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1 **Use of Lentiviral Vectors in Vaccination**

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13 **ABSTRACT (199 words)**

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

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

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

29

30

31 **KEYWORDS**

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

## 34 Article highlights

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

## 54 1. Introduction

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

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

88 Among viral vectors, lentiviral vectors (LVs) have emerged as a particularly powerful platform for  
89 vaccination purposes. LVs exhibit several advantages over other viral vectors, including their combined  
90 capacity to induce both strong and long-lasting T-cell immunity and antibody responses, without being  
91 inflammatory or the target of pre-existing vector-specific immunity [15-19]. LV components, such as the  
92 envelope and capsid proteins, can prime vector-specific immunity [20]. However, pre-existing immunity  
93 against the LV envelope is rarely found in human populations because LVs are predominantly  
94 pseudotyped with the heterologous Vesicular Stomatitis Virus Glycoprotein (VSV-G) envelope, to which  
95 the human population has been barely exposed [21]. Pre-existing immunity against the LV capsid is also  
96 rare, because infections with lentiviruses are less prevalent than those caused by other viruses commonly  
97 used as vectors, such as adenoviruses [22,23]. LVs can be divided into integrating (ILVs) and non-  
98 integrating (NILVs) categories. ILVs are the vector of choice for gene therapy, whereas NILVs are  
99 preferred for vaccination. Both ILVs and NILVs are capable of transducing dividing and non-dividing  
100 cells, notably dendritic cells (DCs) [24-26]. LVs are non-replicative, which means that they only support  
101 one round of host cell infection. The ability of LVs to transduce Antigen Presenting Cells (APCs) ensures  
102 antigen expression throughout the life span of these cells, for instance 5-7 days post-immunization for  
103 plasmacytoid DCs [27]. With respect to vector persistence, it must be considered that promoters may play  
104 a role, as strong promoters that result in higher antigen expression may also trigger more robust immune  
105 responses, in turn capable of eliminating transduced cells that harbor the vector-specific DNA [28]. We  
106 showed that ILV harboring a  $\beta$ 2-microglobulin promoter remained detectable in mice from a few days to  
107 a maximum of three months post-intramuscular immunization, as quantified by a highly sensitive qPCR  
108 assay, detecting vector-specific DNA [29]. Others showed that NILVs harboring the cytomegalovirus  
109 (CMV) promoter, introduced via a single intramuscular injection in rhesus macaques, persisted for at least  
110 six months post-immunization at the site of injection [30]. Similar results were obtained with NILV  
111 harboring the CMV promoter in mice in which vector-specific DNA was only detected at the injection  
112 site, i.e., the muscles, but not at systemic sites, including the spleen and large intestine [31]. Hence,  
113 antigen expression and vector persistence vary on a case-by-case basis, depending on: (i) the route of  
114 vaccination, (ii) the promoter, (iii) the type of cell/tissue, and (iv) type of vector used [32-36]. Similar to  
115 adenoviral vectors, LVs are non-replicative and non-cytopathic vectors but unlike adenoviral vectors,  
116 LVs are negligibly inflammatory, which largely contributes to their safety and which paves the way for  
117 their potential use in mucosal immunization. Overall, these traits make the use of LVs a plausible  
118 opportunity for further vaccine vector development suitable for various clinical applications.

## 119 **2. Methods**

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

123 viral vectors, (iii) were relevant to LVs, and (iv) had a publication dates between 1991 and February  
124 2021.

### 125 **3. Results**

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

#### 129 **3.1. Development of LVs**

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

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

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

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

### 173 **3.2. Non-Integrating LVs**

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



190 Despite many studies demonstrating the excellent safety profile of ILVs, the concern of insertional  
191 mutagenesis prompted the development of NILVs. NILVs carry a missense amino acid in the catalytic  
192 triad of the integrase, that prevents the integration of viral DNA into the host chromosome. The most  
193 commonly reported mutations affecting the catalytic triad are at the D64, D116, and E152 mutation sites  
194 [50]. Without integration, the viral DNA remains in an episomal form, bearing either one or two LTRs,  
195 both equally effective in gene expression. The lack of integration signifies the eventual loss of the  
196 transgene during cell division, which affects the overall transgene level [51]. However, in non-dividing  
197 cells, the transgene expression remains comparable for ILV and NILV, due to the fact that the transgene  
198 is not diluted out by cell division, as shown in muscles and DCs [52,53]. The lack of integration does not  
199 hamper the induction of an effective immune response and only requires adjustment of the vector dose  
200 [17]. A comparison of ILVs and NILVs with various antigens in preclinical models showed that with 10  
201 times more NILV the same immunization efficiency can be achieved with 10 times more NILV as with  
202 ILV; a dose of  $1 \times 10^8$  Transduction Unit (TU)/animal is optimal for NILV, versus  $1 \times 10^7$  TU/animal for  
203 ILV [54,55].

### 204 *3.3. Advantages of LVs as vaccine vectors*

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

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

224 However, anti-envelope immunity can be overcome by pseudotyping LV with heterologous VSV-G  
225 serotypes, without impeding the efficacy of the vector, as demonstrated previously for ILVs and NILVs  
226 [15,17,30,58,59].

227 LVs can accommodate large transgenes of up to in the range of 5 to 7 kb, which further extends the scope  
228 of their application, for example in the generation of multi-antigenic vaccines [60]. Large transgenes  
229 exceeding 10 kb have been reported [60,61]. However, the accommodation of large transgenes may come  
230 at the cost of lower functional viral titers, as previous studies showed a semi-logarithmic reduction of 3 to  
231 4 folds in functional LV titer per kb increase in genome size [60,62]. Aside from these major advantages,  
232 LVs are negligibly inflammatory and induce only minute levels of phenotypic and functional DC  
233 maturation *in vitro* and *in vivo* [63] and our unpublished data. This property largely contributes to the  
234 safety of these vectors and makes their use possible in mucosal applications for vaccination [54,55].

### 235 **3.4. Limitations of LV**

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

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

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

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

### 280 **3.5. APC targeting approaches**

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

#### 287 **3.5.1. Transductional targeting**

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

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

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

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

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

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

### 328 *3.5.2. Transcriptional targeting*

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

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

347 We recently reported the use of a LV harboring the human  $\beta$ 2-microglobulin promoter, which contains  
348 highly conserved cis-regulatory elements, i.e., Interferon (IFN)-Stimulated Response Elements (ISREs),  
349 and SXY modules [29]. ISREs are the binding site for the IFN family of regulatory factors, while SXY  
350 modules interact with a multiprotein complex (Figure 4). These mediators transactivate the and are tightly  
351 regulated by immune mediators, including cytokines, which are upregulated in immune cells [100,101].  
352 In addition, the human  $\beta$ 2-microglobulin promoter contains minimal proximal enhancers [29].  
353 Intramuscular immunization of mice with this LV led to highly efficient *in vivo* transgene expression by  
354 CD11b<sup>+</sup> CD8<sup>-</sup> myeloid, CD11b<sup>-</sup> CD8<sup>+</sup> lymphoid and plasmacytoid DC subsets. In this framework, the *in*  
355 *vivo* transgene expression lasted at least seven days post-immunization and was accompanied by the  
356 induction of long-term memory CD8<sup>+</sup> T-cell responses and complete efficacy in the immune eradication  
357 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- $\alpha$ , 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- $\alpha$  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<sup>+</sup> 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<sup>+</sup>-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- $\kappa$ B signaling pathway in CD11c<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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

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

431 The Ad5 vector is highly inflammatory and can trigger a series of innate immune signaling pathways that  
432 result in substantial secretion of proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-12, IFN- $\gamma$ , and TNF-  
433  $\alpha$  [22]. Ad5 vectors bind to complement proteins C4 and C4-binding protein and activate the complement  
434 system, platelet aggregation, and the secretion of inflammatory cytokines [113,114]. The Ad5 capsid  
435 interacts with TLR2, and the Ad5 DNA is sensed by TLR9, which activates the NF $\kappa$ B and IRFs  
436 pathways, resulting in the production of numerous pro-inflammatory cytokines and chemokines [115].  
437 Ad5 double stranded DNA also activates the inflammasome [116]. Such induction of proinflammatory  
438 cytokines after Ad5 administration leads to a robust humoral and cellular immune response, but can  
439 sometimes also cause drastic adverse effects. A study aimed to evaluate the toxicity of systemic high-dose  
440 Ad5 administration showed the response to be lethal when  $2 \times 10^{11}$  active viral particles (vp) were  
441 administered intravenously to mice with pre-existing Ad5 immunity established by previous  
442 intramuscular injection [117]. Similarly, high doses of Ad5 (i.e.,  $> 10^{13}$  vp per kg), are also lethal for  
443 larger animals [22]. Intravenous administration of a high dose of Ad5, i.e.,  $6 \times 10^{11}$  vp/kg, caused death in  
444 a volunteer participant during a phase I gene therapy clinical trial, due to the triggering of a massive  
445 immune response and multiorgan failures as a result of high-dose administration and pre-existing  
446 immunity. Importantly, the activation of innate immunity by adenoviral vectors is independent of the  
447 transduction because inactive vector particles also interact with the receptors of the innate immunity  
448 [110]. Due to the need for repeated vector administration for gene therapy, the use of the old generation  
449 of Ad5 in gene therapy is facing a sharp decline due to serious adverse effects, such as cellular toxicity  
450 and organ failure, observed in multiple clinical trials [22]. These safety concerns have been studied  
451 extensively and addressed by the development of a new generation of adenoviral vectors that are less  
452 toxic and immunogenic [22].

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



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

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

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

### 507 *3.8. LV-based vaccination in preclinical models*

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

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

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

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

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

566 Aside from the prophylactic vaccination against infectious diseases, we also recently evaluated the onco-  
567 immunotherapeutic impact of LV::OVA using the EG.7 tumor cell line expressing the surrogate OVA  
568 antigen [29]. A single intramuscular administration of LV::OVA to mice bearing large subcutaneous  
569 tumors, i.e., with an average volume of  $\sim 250 \text{ mm}^3$ , led to complete tumor eradication and a high survival  
570 rate of 83%. Only 50% of their Ad5::OVA-vaccinated counterparts were protected in the same  
571 experiment. Therefore, LV onco-therapy provided higher protection and better survival than Ad5 in this  
572 murine immune-therapeutic setting [29].

#### 573 **4. Conclusion/Discussion**

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

#### 588 **5. Expert Opinion**

589 LVs have emerged as a powerful platform for gene therapy and vaccination purposes, and exhibit several  
590 advantages over other viral vectors. Considering the traits of LVs detailed in this review, LVs offers a  
591 promising opportunity for further vaccine development, suitable for a large number of clinical  
592 applications. To date, no adverse effect has been observed in preclinical animal models in diverse settings  
593 or in a clinical trial with LVs against HIV-1. Furthermore, our reported COVID-19 LV-based vaccine  
594 candidate is in progress to proceed to clinical trials. Thus far, a major limitation of the use of LVs in  
595 vaccination has been linked to constraints in technology transfer to manufacturers for their production in  
596 large quantities while maintaining an acceptable production yield. Thus, industrial-scale production for  
597 mass vaccination is still nascent. However, it must be taken into account that, compared to the adenoviral  
598 vaccine vectors that are currently mass produced, and which are injected at doses on the order of  $5\text{--}$   
599  $15 \times 10^{10}$  vp per individual in humans, while ILV-based vaccination only requires the administration of

600 5–500 × 10<sup>6</sup> TU per individual for human use [126,128,142]. Therefore, the much lower effective doses  
601 of LVs should will compensate for their high production cost. LVs have largely shown their  
602 effectiveness, including the mucosal and, in particular, intranasal — vaccination route. With the first  
603 Good Manufacturing Practice (GMP) grade batches, the completion of toxicology studies adapted to the  
604 nasal administration route, will be required before LV can enter the clinic for mucosal immunization.

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

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

624

625 **Legend to the Figures**

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

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

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

641 Reverse transcription is initiated when minus strand DNA synthesis starts from the primer binding site  
642 (PBS) of the single stranded viral RNA (shown in red line), copying the U5 and R region of the 5'  
643 genome. The RNaseH of the reverse transcription enzyme degrades the viral RNA that has been copied  
644 (shown as red dotted line). The minus strand DNA translocated to the 3' end of the viral genome using  
645 the R sequence, and continue to elongate to form a full length minus strand DNA, which now contains the  
646  $\Delta$ U3 (shown as blue line). The remaining viral RNA is degraded by the RNaseH activity, except for  
647 central polypurine tract (cPPT) and PPT, which are resistant to degradation. The cPPT and PPT are used  
648 as primers for second strand plus DNA synthesis (shown as black line). After a second strand transfer,  
649 DNA synthesis continues to the cPPT, displacing ~100 nucleotides of cPPT-primed DNA and terminates  
650 at the central termination sequence (CTS), generating a kink within the three stranded DNA structure  
651 called the DNA flap [143]. At this point, the reverse transcription is completed and both plus and minus  
652 strand of DNAs contain both copies of LTRs that have identical  $\Delta$ U3 at both ends.

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

654 (A) VSV-G-pseudotyped LVs can infect a wide range of cell types via binding to LDLR receptor and  
655 heparan sulfate which are found in all cell types. (B) Measles virus glycoprotein (MVG)-pseudotyped LV  
656 infect APC preferentially via recognition of DC-SIGN or signaling lymphocytic activation molecule  
657 (SLAM) expressed on APC, and mediate entry into cells via direct membrane fusion. (C) The mutated  
658 sindbis virus envelope glycoprotein (muSVG)-pseudotyped LV confer tropism towards DC via binding to  
659 DC-SIGN. (D) LV pseudotyped with MVG-MHC II-specific single-chain antibody (scFv) recognizes  
660 MHC II-expressing APC. (E) LV pseudotyped with VSV-G-fusion DC2.1 or DC1/8-specific nanobody  
661 transduced only DC.  
662

663 **Figure 4. Schematic representation of LV vector containing  $\beta$ 2m internal promoter.**

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

**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 <sup>+</sup> and CD4 <sup>+</sup> responses against Melan-A	[144]
	ILV	New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1)	CD8 <sup>+</sup> response against NY-ESO-1	[145]
	ILV	Hsp70 co-expressed Tyrosine related protein 2 (TRP2)	CD8 <sup>+</sup> response against TRP2	[146]
	ILV	Melan-A	CD8 <sup>+</sup> response against Melan-A	[147]
	ILV	MHC II promoter driving TRP2 expression	CD8 <sup>+</sup> response against TRP2	[35]
	ILV	NY-ESO-1	CD8 <sup>+</sup> , CD4 <sup>+</sup> and antibody response against NY-ESO-1	[148]
	ILV	DC-specific promoter driving expression of NY-ESO-1	CD8 <sup>+</sup> and CD4 <sup>+</sup> response against NY-ESO-1	[99]
	ILV	Tyrosine related protein 1 (TRP1)	CD8 <sup>+</sup> response against TRP1	[149]
	ILV	Human telomerase reverse transcriptase (hTERT)	CD8 <sup>+</sup> response against hTERT	[150]
	NILV pseudotyped with a modified Sindbis virus envelop protein	Melanoma antigen gp100	CD8 <sup>+</sup> response against gp100	[89]
Prostate Cancer	NILV pseudotyped with a modified Sindbis virus envelop protein	NY-ESO-1 <sup>#</sup>	CD8 <sup>+</sup> and CD4 <sup>+</sup> response against NY-ESO-1	[151] [152] [153]
	NILV	Tyrosine related protein 2 (TRP2)	T cells against TRP2	[154]
	ILV	Prostate stem cell antigen (PSCA)	CD8 <sup>+</sup> and CD4 <sup>+</sup> 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 <sup>+</sup> and antibody response against E7	[157]
Malaria	NILV	<i>Plasmodium yoelii</i> Circumsporozoite Protein (Py CSP)	CD8 <sup>+</sup> 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 <sup>+</sup> 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 <sup>+</sup> T cells against Spike	[55,137]
HIV	ILV	Multiple HIV epitopes encoded	Multi-specific CD8 <sup>+</sup> and CD4 <sup>+</sup> response against	[130]

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NILV	Codon-optimized gp120	multiple encoded HIV epitopes CD8 <sup>+</sup> T cell response and antibody response against gp120	[158]
ILV	Codon-optimized SIV gp120	CD8 <sup>+</sup> and CD4 <sup>+</sup> responses	[16]
ILV	HIV Gag, Pol and Rev	CD4 <sup>+</sup> and CD8 <sup>+</sup> 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 <sup>+</sup> and CD4 <sup>+</sup> 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 <sup>+</sup> and CD8 <sup>+</sup> against SIV gag	[59]
ILV <sup>#</sup>	HIV Gag, Pol, Nef	CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells	[128]

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\*Unless otherwise stated, all ILV and NILV vectors stated here are HIV-1 based vector pseudotyped with VSV.G.

<sup>#</sup>Used in clinical trial

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678 **Declaration of Interests**

679 Min-Wen Ku is an employee of TheraVectys, Pierre Charneau is the founder and CSO of TheraVectys,  
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681 financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.  
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685 **Author contribution**

686 All authors substantially contributed to the conception and design of the review article and interpreting  
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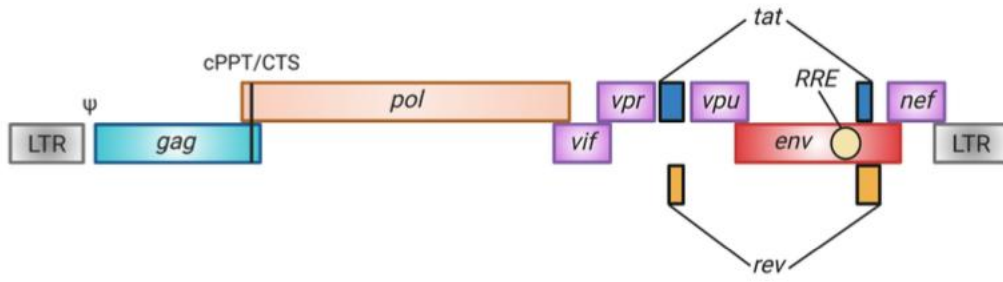
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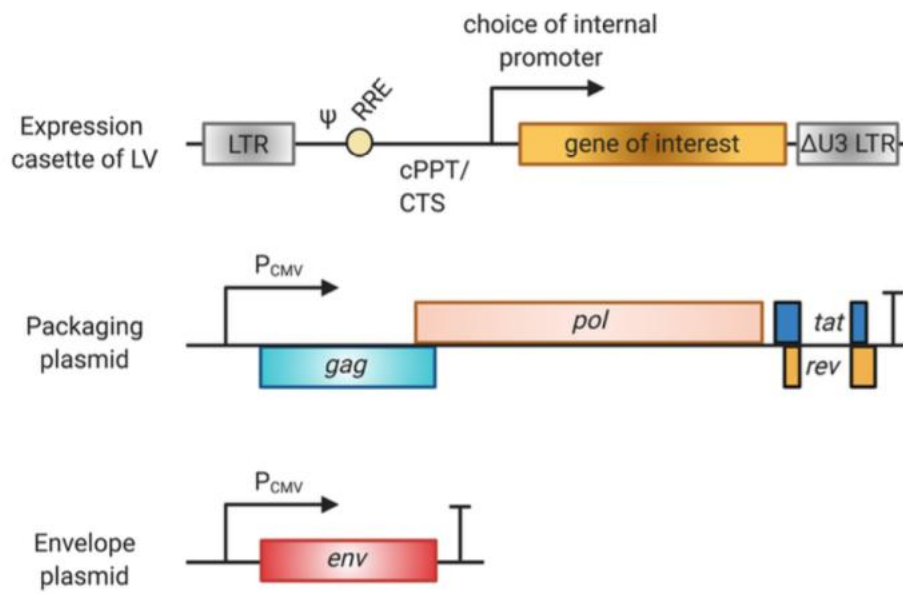
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Figure 1

A

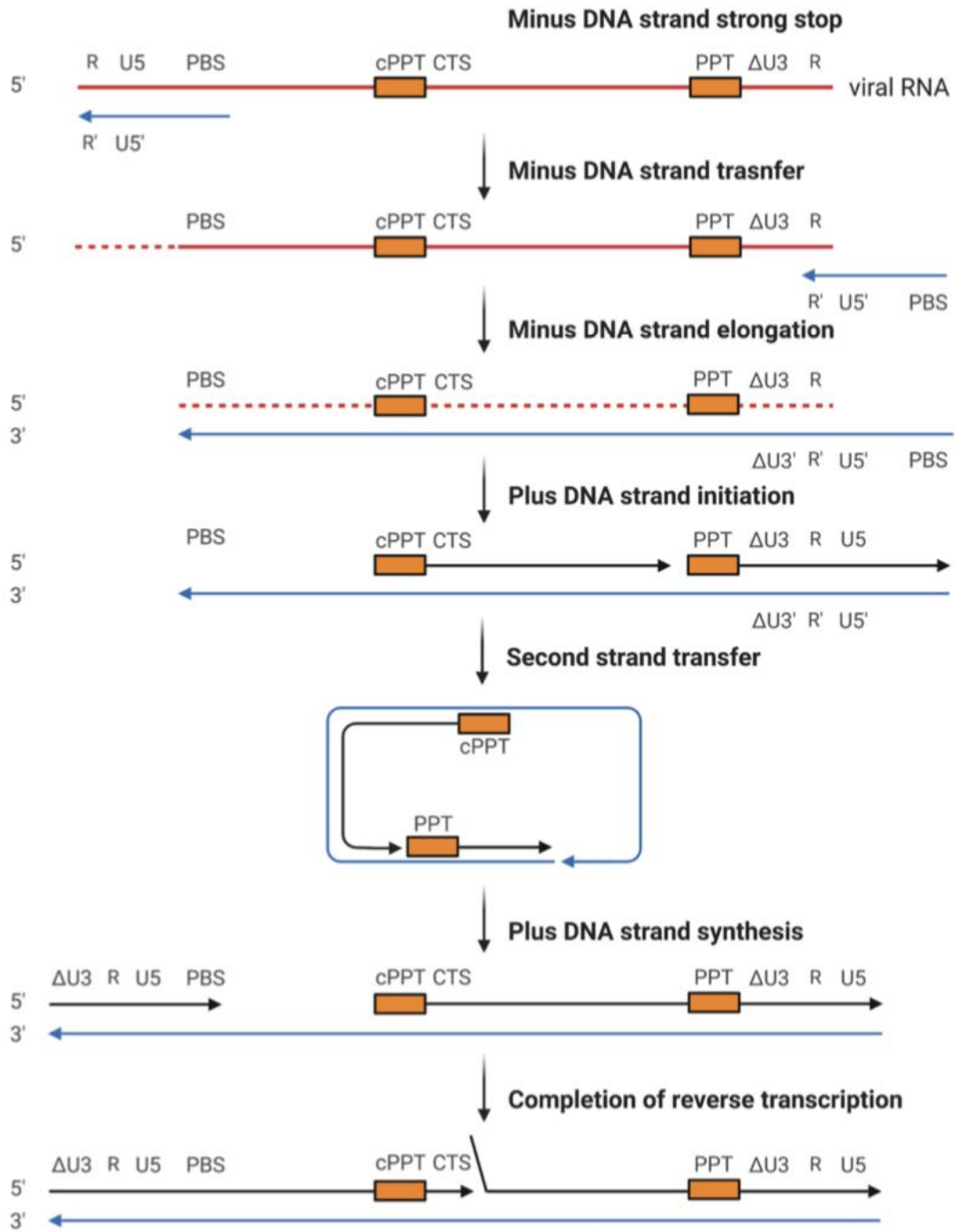


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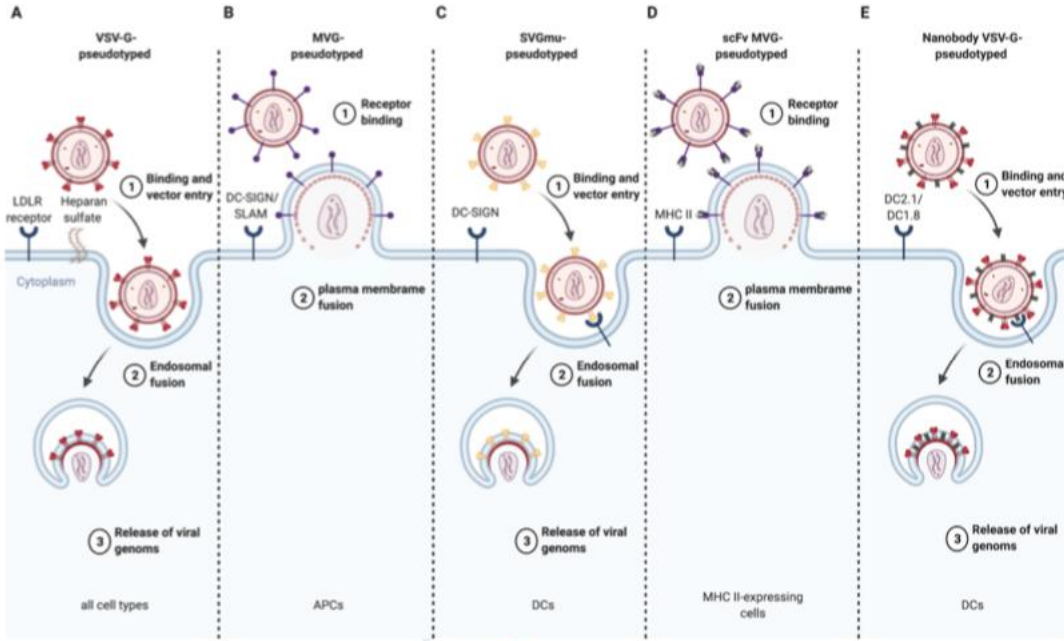
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Figure 2



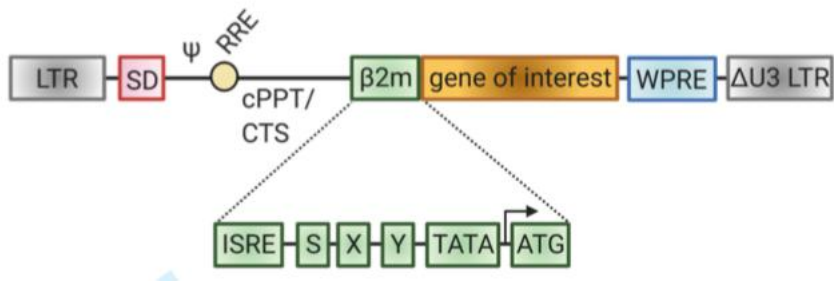
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Figure 3



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Figure 4



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