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Institut Pasteur-TheraVectys Joint Lab, Virology Department, 28 rue du Dr. Roux, Paris F-75015, France

Min-Wen Ku, Ph.D, Post-Doctorate

Pierre Charneau, Ph.D, Head of the Unit

Laleh Majlessi, Ph.D, Associate Professor

*Contact: Laleh Majlessi: laleh.majlessi@pasteur.fr, Phone +33 1 40 61 32 68

ABSTRACT (199 words)

Introduction: Lentiviral vectors have emerged as powerful vectors for vaccination, due to their high efficiency to transduce dendritic cells and to induce long-lasting humoral immunity, $CD8^+$ T cells, and effective protection in numerous preclinical animal models of infection and oncology.

Areas covered: Here, we reviewed the literature, highlighting the relevance of lentiviral vectors in vaccinology. We recapitulated both their virological and immunological aspects of lentiviral vectors. We compared lentiviral vectors to the gold standard viral vaccine vectors, i.e., adenoviral vectors, and updated the latest results in lentiviral vector-based vaccination in preclinical models.

Expert opinion: Lentiviral vectors are non-replicative, negligibly inflammatory, and not targets of pre-existing immunity in human populations. These are major characteristics to consider in vaccine development. The potential of lentiviral vectors to transduce non-dividing cells, including dendritic cells, is determinant in their strong immunogenicity. Notably, lentiviral vectors can be engineered to target antigen expression to specific host cells. The very weak inflammatory properties of these vectors allow their use in mucosal vaccination, with particular interest in infectious diseases that affect the lungs or brain, including COVID-19. Recent results in various preclinical models have reinforced the interest of these vectors in prophylaxis against infectious diseases and in onco-immunotherapy.

KEYWORDS

Antigen Expression *in vivo*, Humoral Immunity, Lentiviral Vectors, Long-Lasting Immune Memory, Non-Integrating Lentiviral Vectors, Transduction, T-Cell Immunity, Vaccination.

Article highlights

- Lentiviral vectors have emerged as a particularly powerful vaccinal platform, as they exhibit a combined capacity to induce both strong and long-lasting T-cell and humoral immunity.
- Lentiviral vectors are not targets of pre-existing vector-specific immunity in human populations.
- Non-integrating versions of lentiviral vectors are highly immunogenic and circumvent the potential genotoxicity associated with the use of integrating vectors.
- The exclusion of all structural and functional HIV genes from the lentiviral genome ensures that the resulting vector is replication defective and only expresses the transgene of interest.
- Pseudotyping of lentiviral vectors with the heterologous Vesicular Stomatitis Virus envelope Glycoprotein (VSV-G) confers them with broad tropism for various cell types, including dendritic cells.
- The potential of lentiviral vectors to transduce non-dividing antigen presenting cells is a favorable characteristic for vaccine development.
- Lentiviral vectors can be engineered to transductionally or transcriptionally target antigen expression to specific host tissue/cells.
- Lentiviral vectors are non-cytopathic and very weakly inflammatory and can therefore be used for mucosal vaccination via the nasal route, with a particular interest in infectious diseases that affect the lungs or brain.
- Recent results in preclinical models have reinforced the relevance of these vectors in both prophylactic vaccination and onco-immunotherapy.

1. Introduction

Recombinant vector-based vaccine platforms rely on engineered bacteria or viruses for immunization. The concept behind using recombinant vectors is to exploit the natural infectivity and immunological properties of live viruses to elicit excellent immune responses and long-lasting immunity against heterologous antigens. A number of bacterial vectors, such as *Mycobacterium bovis* BCG, *Listeria monocytogenes*, *Salmonellae spp* and *Shigellae spp*, have been shown to effectively induce both humoral and cell-mediated immunity in animal models. Although essential virulence factors have been deleted from these bacterial vectors, the possibility of competition with indigenous flora, permeant colonization of the gastrointestinal tract, unintentional horizontal gene transfer, and genetic reversion have raised safety concerns about using such vectored vaccines [1]. Among other important heterologous antigen carriers are viral vectors, engineered to remove virulence factors for reasons of safety. Numerous viral vectors have been used for vaccination, including vectors derived from adenoviruses, retroviruses, vaccinia viruses, poxviruses, alphaviruses, and lentiviruses. Viral vectors can be divided into those that are replicative and non-replicative. Non-replicative viruses have been the most widely tested in clinical trials, due to concerns over safety. Similar to bacterial vectors, viral vectors induce strong humoral and cellular immunity. However, only a low dose is required for vaccination with the best viral vectors, which compensates for their high production cost [2]. One of the major drawbacks of the vast majority of viral vectors is the pre-existing vector immunity in human populations. Therefore, viral vectors for vaccination must be selected with care, taking into account their virological, immunological, and epidemiological characteristics [3].

Viral vectors for vaccination have been derived from poxviruses, alphaviruses, and adenoviruses [4-7]. Poxviruses, including Modified Vaccinia virus Ankara (MVA), canarypox virus (ALVAC), and New York attenuated Vaccinia virus (NYVAC), were the first viral vectors to be evaluated in clinical trials [8]. The use of poxvirus-based vectors has been complicated due to their limited ability to induce memory T cells and the high prevalence of pre-existing anti-vector immunity in human populations [9,10]. The use of alphavirus-based vectors is also limited because they induce strong transgene expression, leading to high toxicity for the host cells [10]. Compared to poxvirus-based vectors, adenoviral vectors trigger stronger T-cell responses, but these vectors, and especially those based on human adenoviruses, are targets of highly prevalent pre-existing adaptive immunity which reduces their persistence in the host organism and thus decreases their immunogenicity [11]. Increasing the administered dose of an adenoviral vector can improve immunogenicity, but can also cause serious “grade 3” adverse events, as recently demonstrated in a phase 2 trial of an Ad5-vectored COVID-19 vaccine [12]. Although such pre-existing immunity can be circumvented by using animal-derived serotypes, there is evidence suggesting that such serotypes are less immunogenic and protective than Ad5 [13,14].

Among viral vectors, lentiviral vectors (LVs) have emerged as a particularly powerful platform for vaccination purposes. LVs exhibit several advantages over other viral vectors, including their combined capacity to induce both strong and long-lasting T-cell immunity and antibody responses, without being inflammatory or the target of pre-existing vector-specific immunity [15-19]. LV components, such as the envelope and capsid proteins, can prime vector-specific immunity [20]. However, pre-existing immunity against the LV envelope is rarely found in human populations because LVs are predominantly pseudotyped with the heterologous Vesicular Stomatitis Virus Glycoprotein (VSV-G) envelope, to which the human population has been barely exposed [21]. Pre-existing immunity against the LV capsid is also rare, because infections with lentiviruses are less prevalent than those caused by other viruses commonly used as vectors, such as adenoviruses [22,23]. LVs can be divided into integrating (ILVs) and non-integrating (NILVs) categories. ILVs are the vector of choice for gene therapy, whereas NILVs are preferred for vaccination. Both ILVs and NILVs are capable of transducing dividing and non-dividing cells, notably dendritic cells (DCs) [24-26]. LVs are non-replicative, which means that they only support one round of host cell infection. The ability of LVs to transduce Antigen Presenting Cells (APCs) ensures antigen expression throughout the life span of these cells, for instance 5-7 days post-immunization for plasmacytoid DCs [27]. With respect to vector persistence, it must be considered that promoters may play a role, as strong promoters that result in higher antigen expression may also trigger more robust immune responses, in turn capable of eliminating transduced cells that harbor the vector-specific DNA [28]. We showed that ILV harboring a β2-microglobulin promoter remained detectable in mice from a few days to a maximum of three months post-intramuscular immunization, as quantified by a highly sensitive qPCR assay, detecting vector-specific DNA [29]. Others showed that NILVs harboring the cytomegalovirus (CMV) promoter, introduced via a single intramuscular injection in rhesus macaques, persisted for at least six months post-immunization at the site of injection [30]. Similar results were obtained with NILV harboring the CMV promoter in mice in which vector-specific DNA was only detected at the injection site, i.e., the muscles, but not at systemic sites, including the spleen and large intestine [31]. Hence, antigen expression and vector persistence vary on a case-by-case basis, depending on: (i) the route of vaccination, (ii) the promoter, (iii) the type of cell/tissue, and (iv) type of vector used [32-36]. Similar to adenoviral vectors, LVs are non-replicative and non-cytopathic vectors but unlike adenoviral vectors, LVs are negligibly inflammatory, which largely contributes to their safety and which paves the way for their potential use in mucosal immunization. Overall, these traits make the use of LVs a plausible opportunity for further vaccine vector development suitable for various clinical applications.

2. Methods

We performed a literature review in PubMed using the following keywords: “vaccine”, “immunization”, “viral vectors”, “lentiviral vectors”, “safety”, “antibody”, “immunology”, and “T-cell vaccines”. Inclusion criteria for manuscript selection were that they: (i) were written in English, (ii) were relevant to

viral vectors, (iii) were relevant to LVs, and (iv) had a publication dates between 1991 and February 2021.

3. Results

In total, 119 original articles and 40 reviews were selected to extract essential information on the virological, immunological, and vaccinological aspects of LVs. Various features of LVs were also compared to those of the gold standard adenoviral vectors.

3.1. Development of LVs

Lentiviruses are a subclass of retroviruses, best known for their ability to perform reverse transcription of their single-stranded RNA genome to a double-stranded DNA. Unlike other retroviruses, lentiviruses can infect both dividing and non-dividing cells, providing the possibility to target cell types that are not infectable by other viral vectors [37]. Based on these characteristics, the use of Human Immunodeficiency Virus (HIV)-1 was proposed as the blueprint for LV development [38]. Today, most of the LVs are derived from HIV-1, HIV-2, and non-human primate lentiviruses, such as Simian Immunodeficiency Virus (SIV), whereas a minority are derived from Feline Immunodeficiency Virus (FIV) and Equine Infectious Anemia Virus (EIAV) [39].

The HIV-1 life cycle and its function rely on three essential genes: *gag*, *pol*, and *env*. The *gag* gene encodes a structural protein, *pol* encodes a set of enzymes required for reverse transcription and integration into DNA, and *env* encodes for the viral surface protein. In addition to these genes, the HIV-1 genome also contains regulatory *tat* and *rev* genes, as well as *vif, vpr, vpu, and nef* accessory genes. These viral genes are flanked by Long-Terminal Repeats (LTRs) consisting of "U3", "R", and "U5". The LTRs are essential for transcription and integration of the viral genome. The transcriptional control elements, such as the promoter and enhancer, are situated in the U3 region of the LTRs [40]. The HIV-1 genome also contains: (i) the packaging signal Ψ, necessary for the recruitment of the viral genome for packaging into the budding viral particle, (ii) the central PolyPurine Tract (cPPT) and Central Termination Sequence (CTS), necessary for "DNA flap" formation and nuclear translocation, and (iii) the Rev Response Element (RRE), important for unspliced RNA transport to the cytoplasm (Figure 1A) [40]. The cPPT and CTS cis-acting elements are unique to LVs, operating coordinately to form a triple-stranded DNA structure known as a "DNA flap", which is indispensable for the nuclear import process [41]. The absence or mutation of cPPT significantly decreases the infectivity of LVs, resulting in the accumulation of linear DNA at the vicinity of the nuclear membrane. As detailed below, the development of recombinant LVs from the pathogenic HIV-1 has been accompanied by several challenges, including the generation of replication-defective LVs and the expansion of the tropism beyond lymphocytes and myeloid cells [42].

To produce a replication-defective LV, only viral structural and functional genes imperative for proper LV function are included. These genes are physically separated onto three distinct plasmids encoding for: (i) packaging, (ii) envelope, both acting in trans, and (iii) the cis-acting elements (necessary for entry, reverse transcription, nuclear import, and integration), which are included in the expression cassette of the LV RNA. These cis-acting elements are the Ψ packaging signal, cPPT/CTS, and the RRE, and are flanked by two LTRs (Figure 1B) [43]. The exclusion of structural and functional genes from the LV genome ensures that the resulting viral particles are replication defective. An additional safety feature has been introduced into LVs by further deleting the promoter enhancer sequence situated in the U3 region of the 3' LTR (ΔU3 LTR) expression cassette, forming the self-inactivated (SIN) vector. The ΔU3 LTR is duplicated and transferred to the 5' LTR of the pro-viral DNA during reverse transcription, thereby abolishing the transcriptional unit in the LTR (Figure 2). This deletion in the 3' LTR also prevents the activation of the promoter, located nearby to the integration site of the vector [43].

To expand the host cell tropism of LVs, the natural HIV-1 envelope has been substituted with VSV-G. The HIV-1 envelope shows restrictive tropism towards lymphocytes and myeloid cells, hence precluding the transduction of other cell types. By contrast, VSV-G shows broad tropism, infecting a wide range of cells, including APCs [21]. VSV-G pseudotyping also increases LV stability and provides the possibility to concentrate the vector for applications that require high viral particle titers [44].

3.2. Non-Integrating LVs

The integrating nature of viral vectors is a double-edged sword; beneficial for long-term transgene expression, it is potentially detrimental with the possibility of inducing oncogenesis. One serious adverse effect of viral vector integration was observed in a clinical trial using a Murine Leukemia Virus (MLV)-based retroviral vector to correct X-linked Severe Combined Immunodeficiency Disease (SCID). The insertional mutagenesis caused by MLV, near the *LMO-2* proto-oncogene led to malignancy in several participants in the clinical trial [45,46]. Unlike MLV, to date, no serious adverse effects have been observed with LV-mediated clinical studies [47]. Detailed profiling of the integration sites of LV and MLV has been performed to better understand vector safety. These studies revealed a non-random integration profile for both vectors, with LVs preferentially integrating near transcriptional units, whereas MLV display a bias towards transcriptional start sites [47]. LVs also exhibit additional criteria for integration site selection, such as favoring recognition sites of cellular factors, i.e., Lens Epithelium-Derived Growth Factor (LEDGF/p75), Barrier-to-Autointegration Factor (BAF), and High-Mobility Group (HMG), which interact with the pre-integration complex of LVs [47,48]. In comparison, MLV was found to cluster preferentially near the enhancer sequence of proto-oncogenes [49]. These studies showed that the integration sites of LVs do not favor the activation of proto-oncogenes and that they are a safer choice for clinical use.

Despite many studies demonstrating the excellent safety profile of ILVs, the concern of insertional mutagenesis prompted the development of NILVs. NILVs carry a missense amino acid in the catalytic triad of the integrase, that prevents the integration of viral DNA into the host chromosome. The most commonly reported mutations affecting the catalytic triad are at the D64, D116, and E152 mutation sites [50]. Without integration, the viral DNA remains in an episomal form, bearing either one or two LTRs, both equally effective in gene expression. The lack of integration signifies the eventual loss of the transgene during cell division, which affects the overall transgene level [51]. However, in non-dividing cells, the transgene expression remains comparable for ILV and NILV, due to the fact that the transgene is not diluted out by cell division, as shown in muscles and DCs [52,53]. The lack of integration does not hamper the induction of an effective immune response and only requires adjustment of the vector dose [17]. A comparison of ILVs and NILVs with various antigens in preclinical models showed that with 10 times more NILV the same immunization efficiency can be achieved with 10 times more NILV as with ILV; a dose of 1×10^8 Transduction Unit (TU)/animal is optimal for NILV, versus 1×10^7 TU/animal for ILV [54,55].

3.3. Advantages of LVs as vaccine vectors

The capability of LV to transduce non-dividing cells, and notably DCs and macrophages, is a favorable characteristic for vaccine development. The ability to efficiently replicate in non-dividing cells is due to the fact that the genome of lentiviruses can be imported into the nucleus, independent of mitosis. This is a distinctive property of LVs, as other retroviral vectors transduce only dividing cells because they are unable to cross the nuclear membrane, except during cell division [56]. Taking advantage of their active nuclear import by lentiviruses, LVs are also used as a gene delivery vectors to target non-dividing cells such as neurons and glia, for genetic correction [57].

Another advantage of LVs is the quasi absence of pre-existing immunity against them in the human populations, which is linked to their: (i) pseudotyping with VSV-G, an envelope glycoprotein to which humans are rarely exposed to and (ii) the low prevalence of lentivirus infection in humans. The presence of pre-existing humoral vector immunity can limit the number of transduced cells, impeding APC transduction, antigen expression, and thus induction of immune responses against the encoded antigens. The presence of pre-existing T-cell vector immunity may rapidly eliminate transduced APCs expressing the antigen. Several studies using viral vectors have reported the negative impact of pre-existing immunity on the induction of cellular and humoral responses. This is, particularly, a limitation for the initial administration of Ad5 and herpes simplex virus type 1 vectors, which have high seroprevalence in the human population [45]. In vaccination, multi-dose administration of vectors is important to reinforce the immune response. However, envelope-specific neutralizing antibodies triggered during the initial LV injection would likely blunt repeated administration of LVs with the same envelope glycoprotein.

However, anti-envelope immunity can be overcome by pseudotyping LV with heterologous VSV-G serotypes, without impeding the efficacy of the vector, as demonstrated previously for ILVs and NILVs [15,17,30,58,59].

LVs can accommodate large transgenes of up to in the range of 5 to 7 kb, which further extends the scope of their application, for example in the generation of multi-antigenic vaccines [60]. Large transgenes exceeding 10 kb have been reported [60,61]. However, the accommodation of large transgenes may come at the cost of lower functional viral titers, as previous studies showed a semi-logarithmic reduction of 3 to 4 folds in functional LV titer per kb increase in genome size [60,62]. Aside from these major advantages, LVs are negligibly inflammatory and induce only minute levels of phenotypic and functional DC maturation *in vitro* and *in vivo* [63] and our unpublished data. This property largely contributes to the safety of these vectors and makes their use possible in mucosal applications for vaccination [54,55].

3.4. Limitations of LV

Large-scale production and purification of viral vectors are often the bottlenecks to successful gene therapies and vaccination development. Each viral vector presents its own sets of manufacturing problems, and LVs are no exception to this [64-66]. The upstream process of LV production involves the transfection of plasmids into packaging cells to produce vector particles. Laboratory-scale LV production is achieved via transiently co-transfection of HEK293T cells, which is economically and technically challenging to scale up for large- or industrial-scale production. Several factors that can limit the productivity of LVs in upstream large-scale production are the adherent nature of the HEK293T cell line and the cytotoxicity incurred by the VSV-G envelope. The adherent HEK293T cell line is traditionally cultured in a T-flask, HyperFlask or multilayer cell factories [67,68]. Although these cell-culture methods are sufficient for clinical-scale LV production, they are economically not viable for large-scale production. To increase cell density for industrial LV production, fixed bed bioreactors, such as iCELLis Nano, iCELLis 500, and Scale-X Hydro, have been used [69,70]. The LV titer in TU using these bioreactors is comparable to the current standard of LV production via adherent cells. Using adherent cell lines for LV production can be costly due to the large amount of disposable materials required, such as flasks and medium. This issue has been circumvented by modifying or adapting variants of HEK293T to suspension culture conditions [71-74]. Current LV production relies on transient transfection of three or four plasmids to provide the building blocks the LV. The production of LV is a cellular process of shedding the viral vector into the media, which lasts only a few days. For large-scale LV production, this classical production method is not cost-effective, due to the large amount of transfection reagents needed and the exhaustion of the producer cells after one cycle of production [64]. Hence, establishing a packaging cell line that expresses some or all of the plasmids of LVs would ease production and enables the harvesting of several supernatants from a single production cycle [64]. However, the cytotoxic VSV-

G envelope does not allow its constitutive expression, and had hampered the establishment of stable packaging cell lines. Instead of using VSV-G, several groups have established a stable cell line with gamma retrovirus RD144 or MLV envelope to overcome the cytotoxicity of VSV-G [75-77]. These constitutive stable cell lines are capable of producing LV titer in the range of $1 – 5 \times 10^6$ TU/mL, and can continuously produce LVs for several months. As an alternative, an inducible packaging cell line expressing VSV-G has been developed, but these cell lines can only produce LVs for a few days [78].

LV supernatants contain contaminants, from the serum that is used for cell culture, the plasmid DNA for transient transfection, and cell-derived proteins. Therefore, the downstream processing of LVs is required to further concentrate and purify the LV before clinical use. The major obstacle during the downstream processing of LVs has been maintaining the bona fide conditions of the LV particles. The low stability of LV particles poses a tremendous challenge to the downstream processes, as LVs are sensitive to environmental changes, such as temperature, pH, ionic strength, and shear stress due to the fragility of the membrane envelope. The recovery of functional LV after purification is typically between 30 and 80%, depending on the type of column and the pH and ionic strength of the buffers [79].

Large-scale production of LV has evolved significantly due to the increase in demand for LV-based products. However, the recovery yields are still far from meeting the current need. Improvements made to the LV production pipeline, such as adaptation of the packaging cell line to suspension culture, can help to increase productivity and reduce the overall production cost. However, much work still needs to be done to achieve a continuous LV production process, otherwise hampered by the cytotoxicity of VSV-G. The downstream processing of LVs using current technological advances is challenging due to the intrinsic fragility of LV. Further transition into a new LV production pipeline, e.g., suspension cells, can overcome specific problems but will add on additional hurdles to the downstream processing of LVs [79].

3.5. APC targeting approaches

Recombinant viral vectors generally show broad specificity and transduce multiple cell types. In vaccine applications, DCs, with their unique ability to activate naïve T cells, are the ideal target for antigen delivery [80,81]. Taking into consideration the growing potential of LVs as a vehicle for T-cell eliciting vaccines, the development of LVs capable of transducing or redirecting expression in APCs *in vivo* could improve the safety and efficacy of LV vaccination. Here, we discuss the current advances in improving LV vaccinal vector targeting of APCs.

3.5.1. Transductional targeting

The pantropism of VSV-G-pseudotyped LVs results from the widespread expression of the VSV-G receptor, Low Density Lipoprotein (LDL) receptor, and the heparan sulfate proteoglycan attachment factor (Figure 3A) [21,82,83]. With the goal of minimizing off-target effects and improving safety,

multiple strategies based on alternative engineered glycoprotein have been described to redirect LV to APC-specific cell surface receptors [80,81,84]. One strategy has been to use an ectopic hemagglutinin glycoprotein, such as Measles Virus Glycoprotein (MVG), which confers tropism towards cells expressing CD46, Signaling Lymphocytic Activation Molecule (SLAM), and DC-SIGN (DC-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin). The SLAM receptor is constitutively expressed on T and B cells, monocytes, and DCs, while DC-SIGN is specifically expressed on DC. Therefore, MVG can be used to target LV towards DC (Figure 3B) [85,86]. The MVG-pseudotyped LV relies on fusion at the plasma membrane for cell entry rather than endocytosis, conferring an advantage over VSV-G-pseudotyped LVs. However, a potential drawback of the use of MVG-pseudotyped LVs is the pre-existing immunity against measles virus in the human populations [87].

Another strategy to direct LVs to APCs can be achieved via pseudotyping with Sindbis Virus envelope Glycoprotein (SVG) following rational domain mutagenesis [88,89]. The standard laboratory-adapted SVG confers tropism towards cells expressing heparan sulfate proteoglycan or DC-SIGN. Taking into advantage the physical separation of the two receptor binding sites on SVG, selective mutations have been introduced to the heparan sulfate binding site, while leaving intact the DC-SIGN binding site. The mutated SVG thus selectively binds to the DC surface protein via DC-SIGN but not to other cell types (Figure 3C). *In vivo* subcutaneous injection of LV pseudotyped with mutated SVG demonstrated enhanced DC transduction, which was accompanied by the induction of strong antigen-specific immune responses [88,89].

Chimeric envelope glycoproteins can also be modified to contain covalently-conjugated ligands or antibodies. This approach of using chimeric LV envelope glycoproteins has been used to couple MVG to a single-chain antibody, scFv, specific to Major Histocompatibility Complex class II (MHC-II) α chain to direct the LV and thus its transduction into DCs (Figure 3D) [81,90]. An LV pseudotyped with a fusion of this scFv and MVG resulted in effective T-cell responses, albeit lower in magnitude and quality than those of VSV-G-pseudotyped LV. The reduced immunogenicity induced by LV pseudotyped with MVG-scFv fusion can be explained by the lower transduction efficiency and reduced stability of the vector [91]. The use of chimeric proteins has been shown to facilitate retargeting of the vector to specific moieties but nonetheless, remains difficult, as such chimeric envelope glycoproteins often compromise the stability and immunogenicity of the vaccine vectors [92,93].

An alternative strategy has been developed by using the nanobody display technology, for directing LVs towards APCs. Nanobodies, single monomeric variable antibody domains devoid of light chains, and similar to a conventional antibodies, are able to bind to specific antigens [94]. A nanobody specific to DC2.1 and DC1.8 surface DC receptors was incorporated, together with a fusogenic but binding-defective VSV-G, to the surface of LVs (Figure 3E). The resulting LV was thus specifically targeted to DCs to

which it fused via the action of the mutated VSV-G. The use of such nanobody-engineered LVs demonstrated the feasibility of selective APC transduction and allowed the induction of an effective immune response, but again to a lesser extent than conventional VSV-G-pseudotyped LVs [84,85].

3.5.2. Transcriptional targeting

Another strategy for to program LVs for selective expression in APCs is via the insertion of tissue-specific promoters in the LV expression cassette. The most commonly used promoters in LVs are strong and constitutive in nature and include CMV, Spleen Focus-Forming Virus (SFFV), and human Phospho-Glycerate Kinase (PGK) promoters [95]. The nonselective feature of these promoters has significant shortcomings, especially affecting the safety of the vectors. These promoters are more prone to inactivation than cell-specific promoters, due to increased methylation at the CpG site of promoter enhancer sequences or viral LTR promoters [96]. They are also subjected to cytokine-induced inactivation following a strong immune activation [97]. Therefore, restrictive promoters have been explored to drive LV-mediated transgene expression in APCs.

An LV harboring the MHC-II-specific human HLA-DRα promoter induced restricted antigen expression in MHC-II$^+$ APCs, but failed to stimulate antigen-specific T-cell responses [35,98]. Indeed, immunization with this vector induced DC maturation, which led to down-regulation of the HLA-DRα promoter. Under these conditions, reduced antigen expression by mature DCs and selective antigen expression/presentation, primarily by immature DCs led to immune unresponsiveness and, seemingly, to T-cell anergy [35]. Another DC-specific promoter is the dectin-2 promoter, which is notably active in cells of the myeloid lineage. Incorporation of the dectin-2 promoter in the LV successfully restricted the transgene expression into APCs. An LV encoding for the melanoma antigen NY-ESO-1 under the transcriptional regulation of the dectin-2 promoter induced robust antigen-specific T-cell responses [99].

We recently reported the use of a LV harboring the human β2-microglobulin promoter, which contains highly conserved cis-regulatory elements, i.e., Interferon (IFN)-Stimulated Response Elements (ISREs), and SXY modules [29]. ISREs are the binding site for the IFN family of regulatory factors, while SXY modules interact with a multiprotein complex (Figure 4). These mediators transactivate the and are tightly regulated by immune mediators, including cytokines, which are upregulated in immune cells [100,101]. In addition, the human β2-microglobulin promoter contains minimal proximal enhancers [29]. Intramuscular immunization of mice with this LV led to highly efficient *in vivo* transgene expression by CD11b$^+$ CD8$^-$ myeloid, CD11b$^-$ CD8$^+$ lymphoid and plasmacytoid DC subsets. In this framework, the in vivo transgene expression lasted at least seven days post-immunization and was accompanied by the induction of long-term memory CD8$^+$ T-cell responses and complete efficacy in the immune eradication of large solid tumors expressing the model antigen [29].

3.6. Mechanisms of T-cell induction by LVs

As mentioned above, LVs induces strong antigen-specific cytotoxic T-cell responses due to their marked ability to transduce DCs [25,26]. LVs can transduce murine plasmacytoid, myeloid, lymphoid, or bone-marrow-derived DCs and human plasmacytoid, myeloid, or monocyte-derived DCs [26,102,103]. In humans, the plasmacytoid DC subset is the least and the monocyte-derived DC the most susceptible to LV transduction. However, the permissiveness of monocyte-derived DCs to LVs decreases as they differentiate [104]. The mechanism underlying the strong induction of the cytotoxic T-cell response induction by LV immunization is yet to be unraveled. As LVs are RNA viral vectors, much attention has been focused on possible innate single-stranded RNA sensing mechanisms in DCs following their interaction with LVs. The LV RNA genome was reported to stimulate intracellular innate pathways via the Toll-Like Receptor (TLR) 7 in human plasmacytoid DCs, resulting in the production of IFN-I and TNF-α, which in turn activated bystander myeloid DCs [104,105]. However, the impact on the induction of the T-cell response was not assessed in these studies. In mice, myeloid DCs produce IFN-I and TNF-α following interaction with LVs and through TLR3 and TLR7 signaling. In this study, mice deficient for TLR3 and TLR7 signaling showed weaker T-cell responses than their wild-type counterparts [102]. TLR7 is probably not the only pathway to trigger IFN-I production, because a TLR7 antagonist was not sufficient to prevent IFN-I induction upon LV transduction, suggesting the presence of alternative pathways. However, it was shown that IFN-I was not critical for LV-mediated T-cell activation, which was further confirmed by the use of IFNAR KO mice [63,103]. Recently, we also demonstrated that conditional deletion of IFNAR in $CD11c^+$ cells in vivo does not affect the induction of the T-cell response LVs (our unpublished data).

Conversely, a deficiency of IFN-I was demonstrated to increase the transduction efficiency of LVs in the liver, therefore increasing the gene expression for a longer duration [103]. This ability to mediate long-term gene transfer is critical in the context of gene therapy but not vaccination. One explanation is that in the context of vaccination, a transient but adequate transgene expression is sufficient for the induction of a high-quality T-cell response. In this context, it is still a challenge to determine the level and duration of transgene expression needed for a high-quality T-cell response in LV immunization, as the concept of "one size fits all" transgene expression may not be applicable in T-cell induction due to differences in the nature of the epitopes. Another study aiming to decipher the mechanism of immune induction by LVs showed that only conventional DCs but not plasmacytoid DCs, were required for T-cell induction in a murine model, and that the $CD8^+$-T cell induction was independent of the TLRs, Myeloid differentiation primary response 88 (MyD88) adaptor, Interferon Regulatory Factor (IRF), Retinoic acid Induced Gene I (RIG-I), and Stimulator of Interferon Genes (STING) signaling pathways. Only the blockade of the central NF-κB signaling pathway in $CD11c^+$ DCs inhibited the CTL induction [63]. In summary, these results should be evaluated with care, as LV production in the laboratory setting is often prone to contamination from producer cells and DNA/RNA, resulting in activation of the innate sensing pathways

[106]. In addition, the experimental conditions such as different multiplicities of infection, LV preparations, doses, and immunization routes can also lead to different outcomes in T-cell induction.

3.7. Comparison of LVs to adenoviral vectors

Adenoviruses are non-enveloped icosahedral viruses that contain a linear double-stranded DNA genome. There are 57 various serotypes of human adenoviruses, classified into species A to G with distinct tropism and receptors to target host cells. Human adenovirus subgroup C, and in particular Ad5, is by far the best characterized and widely used vector for clinical applications. Adenoviral vectors, and notably Ad5, are still very attractive vectors for gene delivery applications for several reasons. They (i) infect a variety of dividing and quiescent cells, with their main tropism directed to epithelial cells, fibroblasts, hepatocytes and endothelial cells, (ii) exhibit a minimal risk of insertional mutagenesis, as the genome of Ad5 remains as episomal elements after entering the host cell nucleus, (iii) are highly stable and can be produced at very high titers, due to their non-enveloped nature, (iv) are replication-defective, and (v) accept large DNA payloads of up to 35 kb [107]. Despite these advantages, Ad5 poorly infects certain cell types and tissues, including brain tissue, skeletal muscles, hematopoietic cells and DCs, which are important targets for gene therapy and vaccination [108]. However, the viral tropism of adenoviral vectors can be modified to target other cell types or tissues by incorporating peptide ligands within their surface protein or by pseudotyping with surface proteins of non-human adenovirus species [109].

The major drawback of adenoviral vaccination vectors is the pre-existing anti-adenoviral immunity in the human populations. More than 80 % of the human population has been pre-exposed to at least one serotype of human adenoviruses and has developed adenoviral serotype-specific immunity. Adenoviral-specific neutralizing antibodies are directed against the capsid and hexon proteins of the viral structure. In addition, both $CD4^+$ and $CD8^+$ T cells specific to adenoviruses are found in pre-exposed individuals. Such pre-existing humoral immunity interferes with uptake of the adenoviral vector, while the pre-existing cell-mediated immunity can dampen the efficacy of adenoviral-mediated gene transfer, thus shortening the duration of transgene expression [22,107]. Ad5-specific antibodies can opsonize the vector, inducing uptake of these complexes via Fc receptors by macrophages, DCs, and neutrophils, which results in rapid vector clearance and decreased tissue transduction, triggering intense inflammatory responses [110,111]. Even in individuals without pre-existing adenoviral immunity, the first administration of adenoviral vectors induces specific humoral and cellular immune responses. The repeated administration of the same vector for prime-boost or distinct vaccination purposes leads to the same issues as for pre-existing natural immunity. To circumvent the problem of pre-existing adenoviral immunity in humans, chimpanzee adenovirus-based vectors have been developed [112]. In addition to their property of being culturable in human cell lines, they also have a low seroprevalence in the human populations, hence significantly decreasing the effect of pre-existing immunity. Although serum

neutralizing antibodies against chimpanzee adenoviruses are detectable in only 0 to 4% of human populations in Europe and the United States, the prevalence can be significant, i.e., up to 20% in human populations in developing countries, including sub-Saharan Africa [112].

The Ad5 vector is highly inflammatory and can trigger a series of innate immune signaling pathways that result in substantial secretion of proinflammatory cytokines, such as IL-1β, IL-6, IL-12, IFN-γ, and TNF-α [22]. Ad5 vectors bind to complement proteins C4 and C4-binding protein and activate the complement system, platelet aggregation, and the secretion of inflammatory cytokines [113,114]. The Ad5 capsid interacts with TLR2, and the Ad5 DNA is sensed by TLR9, which activates the NFκB and IRFs pathways, resulting in the production of numerous pro-inflammatory cytokines and chemokines [115]. Ad5 double stranded DNA also activates the inflammasome [116]. Such induction of proinflammatory cytokines after Ad5 administration leads to a robust humoral and cellular immune response, but can sometimes also cause drastic adverse effects. A study aimed to evaluate the toxicity of systemic high-dose Ad5 administration showed the response to be lethal when 2×10^{11} active viral particles (vp) were administrated intravenously to mice with pre-existing Ad5 immunity established by previous intramuscular injection [117]. Similarly, high doses of Ad5 (i.e., $> 10^{13}$ vp per kg), are also lethal for larger animals [22]. Intravenous administration of a high dose of Ad5, i.e., 6×10^{11} vp/kg, caused death in a volunteer participant during a phase I gene therapy clinical trial, due to the triggering of a massive immune response and multiorgan failures as a result of high-dose administration and pre-existing immunity. Importantly, the activation of innate immunity by adenoviral vectors is independent of the transduction because inactive vector particles also interact with the receptors of the innate immunity [110]. Due to the need for repeated vector administration for gene therapy, the use of the old generation of Ad5 in gene therapy is facing a sharp decline due to serious adverse effects, such as cellular toxicity and organ failure, observed in multiple clinical trials [22]. These safety concerns have been studied extensively and addressed by the development of a new generation of adenoviral vectors that are less toxic and immunogenic [22].

Adenoviral vectors have been explored for use in vaccination due to their ability to induce a robust immune response. These vectors have reached the clinical stage and have shown promising results in the induction of antibody and $CD8^+$ T-cell responses to resolve infections with viruses, other intracellular pathogens and cancer cells. During the Ebola outbreak, three adenoviral-based vaccine approaches were explored: (i) the chimpanzee adenovirus type 3 encoding the Ebola virus glycoprotein (ChAd3-ZEBOV1) from GlaxoSmithKline, (ii) a prime-boost regimen combining human adenovirus type 26 (Ad26-ZEBOV) and an MVA vector encoding the vector EBOV, Sudan virus, and Marburg virus glycoproteins and Tai Forest virus nucleoprotein (MVA-BN-Filo2) from Johnson & Johnson, and (iii) Ad5 expressing the Ebola virus makona variant glycoprotein from the Chinese Federal Agency (FDA). These vaccines were developed in a short time and progressed rapidly into phase I clinical trials [118]. Among them, Ad26-

ZEBOV/MVA-BN-Filo2 showed the presence of EBOV glycoprotein–specific IgG in 80 % of the participants with limited adverse events. Hence, this candidate vaccine further progressed into phase II and III clinical trials (NCT04028349) [119]. On the other hand, the Ad5-based Ebola vaccine from the Chinese FDA showed poor efficacy in the phase I clinical trial, especially in individuals with pre-existing adenoviral immunity, adding further credence to the issue of pre-existing adenoviral immunity in vaccination [118]. During the COVID-19 pandemic, human Ad26- and/or Ad5-based vaccines (Sputnik-V and Ad26.COV2.S, Johnson & Johnson) and the simian ChAdOx1 nCoV-19 vaccine (AZD1222) were also among the very first viral vectors approved for vaccine use [120]. Both Ad26 and ChAdOx1 were well tolerated and most subjects receiving these vaccines experienced mild to moderate side effects, such as pain at the injection site, headache, fatigue and muscle pain. For both vaccines, the reactogenicity was higher in groups receiving a higher dose of vaccine, but a decrease in reactogenicity was observed with increasing age [121,122]. Following administration of Ad26 or ChAdOx1 to the public, rare cases of thrombocytopenia were reported but the exact correlation between the adenoviral vector vaccination and the incidence of thrombocytopenia is still uncertain [123]. Most adenoviral vector-based vaccines have demonstrated good immunogenicity and safety, but a small number of the trials have indicated certain serious adverse effects when using these vectors. One of the examples of such a trial is the first cellular prophylactic Ad5 vaccine trial against HIV. This trial was suspended due to unexpected enhancement of HIV infection in vaccinated volunteers [124]. Activation of the DC-T cell axis by Ad5 immune complexes appears to have been responsible for the increased incidence of HIV acquisition among vaccinated individuals, indicating a serious side effect of the highly inflammatory Ad5 when used in HIV patients [125]. Asides from HIV, Ad5 vectors have also been developed against many other pathogens, such as Zika Virus (ZIKV), Influenza virus, *Mycobacterium tuberculosis*, plasmodia, and SARS-CoV-2, and have demonstrated strong immunogenicity against these diseases [118,126].

Adenoviral vectors are easy to design and can be rapidly produced in mass quantity. The ease of mass production is of supreme importance during an emergency outbreak to supply the demand within a short period of time, as demonstrated by adenoviral vectors during the COVID-19 pandemic. In contrast to Ad, the high cost and difficulties in mass production of LVs impede the worldwide implementation of LV vaccines. However, the extensive improvements in industrial-scale LV production have further advanced the approval of LV-based therapies in clinical trials and for human use. The large payload capacity of adenoviral vectors relative to LVs is another advantage. Adenoviral vectors can accommodate large foreign genes of up to 35 kb, otherwise difficult for viral vectors with smaller payload capacities. In contrast to adenoviral vectors, LVs only minimally induce only minute levels of phenotypic and functional DC maturation *in vitro* and *in vivo* [63,127] (our unpublished data) in murine models, indicative of their negligible pro-inflammatory properties. The LV immunization doses in non-human primates and humans are from 10^6 to 10^8 TU, which are significantly lesser than the above-mentioned

doses used for adenoviral vectors [15]. LVs are also suitable for repeated administration in gene therapy and vaccination due to the ease of pseudotyping with heterologous VSV-G and their weak inflammatory property. The safety profile of LVs, despite not being as widely studied as adenoviral vectors, has so far been shown to be excellent in vaccinated animals and humans with priming and boosting [128]. On the other hand, the safety profile of adenoviral vectors in terms of inflammation, toxicity, and pre-existing immunity in humans is likely to be continuously be improved. The favorable scientific and practical features of both vectors clearly show their ability to address many diseases. However, each vector has its own shortcomings that need to be addressed to unlock their potential of these viral vectors for vaccination.

3.8. LV-based vaccination in preclinical models

The field of LV-based vaccination is still in its infancy compared to LV-based gene therapy. One of the reasons that LVs have come to the fore in vaccination in recent years is their ability to transduce DCs. Multiple LV vaccine candidates are being tested for tumor immunotherapy and a small handful are being tested for prophylactic T-cell immunity against infectious diseases [15,129,130]. Most attention on LV-based prophylactic T-cell immunity has focused on its use to target HIV. Numerous preclinical studies in animal models have shown great success in strongly eliciting HIV-specific antibodies and cytotoxic $CD8^+$ T cells. A prime-boost regimen of ILVs encoding SIV macaque 239 Gag (SIVmac239 Gag) efficiently protected Cynomolgus macaques when they were challenged with SIVmac251. This protection was accompanied by a reduction in the viral load of two orders of magnitude and full preservation of $CD28^+$ $CD95^+$ memory $CD4^+$ T cells during the primo-infection [15]. Another study that used a SIV-based NILV encoding EnvC.1086 gp140 showed durable antibody and T-cell responses after one year of a single immunization in rhesus macaques, with most of the vaccine-induced T cells being polyfunctional $CD4^+$ central memory cells [131]. Therapeutic vaccination of macaques, chronically infected with Simian Human Immunodeficiency Virus (SHIV), with a NILV encoding the SIV gag, induced durable T-cell responses, leading to sustainable virus control for more than 20 weeks [59]. Overall, these data suggest a durable and protective immune response following LV vaccination, in particular for complex diseases such as HIV, further demonstrating the potential of LV-based vaccines to advance to human clinical trials. An LV-based DC vaccine has been developed in which HIV-1 or LCMV antigens are co-expressed with CD40 ligand (CD40L) and possibly with a soluble Programmed cell Death 1 (PD-1) dimer. This vector activates DCs via CD40 and blocks the checkpoint PD Ligand 1 (PD-L1). Injection of appropriate mice with DCs transduced with such LV-based DC vaccines have shown induction of T-cell effector and memory cells and marked degrees of anti-viral protection [91,132,133].

Notably, LVs have also demonstrated superior immunogenicity relative to other vaccine platforms, such as those based on DNA, protein, and Ad5 [58,134,135]. The absence of any adverse effects of LVs was

also established in humans in a phase I HIV therapeutic clinical trial [136]. Although LV-based vaccine candidates are at their early stage of clinical development, increasing evidence of their efficacy and safety reinforce the potential and interest of these vectors in vaccination. LV vaccinations against malignancy and infectious diseases have consistently demonstrated the induction of strong humoral and cellular immune responses, accompanied by highly significant protection in preclinical animal models (Table 1).

Our recent work on anti-flaviviral immunity, notably with ZIKV, illustrates the anti-viral efficacy of LV-based vaccination. ZIKV caused an unprecedented global health crisis in 2016 due to its potential to induce neurological defects in the developing fetus. As part of the concerted effort to combat ZIKV infection, we developed a NILV-based vaccine candidate encoding the ZIKV pre-membrane (prM) and envelope glycoprotein (E). A single systemic immunization of: (i) IFN α/β receptor knockout A129 mice, particularly susceptible to Zika, or (ii) immunocompetent mice, induced strong serum neutralizing antibody titers, which correlated with full and long-term protection, measurable as early as seven days up to the last time point studied, i.e. six months post-vaccination [135].

More recently, we developed a NILV-based vaccine candidate against COVID-19, that encodes for the full-length Spike glycoprotein of SARS-CoV-2 (S_{CoV-2}) and elicits high titers of neutralizing antibodies, as well as strong and poly-specific $CD8^+$ T-cell immunity [55]. Due to the non-replicative, non-integrating, non-cytopathic, negligibly inflammatory features of NILV, it was possible to use this vaccine candidate for intranasal administration. By inducing mucosal immunity, and notably the production of lung IgG, IgA, and $CD8^+$ T cells, and targeting the immune responses to the respiratory tract, at the entry site of the virus, it was possible to achieve full protection, characterized by the absence of detectable replicating virus, and the inhibition of deleterious inflammation and tissue injury in the lung [55]. This was demonstrated in mice, in which the expression of the SARS-CoV-2 receptor human Angiotensin Converting Enzyme 2 (hACE2) was induced by *in vivo* transduction of respiratory tract cells by an adenoviral vector, and in the highly susceptible golden hamsters, which are naturally permissive to SARS-CoV-2 replication, mimicking the human COVID-19 physiopathology. We also developed a hACE2 transgenic mouse model with unprecedented brain permissiveness to SARS-CoV-2 replication leading to a lethal disease in <4 days post infection. We used this highly stringent transgenic model to provide the proof of principle that an intranasal booster immunization with the NILV::S_{CoV-2} vaccine candidate achieves full protection of both respiratory tract and brain against SARS-CoV-2 [137]. Given the neurotropism of SARS-CoV-2, the COVID-19-associated symptoms such as headache, anosmia, dysgeusia, impaired consciousness and cerebrovascular disease, the presence of SARS-CoV-2 in the nasopharynx and brain, and viral entry into the brain via the olfactory mucosa, the feasibility of nasal vaccination by NILV merits consideration for vaccine development [54,138-141]. Other vaccine strategies currently being developed do not take into account the protection of the central nervous system.

Aside from the prophylactic vaccination against infectious diseases, we also recently evaluated the onco-immunotherapeutic impact of LV::OVA using the EG.7 tumor cell line expressing the surrogate OVA antigen [29]. A single intramuscular administration of LV::OVA to mice bearing large subcutaneous tumors, i.e., with an average volume of ~250 ^{mm3}, led to complete tumor eradication and a high survival rate of 83%. Only 50% of their Ad5::OVA-vaccinated counterparts were protected in the same experiment. Therefore, LV onco-therapy provided higher protection and better survival than Ad5 in this murine immune-therapeutic setting [29].

4. Conclusion/Discussion

LVs are potentially powerful vaccine vectors by virtue of their efficiency in transducing DCs *in vivo* and in inducing both strong and long lasting humoral and T-cell responses and effective protections in multiple preclinical models of infection and oncology. In addition to their high immunogenicity, integrase-deficient NILVs present a notable safety profile. These vectors are negligibly pro-inflammatory and non-replicative. Here, we reviewed the literature relating to the virological and immunological aspects of these vectors and provided elements of comparison with the more widely used, gold standard adenoviral vectors. We stress the quasi-absence of pre-existing immunity against LVs pseudotyped by the envelope glycoprotein of VSV, to which human populations have only been very rarely exposed, unlike adenoviral-based vectors, against which more than 80% of humans may have immunity, with the limitations that the pre-existing humoral and T-cell responses can have on the efficacy and safety of these vectors. The very weak inflammatory properties of LVs, as well as their non-cytopathic nature, pave the way for their use in mucosal vaccination, with particular interest in infectious diseases that affect the respiratory tract or brain. Recent results in various preclinical models reinforce the interest of these vectors in the prevention of infectious diseases and in onco-immunotherapy.

5. Expert Opinion

LVs have emerged as a powerful platform for gene therapy and vaccination purposes, and exhibit several advantages over other viral vectors. Considering the traits of LVs detailed in this review, LVs offers a promising opportunity for further vaccine development, suitable for a large number of clinical applications. To date, no adverse effect has been observed in preclinical animal models in diverse settings or in a clinical trial with LVs against HIV-1. Furthermore, our reported COVID-19 LV-based vaccine candidate is in progress to proceed to clinical trials. Thus far, a major limitation of the use of LVs in vaccination has been linked to constraints in technology transfer to manufacturers for their production in large quantities while maintaining an acceptable production yield. Thus, industrial-scale production for mass vaccination is still nascent. However, it must be taken into account that, compared to the adenoviral vaccine vectors that are currently mass produced, and which are injected at doses on the order of 5–15×10^{10} vp per individual in humans, while ILV-based vaccination only requires the administration of

5–500 × 10^6 TU per individual for human use [126,128,142]. Therefore, the much lower effective doses of LVs should will compensate for their high production cost. LVs have largely shown their effectiveness, including the mucosal and, in particular, intranasal — vaccination route. With the first Good Manufacturing Practice (GMP) grade batches, the completion of toxicology studies adapted to the nasal administration route, will be required before LV can enter the clinic for mucosal immunization.

A promising area to further optimize the immunogenicity of LVs will be to engineer LVs to: (i) route antigens to the MHC-II presentation machinery for $CD4^+$ T-cell induction, (ii) co-encode antigens and cytokines/chemo-attractants, and (iii) co-encodes antigens and elements for targeting them to relevant DC subtypes. Additional applied research also needs to be conducted to assess the effectiveness of LVs in the prevention and/or therapy of chronic infectious diseases, such as malaria and tuberculosis, as well as cancers. In the cancer field, the use of preclinical models, other than subcutaneous solid tumors, for example models of tumor dissemination will be essential. A head-to-head comparison of LV- and RNA-based vaccines in terms of antibody affinity, T-cell responses, pathogen neutralizing potential, immune memory characteristics, and the duration of the protective immunity, as well as side effects, will be valuable.

LVs meet all the criteria for efficacy, as they trigger the various arms of adaptive immunity, with a high protective potential, without any side effects linked to innate immune reactions, as tested in numerous preclinical models by independent expert groups in the field, and pre-existing vector-specific immunity is absent in human populations. Overall, we believe that LVs have the potential to offer prophylactic and therapeutic effects against infectious diseases and cancer. In the near future, following their validation in regulatory preclinical trials, LVs have a realistic potential to be implemented in clinical practice, with significant use in vaccination. The next boost for LV-based vaccines will rely on niche technologies that will enable affordable large-scale production of LV doses, bringing us one step closer to the mass use of LVs in vaccination.

Legend to the Figures

Figure Erreur ! Il n'y a pas de texte répondant à ce style dans ce document.**. Schematic representation of HIV-1 genome and development of LV system.**

(A) The essential (*gag, pol* and *env)*, accessory (*vif, vpr, vpu* and *nef)* and regulatory (*tat* and *rev)* genes of HIV-1 are flanked by two identical long terminal repeats (LTRs). Ψ represents the packaging signal, while the central polypurine tract (cPPT) and central termination sequence (CTS) were represented as black line. The cPPT and CTS forms a triple helical DNA flap, ensuring proper nuclear translocation of the pre-integration complex. RRE, represented in yellow circle, is the rev response element, responsible for transporting the unspliced RNA into cytoplasm. **(B)** Production of recombinant LV is separated into 3 distinct plasmid constructs. The expression cassette of LV contains cis-acting sequences (Ψ, RRE and cPPT/CTS) and gene of interest under the control of an internal promoter which are flanked by two LTRs. The packaging plasmid encodes for *gag, pol, tat and rev* while the envelope plasmid encodes for the envelope protein, both plasmid expression under the transcriptional control of P_{CMV}. LTR, long terminal repeats; Ψ, packaging signal; RRE, rev response element; cPPT, central polypurine tract; CTS, central termination sequence; $P_{CMV,}$ cytomegalovirus promoter.

Figure 2. Reverse transcription of self-inactivated (SIN) vector.

Reverse transcription is initiated when minus strand DNA synthesis starts from the primer binding site (PBS) of the single stranded viral RNA (shown in red line), copying the U5 and R region of the 5' genome. The RNaseH of the reverse transcription enzyme degrades the viral RNA that has been copied (shown as red dotted line). The minus strand DNA translocated to the 3' end of the viral genome using the R sequence, and continue to elongate to form a full length minus strand DNA, which now contains the ΔU3 (shown as blue line). The remaining viral RNA is degraded by the RNaseH activity, except for central polypurine tract (cPPT) and PPT, which are resistant to degradation. The cPPT and PPT are used as primers for second strand plus DNA synthesis (shown as black line). After a second strand transfer, DNA synthesis continues to the cPPT, displacing ~100 nucleotides of cPPT-primed DNA and terminates at the central termination sequence (CTS), generating a kink within the three stranded DNA structure called the DNA flap [143]. At this point, the reverse transcription is completed and both plus and minus strand of DNAs contain both copies of LTRs that have identical ΔU3 at both ends.

Figure 3. Strategies for redirecting LV vectors to APC via engineered glycoproteins.

(A) VSV-G-pseudotyped LVs can infect a wide range of cell types via binding to LDLR receptor and heparan sulfate which are found in all cell types. **(B)** Measles virus glycoprotein (MVG)-pseudotyped LV infect APC preferentially via recognition of DC-SIGN or signaling lymphocytic activation molecule (SLAM) expressed on APC, and mediate entry into cells via direct membrane fusion. **(C)** The mutated sindbis virus envelope glycoprotein (muSVG)-pseudotyped LV confer tropism towards DC via binding to DC-SIGN. **(D)** LV pseudotyped with MVG-MHC II-specific single-chain antibody (scFv) recognizes MHC II-expressing APC. **(E)** LV pseudotyped with VSV-G-fusion DC2.1 or DC1/8-specific nanobody transduced only DC.

Figure 4. Schematic representation of LV vector containing β2m internal promoter.

LTR: Long Terminal Repeats; SD, Shine-Dalgarno sequence; RRE, Rev Response Element; cPPT, central PolyPurine Tract; CTS, Central Termination Sequence; ISRE, IFN-Stimulated Response Element; WPRE, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element.

667 **Table 1. LV-based vaccines against malignancies and infectious diseases.**

Disease	Vector Type*	Antigen(s)	Characteristics of the immune response	Reference
Melanoma	ILV	Melan-A	$CD8^+$ and $CD4^+$ responses against Melan-A	[144]
	ILV	New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1)	$CD8^+$ response against NY-ESO-1	[145]
	ILV	Hsp70 co-expressed Tyrosine related protein 2 (TRP2)	$CD8^+$ response against TRP2	[146]
	ILV	Melan-A	$CD8^+$ response against Melan-A	[147]
	ILV	MHC II promoter driving TRP2 expression	$CD8^+$ response against TRP2	[35]
	ILV	NY-ESO-1	$CD8^+$, $CD4^+$ and antibody response against NY-ESO-1	[148]
	ILV	DC-specific promoter driving expression of NY-ESO-1	$CD8^+$ and $CD4^+$ response against NY-ESO-1	[99]
	ILV	Tyrosine related protein 1 (TRP1)	$CD8^+$ response against TRP1	[149]
	ILV	Human telomerase reverse transcriptase (hTERT)	$CD8^+$ response against hTERT	[150]
	NILV pseudotyped with a modified Sindbis virus envelop protein	Melanoma antigen gp100	$CD8^+$ response against gp100	[89]
	NILV pseudotyped with a modified Sindbis virus envelop protein	NY-ESO-1[#]	$CD8^+$ and $CD4^+$ response against NY-ESO-1	[151] [152] [153]
	NILV	Tyrosine related protein 2 (TRP2)	T cells against TRP2	[154]
Prostate Cancer	ILV	Prostate stem cell antigen (PSCA)	$CD8^+$ and $CD4^+$ response against PSCA	[155]
Hepatitis B virus (HBV)	NILV	secreted HBsAg	Antibody and T cells against HBsAg	[34]
Hepatitis cytomegalovirus	NILV	Prime with Ad5 harboring envelope 1 (E1) and envelope 2 (E2) glycoproteins, boosted with NILV pseudotype with E1 and E2, harboring the non-structural protein 3 (NSP3)	Broadly neutralizing antibodies against E1, E2 and NS3	[156]
Human Papilloma Virus	NILV	E7 fused to calreticulin to enhance MHC I presentation	$CD8^+$ and antibody response against E7	[157]
Malaria	NILV	*Plasmodium yoelii* Circumsporozoite Protein (Py CSP)	$CD8^+$ response against CSP	[17]
West Nile Virus	ILV	secreted soluble form of the envelope E-glycoprotein (sE)	Antibody against sE	[19]
	NILV	secreted soluble form of the envelope E-glycoprotein (sE)	Antibody against sE	~~[17]~~ [24]
Influenza	NILV	hemagglutinin (HA) and nucleoprotein (NP)	$CD8^+$ response against NP; Antibodies against HA and NP	[18]
Zika	ILV and NILV	pre-membrane envelope (prM-E)	Antibody against prM-E	[135]
SARS-CoV-2	ILV and NILV	Full length Spike protein	Antibody and $CD8^+$ T cells against Spike	[55,137]
HIV	ILV	Multiple HIV epitopes encoded	Multi-specific $CD8^+$ and $CD4^+$ response against	[130]

		multiple encoded HIV epitopes	
NILV	Codon-optimized gp120	$CD8^+$ T cell response and antibody response against gp120	[158]
ILV	Codon-optimized SIV gp120	$CD8^+$ and $CD4^+$ responses	[16]
ILV	HIV Gag, Pol and Rev	$CD4^+$ and $CD8^+$ T cell response against Gag, Pol and Rev	[159]
SIV-based NILV	1086 gp140 and 1176 gp140	Antibodies against 1086 and 1176 gp140	[30]
SIV-based NILV	HIV-1 EnvC.1086 gp140	Antibodies, $CD8^+$ and $CD4^+$ against gp120	[131]
SIV-based NILV	HIV-1 EnvC.CH505 gp140	Antibodies and T cells against gp140	[58]
SIV- and HIV-based NILV	SIV-gag and (bnAb) PGT121	$CD4^+$ and $CD8^+$ against SIV gag	[59]
ILV[#]	HIV Gag, Pol, Nef	$CD4^+$ and $CD8^+$ T cells	[128]

*Unless otherwise stated, all ILV and NILV vectors stated here are HIV-1 based vector pseudotyped with VSV.G.
#Used in clinical trial

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Declaration of Interests

Min-Wen Ku is an employee of TheraVectys, Pierre Charneau is the founder and CSO of TheraVectys, Laleh Majlessi has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Author contribution

All authors substantially contributed to the conception and design of the review article and interpreting the relevant literature, and have been involved in writing the review article or revised it for intellectual content.

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* This work demonstrates that a single therapeutic immunization with non-integrative LV encoding HPV16 E7 is able to completely eradicate tumor in mice and can induce long lasting T cells at least up to one year after immunzation.
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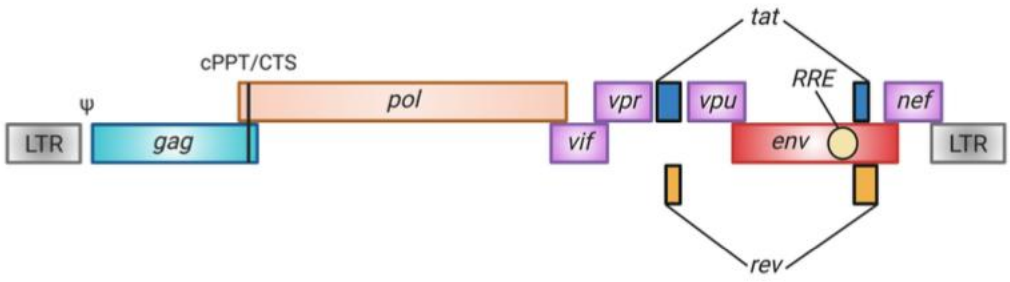
Figure 1

A


tat
cPPT/CTS
RRE
ψ
pol
vpr
vpu
nef
LTR
gag
vif
env
LTR
rev

B

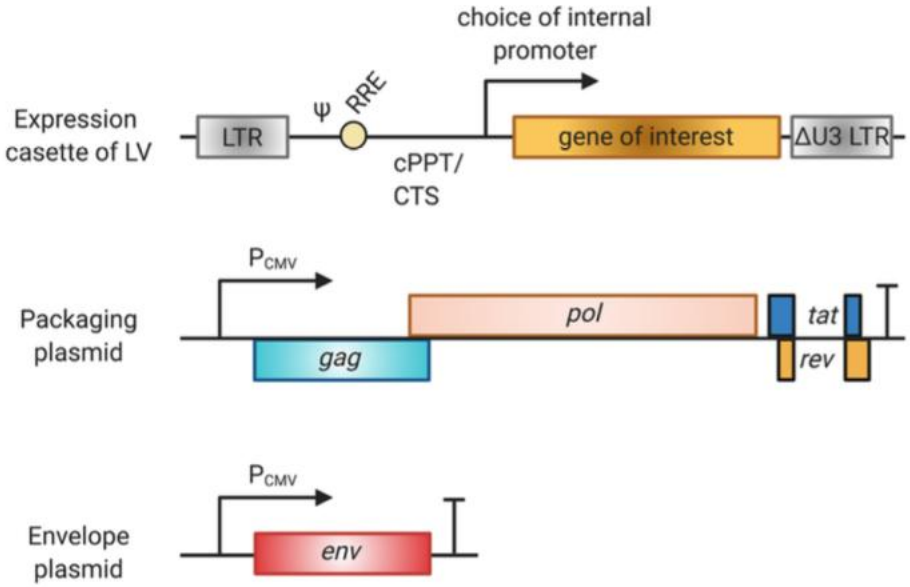

choice of internal promoter
Expression casette of LV
LTR
ψ
RRE
cPPT/ CTS
gene of interest
ΔU3 LTR
Packaging plasmid
PCMV
pol
tat
gag
rev
Envelope plasmid
PCMV
env

Figure 2

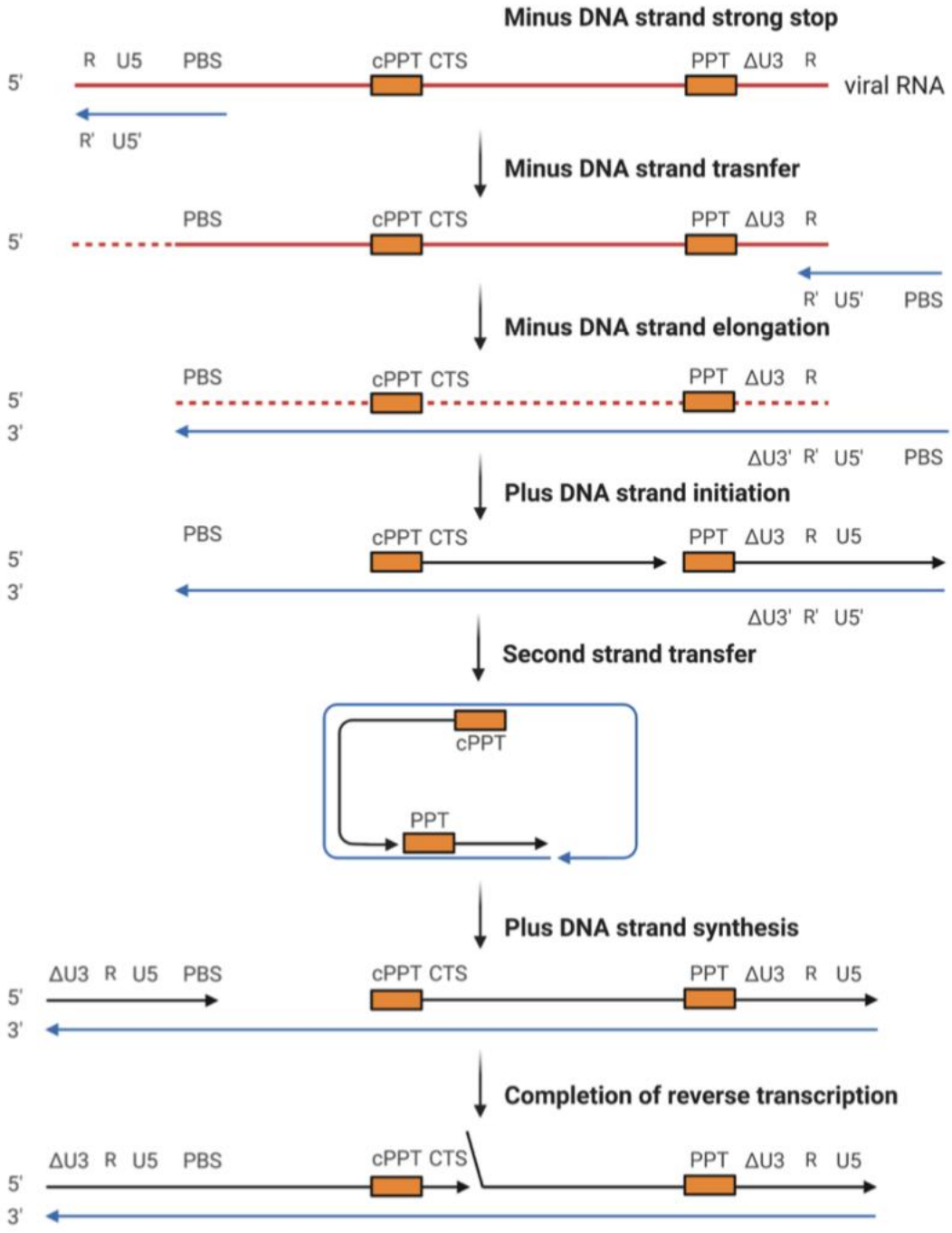

Minus DNA strand strong stop
R U5 PBS
cPPT CTS
PPT ΔU3 R
5'
viral RNA
R' U5'
Minus DNA strand trasnfer
PBS
cPPT CTS
PPT ΔU3 R
5'
R' U5' PBS
Minus DNA strand elongation
PBS
cPPT CTS
PPT ΔU3 R
5'
3'
ΔU3' R' U5' PBS
Plus DNA strand initiation
PBS
cPPT CTS
PPT ΔU3 R U5
5'
3'
ΔU3' R' U5'
Second strand transfer
cPPT
PPT
Plus DNA strand synthesis
ΔU3 R U5 PBS
cPPT CTS
PPT ΔU3 R U5
5'
3'
Completion of reverse transcription
ΔU3 R U5 PBS
cPPT CTS
PPT ΔU3 R U5
5'
3'

Figure 3

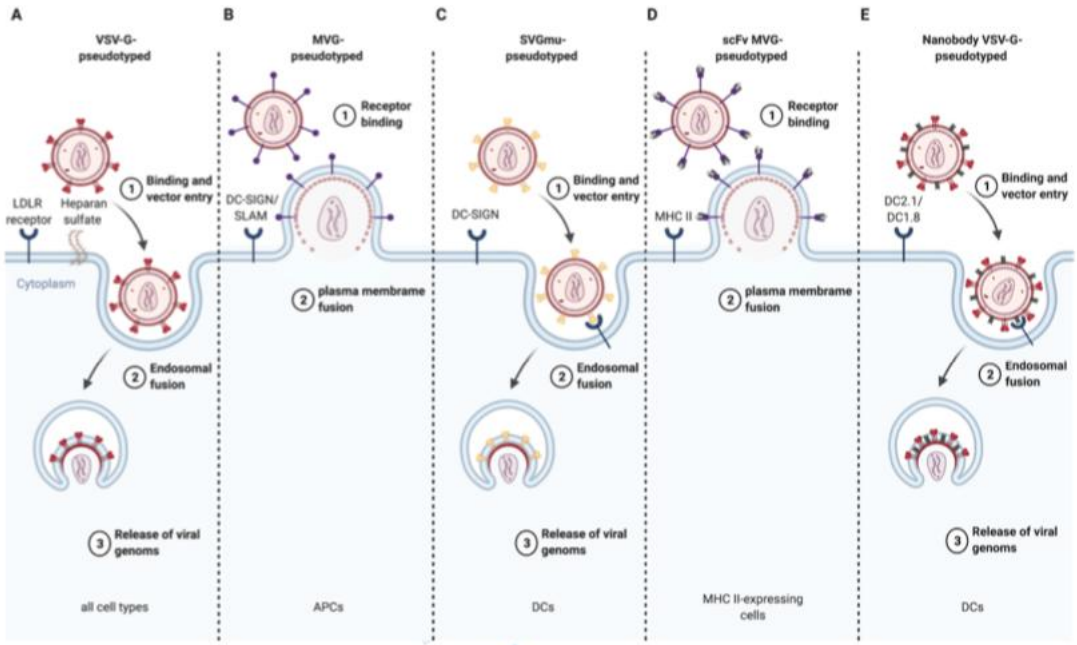
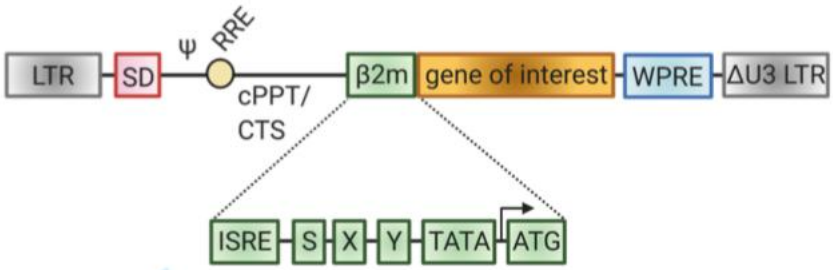

A
VSV-G-pseudotyped
1 Binding and vector entry
LDLR receptor
Heparan sulfate
Cytoplasm
2 Endosomal fusion
3 Release of viral genoms
all cell types
B
MVG-pseudotyped
1 Receptor binding
DC-SIGN/ SLAM
2 plasma membrame fusion
APCs
C
SVGmu-pseudotyped
1 Binding and vector entry
DC-SIGN
2 Endosomal fusion
3 Release of viral genoms
DCs
D
scFv MVG-pseudotyped
1 Receptor binding
MHC II
2 plasma membrame fusion
MHC II-expressing cells
E
Nanobody VSV-G-pseudotyped
1 Binding and vector entry
DC2.1/ DC1.8
2 Endosomal fusion
3 Release of viral genoms
DCs

Figure 4



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