale work. Future developments are likely to include improved vector systems with even greater safety profiles, such as vectors with targeted integration systems that reduce the risks of insertional mutagenesis. Continuous personnel education and training remain essential components of an effective biosafety program, as human factors continue to be the most significant

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variable in preventing laboratory accidents and exposures. Through diligent application of current guidelines and adaptive response to new challenges, the scientific community can continue to harness the power of LVV technology while maintaining the highest standards of safety for both laboratory personnel and patients.

Enhanced and collaborative efforts between medical centers, the biotechnology industry, pharmaceutical companies, and nanotechnology institutes are poised to expedite the translation of these cutting-edge approaches into transformative clinical interventions, such as the *in vivo* generation of CAR-T cells, potentially reshaping the landscape of cancer and other immune-related diseases. As we navigate these frontiers, an exciting era of precision immunotherapy emerges, promising personalized, potent, and durable treatments for patients in need. This potential inspires us to continue pushing the boundaries of scientific research.

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Abbreviations

The following abbreviations are used in this manuscript:

4-1BB or CD137	A surface glycoprotein that belongs to the tumor necrosis factor receptor family
AAVs	Adeno-associated virus
ALL	Acute lymphoblastic leukemia
BSCs	Biological Safety Cabinets
BSL-2+	Biological Safety Level 2+
BSL-3	Biological Safety Level 3
BSO	Biosafety Office
BCMA	Anti-B cell maturation antigen
CA	Capsid
CAR-CIK	CAR-cytokine-induced killer cell
CAR-MΦ	CAR-macrophage
CAR-NK	CAR-natural killer cell
CAR-T	Cell chimeric antigen receptor T cell

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CD19	Cluster of differentiation 19 .
CD28	Cluster of differentiation 28.
CD3ζ	Accessory signaling molecule
CRISPR	Clustered regularly interspaced palindromic repeats
DLBCL	Diffuse large B-cell lymphoma.
Env	Envelop
EHS	Environmental Health and Safety
EIAV	Equine infectious anemia virus
FDA	Food and Drug Administration (USA)
FIV	Feline immunodeficiency virus
FL	Follicular lymphoma
Gag	Group-specific antigen
GMP	Good manufacturing practice
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
IN	Integrase
IBC	Institutional Biosafety Committee
kb	Kilo bytes
KI	Knocking-In
KO	Knocking Out
LTR	Long terminal repeat
LV	Lentivirus
LVVs	Lentivirus vectors
MA	Matrix
MCL	Mantle cell lymphoma
MM	Multiple myeloma
NC	Nucleocapsid
NMPA	National Medical Products and Drug Administration (PR China)
PPE	Personal Protective Equipment
PA	Phosphatidic Acid
PI	Principal Investigator
Pol	Polymerase
Poly A	Poly-Adenine tail
PR	Protease
R	Repeat region
RCL	Replication-competent lentivirus
Rev	Regulator of the expression of viral protein
RNA	Ribonucleic acid
RRE	Rev response element
RT	Reverse transcriptase
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SOP	Standard Operating Procedure
SIV	Simian immunodeficiency virus
TAA	Tumor-associated antigens
Tat	Trans-activator of transcription

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U3	Unique 3′ region
U5	Unique 5′ region
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus G
Ψ	Retroviral psi packaging element

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