Published in final edited form as:
Immunol Rev. 2011 January ; 239(1): 45–61. doi:10.1111/j.1600-065X.2010.00967.x.

Immunization Delivered by Lentiviral Vectors for Cancer and Infectious Diseases

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Summary

The increasing level of understanding of the lentivirus biology has been instrumental in shaping the design strategy of creating therapeutic lentiviral delivery vectors. As a result, lentiviral vectors have become one of the most powerful gene transfer vehicles. They are widely used for therapeutic purposes as well as in studies of basic biology, due to their unique characteristics. Lentiviral vectors have been successfully employed to mediate durable and efficient antigen expression and presentation in dendritic cells both *in vitro* and *in vivo*, leading to the activation of cellular immunity and humoral responses. This capability makes the lentiviral vector an ideal choice for immunizations that target a wide range of cancers and infectious diseases. Further advances into optimizing the vector system and understanding the relationship between the immune system and diseases pathogenesis will only augment the potential benefits and utility of lentiviral vaccines for human health.

Keywords

lentiviral vector; vaccine; immunization; cancer; infectious diseases

Introduction

After the first promising report of a vaccination regime utilizing a recombinant vaccinia virus expressing the hepatitis B surface antigen (1-2), clinically suitable viral vector vaccines have been investigated. The optimal vector vaccine would maximize the immune response and be capable of targeting the most difficult antigens, including human immunodeficiency virus-1 (HIV-1) and cancers, while remaining safe for human applications (3). Lentiviral vectors (LVs) are uniquely suited for vectored vaccine applications due to their capacity for encoding large transgenes (up to 8kb) (4), efficient transduction of both diving and non-dividing cells (5), ability to maintain persistent gene expression by integrating the transgenes into the host cell genomes, absence of preexisting anti-vector immunity (6), low anti-vector host immunity (7-8), and low potential for genotoxicity due to insertional mutagenesis (9-10).

LVs have been shown to be excellent delivery vehicles for antigens of infectious diseases or cancers for vaccination purposes to elicit effective cellular immunity and humoral responses. Their large capacity allows for the delivery of large antigens and even multiple antigens to aid in the manipulation of antigen-presenting cells (APCs) to optimize the induction of antigen-specific immunity. Another key feature of LVs is the low anti-vector immunity

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inherent in host organisms, which is critical for the LVs to avoid the rapid clearance of the vectors and the transgene-expressing cells by the host. This results in efficient antigen expression and presentation *in vivo* and allows for the possibility of multiple rounds of immunizations. Collectively, these features make LVs a more attractive vector system than adenoviral (11) and adeno-associated viral (12-15) vectors. γ-Retroviral vectors (γ-RVs), another vector system that has been studied, carry the risk of severe insertional mutagenesis in patients (16); however, LVs have been shown to provide a lower risk of genotoxicity from transgene integration and are therefore favored (10). In addition, the recent development of integrase-defective LVs (IDLVs) further reduces the risk of insertional mutagenesis, while the vector retains the ability to efficiently transduce dendritic cells (DCs) and deliver antigens to generate an immune response (17-23).

The immune responses resulting from LV vaccines have been studied using various model antigens as well as viral and tumor antigens. Vaccinations by LV-transduced DCs or the direct injection of LVs have resulted in high levels of T-cell immunity and antibody responses. Several recent reviews (24-29) have been published that describe the progress and applications of LVs for vaccination purposes. In this review, we focus on the immunogenicity of antigen-encoding LVs, common strategies for LV-based immunizations, and summarize the progress of ongoing research in LV vaccines against cancer and infectious diseases.

Lentiviral vectors

What are the components of LVs?

LVs are derived from the lentivirus, which is a type of retrovirus. Other types of retroviruses include oncoretroviruses and spumaviruses. Retroviruses are enveloped RNA viruses that contain three main genes, *gag*, *pol*, and *env*, while lentiviruses contain three to six more viral proteins that are responsible for viral replication and a persistent infection. For example, most LVs are based on the HIV-1, which includes six accessory proteins: *tat*, *rev*, *vif*, *vpr*, *nef*, and *vpu* (6).

The third-generation HIV-1-based LV

The currently used HIV-1-based LV is a third-generation vector with significant changes to improve the safety and efficiency of the vector. Nonessential viral genes were removed from the construct, including *vif*, *vpr*, *nef*, and *vpu*. The use of a CMV/LTR hybrid construct led to *tat*-independent vector production and an increase in vector production efficiency (30). In addition, the design of a complete codon-optimized HIV-1 *gag-pol* gene resulted in a *rev*-independent LV (31). To further improve the safety of the vector, self-inactivating (SIN) LVs were developed (4). Deletions in the transcriptional activation unit in the 3' U3 region of the LTR lead to the inactivation of the LTR, lowering the risk of recombination with the wildtype virus. However, studies have shown that even SIN LVs are capable of producing full-length genomic transcripts that can be encapsulated and integrated (32). Another sequence, the *rev*-responsive element (RRE) is also included in LVs. This section of the viral RNA is responsible for binding to the rev protein, provided in *trans*, to export the full-length viral RNA genome out of the nucleus. Recently, the central polypurine tract (cPPT), which is another *cis*-acting sequence, has been added to the *pol* ORF (33). This sequence is able to increase transduction efficiency by improving the nuclear import of the proviral DNA. To bypass the restrictive host range of the HIV-1 *env* glyocoprotein, LVs have been pseudotyped with various viral glycoprotiens such as vesicular stomatitis virus glycoprotein (VSV-G) with great success (34).

Recent advances in LV designs and applications

LVs have been studied and shown to be potent for both *ex vivo* and *in vivo* gene transfer into dividing and non-dividing cells. HIV-1-based LVs have been successfully used for *ex vivo* gene delivery into stem cells and also for the generation of induced pluripotent stem cells (23). In addition, targeting LVs have been created with specific ligands or antibodies incorporated into the vector envelope and integration-deficient LVs have been studied to reduce the risk of insertional mutagenesis. Hybrid LVs have also been designed utilizing transposon and finger nuclease technology. MicroRNA-regulated vectors have been successful in suppressing immune responses towards the transgene products and the transduced cells (23).

Production of LVs

LVs are typically produced by transiently transfecting producer cells with the vector construct and the packaging constructs. *Gag* and *gag/pol* precursor proteins then package the RNA genomes at the cellular membrane, and vector particles leave the producer cells by budding through the cellular membrane, taking up envelope glyocproteins in the process. Although this method allows for the production of high-titer LVs, it is impractical for large-scale manufacturing processes and regulatory considerations due to its cumbersome nature and difficulty to scale up (6). To address these concerns, stable packaging cell lines have been developed that are able to stably express the viral genes that are required for vector production. However, new limitations arise with this vector production system (23). First, the viral protease encoded in the *pol* gene is intrinsically cytotoxic. Second, the envelope glycoprotein, for example, VSV-G, is also toxic when it is expressed in the cells. To combat these concerns, Rev and VSV-G expression are regulated at the transcriptional level with a Tet-On, Tet-Off, or cumate switch. With these modifications, stable packaging cell lines have consistently produced high-titer LVs ($>10^7$ TU/ml) for months with no sign of vector rearrangements (23). For SIN vectors, high titers can be achieved by stably transfecting packaging cells by concatemeric array transfection (6,23,35).

Antigen presentation through DC activation and maturation

DCs have been found to be the most powerful APC, capable of controlling autoimmunity to self-antigens and initiating immune responses by stimulating both T cells and B cells (36-37). In early studies using DCs to develop immune resistances against infectious diseases and tumors, the primary strategy was to generate DCs *ex vivo*, load them with antigens and peptides, and re-infuse them back into the host (38-39). However, the discovery that viral vectors could be effectively used for gene delivery led to a new strategy, one in which antigens are genetically expressed in DCs to provide more long-lasting antigen presentation and more potent T-cell-mediated immune responses. LVs have been shown to be very effective in delivering genes into DCs (40-44), and DCs that are transduced by antigen-encoding LVs are able to efficiently present the antigens and provide antigen-specific responses either *ex vivo* (45-48) or through re-injection to the host (47,49-51). The *ex vivo* strategy faces some limitations. For example, a small number of the injected DCs migrate to draining lymph nodes (52) and the preparation of *ex vivo* antigen-loaded DCs is a time-consuming process. However, the direct injection of antigen-encoding LVs for *in vivo* immunization is a strategy that can bypass these limitations (25). LVs have been shown to have great potential for immunizations, due to the high immunogenicity of antigens delivered by both *ex vivo* DC delivery or *in vivo* DC transduction, and should therefore be examined for further applications in vaccinations against infectious diseases and tumors.

To efficiently present transduced antigens on DCs and generate antigen-specific responses, LVs must not only transduce the DCs but also stimulate the DCs to mature to initiate the

immune responses, as opposed to generating tolerance to the antigen. It has been previously shown that LV transduction of DCs does not affect the process of DC maturation, permitting efficient and TAP-dependent major histocompatibility complex (MHC) class I antigen presentation (48). To target the MHC class II presentation pathway, antigens were fused with a part of the transferrin receptor that included a membrane-anchoring region or the C-terminal of the invariant chain (53). This strategy resulted in the efficient stimulation of $CD4^+$ cells both *in vitro* and *in vivo*. As the LV-delivered antigens are able to provide immunogenicity through DCs, it is believed that the DCs still function effectively as APCs and may possibly even be activated by the LV transduction through innate pattern recognition receptors (PRRs) (25). For example, one study reported that transduction of human monocyte-derived DCs by LV at a high MOI (multiplicity of infection) resulted in the phenotypical and functional maturation of the immature DCs, evidenced by the upregulation of CD83, MHC class II, and costimulatory molecules, an enhanced allo-stimulatory capacity, and an increase in the secretion of interleukin-1β (IL-1β), IL-6, and IL-12 (54). It was shown that the protein kinase R (PKR) pathway was activated by the LV transduction, which in turn resulted in the activation of the nuclear factor- κB (NF-κB) pathway. Other studies have shown that wildtype HIV-1 is able to activate plasmacytoid DCs (pDCs) *in vitro* to secrete type I interferon (IFN), which is a powerful antiviral cytokine able to induce the maturation of both pDCs and other DC subsets (55-57). Toll-like receptor 7 (TLR7), a PRR for single stranded RNA (ssRNA), is also involved in this DC activation mechanism. It is therefore believed that LVs are also capable of stimulating similar innate immune responses in hosts. Brown *et al*. (58) were able to demonstrate that type I IFN was produced by pDCs after an *in vivo* administration of LVs; this result depended on vector infectivity and increased vector clearance. Although vector clearance is a result of activated innate immune responses against LVs and is therefore undesirable for efficient gene delivery, the activation and maturation of DCs can help trigger and enhance the immune responses towards the delivered antigens and is thus a powerful tool for the manipulation of the immune system. A recent study by the Collins' group (59) investigated the effect of LVs on myeloid DCs (MDCs), the major subset of transduced DCs after a subcutaneous immunization. Both cell entry and reverse transcription were shown to affect the activation of DCs *in vitro*. DCs transduced *in vivo* were able to secrete tumor necrosis factor-α (TNF-α) and stimulate naive $CD8^+$ T cells, displaying both the mature phenotype and functions. They determined that the DC activation by LVs was mediated by TLR3 and TLR7 and that a lack of TLR3 or TLR7 hindered the ability of the transduced DCs to trigger antigen-specific $CD8^+$ T-cell responses.

Ex vivo LV-transduced DCs for tumor and viral vaccines

DCs have now been generated and transduced by LVs encoding antigen transgenes *ex vivo* for use as immunity-boosting vaccines with positive results. It had been found that LVs were able to deliver genes to DCs in a persistent, nontoxic, and non-immunogenic manner, which contrasts greatly with the high MOI requirement and highly immunogenic effects resulting from the use of adenoviruses for the transduction of DCs (29). DC-delivered antigens have been shown to be immunogenic in patients with cancer or chronic HIV infection, proving that DCs can be successfully employed in a vaccine regime (39). To perform the clinical studies on DCs for vaccine purposes, methods to generate large quantities of DCs *ex vivo* had to be developed (36). Human DCs have been generated from $CD34^+$ hematopoietic progenitors that were purified from either cord blood or bone marrow, cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) and TNF-α. Human peripheral blood mononuclear cells (PBMCs) and murine bone marrow cells have been also developed into DCs with the aid of GM-CSF and IL-4.

It has been studied and previously shown that DCs transduced by LVs to express tumor-associated antigens (TAAs) are able to activate cytotoxic T-lymphocyte (CTL) responses *in vivo* and/or provide protection against tumor challenges. Compared with using electroporation to deposit mRNA into murine DCs, LV transduction of the DCs was more effective in inducing ovalbumin (OVA)-specific CTLs *in vivo*, without negatively impacting the viability, phenotype, or allostimulatory capacity of the DCs (60). Mouse models have also been used to test and confirm the ability of *ex vivo* transduced, bone marrow-derived DCs (BMDCs) to express melanoma antigens [murine tyrosinase-related protein 2 (mTRP-2) and melanoma antigen recognized by T cell 1 (MART-1)], hepatoma TAAs [stem cell antigen-2 (Sca-2), glycoprotein 38 (GP38), and cellular retinoic acid-binding protein 1 (RABP1)], and a prostate tumor antigen (the kinase-deficient form of erbB2, erbB2tr), and the subsequent successful development of antitumor immunity (Table 1). Another study performed by Koya *et al*. (61) also investigated an immunization strategy where mouse bone marrow cells were co-transduced by LVs expressing both GM-CSF and IL-4 and LVs encoding the melanoma antigens MART-1 or TRP-2. This combination of LVs was intended to drive the autonomous differentiation of the bone marrow cells into DCs. When human APCs were transduced by LVs that expressed the melanoma-associated antigen tyrosinase, it was discovered that monocyte-derived DCs were more potent for *in vitro* T-cell recognition than both CD40L-activated B lymphocytes and Epstein Barr virus (EBV)-transformed B lymphocytes (44).

LVs have been used to deliver viral antigens to DCs generated *ex vivo*, which has resulted in efficient antigen presentation by the DCs to generate antigen-specific antiviral immunity. Mouse BMDCs displaying the lymphocytic choriomeningitis virus (LCMV) glycoprotein and monkey monocyte-derived DCs displaying the simian immunodeficiency virus (SIV) gag protein have been shown to produce *in vitro* CTL responses and antigen-specific $CD8^+$ T-cell responses (Table 1). In addition, human monocyte-derived DCs have been transduced *in vitro* with LVs incorporating either the flu peptide sequence or HCV non-structural genes to successfully generate flu-specific CTL and antigen-specific $CD8^+$ T-cell responses to HCD-NS3, NS4a, and NS5b. Another study reported that an immunization of mouse BMDCs using LVs that incorporated LCMV gp_{33-41} was able to provide protection against both intravenous (IV) and peripheral routes of LCMV infection (Table 1).

Direct injection of LV for *in vivo* immunization

Immunization through different routes

The success of the *ex vivo* vaccination studies using DCs transduced by LVs has encouraged studies of direct immunizations using LVs as vaccine carriers. A recent study has shown that LVs delivered subcutaneously resulted in the direct transduction in skin-derived DCs with potent and prolonged antigen presentation (62). These DCs then migrate to skin-draining lymph nodes and prime naive T cells, causing strong antigen-specific lytic activity *in vivo*, resulting in effective antitumor immunity. This phenomenon was also confirmed by other studies in which DCs modified by green fluorescent protein (GFP)-encoding LVs (GFP-LVs) were detected in skin-draining lymph nodes (63-66). Furthermore, a recent study revealed skin-derived dermal DCs (dDCs) as the major active APCs for antigen presentation and $CD8^+$ T-cell priming following intradermal immunization with LVs, whereas skin langerin-expressing positive dermal DCs exhibited dispensable minor contribution and skin Langerhans cells were not involved (67). It was also shown that antigen expression peaked during the two weeks after immunization, and migration of skin dDCs in the initial 10 days post-immunization was critical for optimal induction of the $CD8^+$ T-cell response. Subcutaneous immunization by LVs incorporating the model antigen OVA (24,66) and the human melanoma antigen NY-ESO-1 (64) resulted in effective $CD8^+$ and $CD4^+$ T-cell immune responses. When LVs encoding the mouse melanoma-associated antigen TRP-2 and

NeuED were subcutaneously injected, significant antitumor activity was observed in a B16 subcutaneous melanoma model, a GL-26 subcutaneous glioblastoma tumor model, and a BALB/c-Neu transgenic mouse-based spontaneous breast carcinoma model (63).

Although DCs have a relatively short lifespan (1-2 weeks), the systemic delivery of LVs resulted in persistent and long-lived gene expression in APCs *in vivo*. A study in adult severe combined immunodeficiency (SCID) mice demonstrated efficient and stable gene transfer into the liver and spleen through IV administration of GFP-LV. Normal BALB/c mice also experienced efficient gene transfer to MHC class II^+ APCs (including DCs and B cells) in the spleen (33). In another study, LVs expressing GFP were injected through the IV route, and splenic DCs, including mDCs and pDCs, were transduced (53). However, this transduction was not particularly DC specific, as 5.96% of the cells that were transduced were $CD11c^-$, including 4.07% B cells. Studies using firefly luciferase as a gene reporter and noninvasive optical imaging analysis have demonstrated the persistence of the gene transfer; the gene of interest was shown to be expressed for several months following transduction (68). Thus, IV immunization by antigen-expressing LVs can be efficiently utilized in stable gene transfer to splenic APCs, and DC precursors can be transduced to maintain long-term antigen presentation in the DCs and induce effector memory T cells. In one study, GFP-LVs were found in lymphocytes, macrophages, and all subsets of DCs in the spleen 5 days post-IV injection (65). Bromodeoxyuridine (BrdU) incorporation tests showed that these DCs were the progeny of dividing precursor cells and that they remained detectable 2 months post-injection, resulting in prolonged antigen presentation and effective T-cell memory. NY-ESO-1 LVs injected by IV have also been shown to induce NY-ESO-$1_{157\text{-}165}$-specific $CD8^+$ T cells, which were expandable by a boost with an NY-ESO-1 vaccinia virus and were capable of killing NY-ESO-$1_{157\text{-}165}$ peptide-pulsed targets *in vivo* (64,69).

Another route of injection, the intramuscular (IM) route, has been shown to be an effective means to induce strong cell-mediated immune responses against secreted proteins. Polymerase chain reaction (PCR) amplification of DNA extracted several months after an immunization showed vector persistence at the injection site, suggesting that sustained antigen expression and prolonged immune responses could be achieved through this route (70-71). One study utilized SIN LVs that incorporated either the full-length HIV-1HXB2 Rev/Env (TY2-IIIBEnv) or codon-optimized HIV-$1_{JR\text{-}FL}$ gp120 (TY2-JREnv) to perform a single IM immunization that resulted in durable CTL responses (72). Although the LVs encoding the codon-optimized TY2-JREnv generated detectable anti-gp120 IgG responses, there was a notable absence of neutralizing antibodies. Another study also demonstrated that a single IM injection of an SIN HIV-based LV encoding a codon-optimized SIV gag sequence was capable of inducing cellular and humoral immune responses *in vivo* (73). Other studies examined the use of IDLV in a single IM immunization regime. For example, an IDLV encoding a codon-optimized HIV-1 gp120 envelope sequence with a murine GM-CSF sequence was found to be efficient in inducing persistent $CD8^+$ T-cell responses, resulting in CTLs with activity specific to Env and multicytokine-secreting capabilities (70,74). Another study used an SIV-based IDLV and chose GFP as the model antigen (75). These IDLV were shown to be efficient in eliciting both specific and persistent polyfunctional $CD8^+$ T-cell responses. A recent study examined the IM injection of IDLV expressing the secreted hepatitis B virus (HBV) surface antigen and demonstrated both antibody and systemic $CD8^+$ T-cell responses to the antigen (71).

The intraperitoneal (IP) injection route is also applied in LV immunization studies. For a study investigating an LV-based vaccine incorporating HIV-1 gag, we compared the efficiencies of various routes in inducing gag-specific $CD8^+$ T-cell responses. It was observed that the IP route resulted in measurable gag-specific $CD8^+$ T cells and gag-specific

antibody titers (76). In a study utilizing the IP immunization route, polyepitope-encoding LVs were successful in inducing strong, diversified, and long-lasting CTL responses in both HLA-A2 and HLAB7 transgenic mice models (77). A single IP immunization with LVs or IDLVs carrying a secreted form of the WNV envelope protein was sufficient to induce strong antibody responses and protected the immunized mice against a lethal dose of WNV (78-79).

Prime/boost regimens

LVs are powerful tools for immunizations, because they are able to transduce both DCs and other APCs efficiently, resulting in long-term antigen expression and presentation. On the other hand, peptide or protein vaccinations can only provide temporary antigen presentation. The durability of antigen presentation after a direct injection of LVs has been the subject of several studies; it was determined that antigen presentation was measurable up to several weeks post-immunization (62,65). The duration of antigen presentation is positively correlated with the effectiveness of generating antigen-specific T-cell responses and memory T cells, which is likely due to the extended interaction between the APCs and the T cells. Although it is currently unknown why LV immunizations and chronic infections produce different durations of antigen presentation, LV immunizations usually generate long-term, functional, antigen-specific $CD8^+$ T cells (69,80) that display memory phenotypes (81).

One particular difficulty in generating tumor-specific T-cell responses is the high frequency of responses required to slow tumor progression, coupled with the poor immunogenicity of tumor/self-antigens (82). Multiple rounds of vaccination are one method used to counter this problem and produce protective memory T cells. The prime/boost strategy is also employed in vaccinations against viral infections, as it has been shown that a higher number of memory $CD8^+$ T cells could provide better protection against infections in animal models (83).

Prime/boost immunizations with LVs are attractive because the rarity of pre-exposure to lentiviruses in the hosts results in a general lack of pre-existing immunity against LVs (6). In addition, LVs themselves do not induce vector-specific immunity; they only experience weak host immunity against the pseudotyping envelope (84). One method, the homologous prime/boost regimen, has been shown to be successful for LVs encoding the model antigen OVA (80), Melan-A minigene (85), and HIV-1 polyepitope (77). We have previously demonstrated that an immunization strategy using a boost with the same LV encoding HIV-1 gag resulted in both a significant increase in gag-specific $CD8^+$ T cells and T cells reactive against a larger range of gag-derived epitopes (76). This homologous prime/boost regimen also significantly increased the functionality of gag-specific T cells in secreting multiple cytokines and enhanced the gag-specific antibody response compared with the DNA prime/adenovirus boost and DNA prime/LV boost strategies. Although enhancement in the immune response was only seen in increase of the number of responding mice in a previous study utilizing LVs encoding an HIV-1 polyepitope (77), this was probably a result of the different levels of anti-vector immunity experienced by the hosts.

To avoid the possibility of host anti-vector immunity, heterologous prime/boost strategies are used to broaden or strengthen a vaccination response. For example, an LV prime could be followed by boosting with a peptide (81,86), vaccine virus (69), adenovirus (87), or LVs with an envelope from a different serotype (84). In one study, a heterologous boosting vaccination using the hTERT peptide pY572 with hepatitis B-derived helper peptide emulsified incomplete Freund adjuvant (IFA) greatly expand the number of tumor-specific $CD8^+$ T cells and improve their functional activity to kill tumor cells. This led to an enhanced therapeutic and protective effect against tumor progression (86).

Adjuvants and coexpression of immunostimulatory molecules

Adjuvants are agents that supplement antigens and are widely used in immunization to improve adaptive immune responses to antigens (88). Adjuvants interact with the innate immunity to trigger signaling cascades that boost both T and B-cell responses. It has been shown that microbial adjuvants interact with innate immunity receptors, such as TLRs, and that T-cell adjuvants have varying effects on the programming of DC maturation, which subsequently affects T-cell priming. Cytokines, chemokines, costimulatory molecules, and other immunomodulators that boost and guide immune responses have been recently used as adjuvants in peptide vaccinations (89). Some studies have shown that LVs coexpressing both GM-CSF and IL-4 were capable of driving the differentiation of bone marrow cells (61) or $CD14^+$ monocytes (90) into DCs, while other studies have shown that LVs expressing CD40L (91) and gp34/OX40L (92) induced the self-maturation of DCs *ex vivo* and enabled alloreactive $CD4^+$ T-cell responses *in vitro*, respectively. Thus, antigen-bearing LVs that target DCs to generate immune responses can also benefit from the use of adjuvants that enhance the ability of DCs to prime T and B-cell responses.

Human hsp70, which is believed to promote DC function, was fused to tumor antigens in one study to generate tumor-specific $CD8^+$ T-cell responses and therapeutic antitumor immunity (63). An improved antitumor immunity was seen with the LV-delivered, hsp70-fused tumor antigens in comparison with LVs delivering tumor antigens alone, suggesting that hsp70 had an adjuvant effect on the vaccination. Another study examined IL-7 treatment post-LV immunization and showed that the adjuvant could also be targeting antigen-specific $CD8^+$ memory T cells that expressed the survival/memory marker CD127 [IL-7 receptor α chain (IL-7Rα)] (81,93). IL-7 can also be used to upregulate the survival molecule Bcl-2 to expand the numbers of antigen-specific effector and memory $CD8^+$ T cells.

Another strategy to enhance immune responses is to target elements of the DC signaling pathway. For example, the activation of DCs through p38 resulted in a greatly enhanced level of antitumor immune response and the subsequent prolonged survival of tumor-bearing mice. In contrast, the activation of extracellular signal-regulated kinase (ERK) increased the expression of both TGF-β and a constitutively activated interferon regulatory factor-3 (IRF3), resulting in the stimulation of IL-10-secreting DCs. This effectively suppressed the immune response and also stimulated the expansion of regulatory T (Treg) cells (94). In another study, the expression of vFLIP from Kaposi's sarcoma-associated herpesvirus (KSHV) on an LV was shown to activate NF-κB in mouse bone marrow-derived DCs *in vitro*, leading to the maturation of DCs (95). This was evidenced by the upregulation of costimulatory molecules and the secretion of both TNF-α and IL-12. This effect was also seen on DCs *in vivo*, and the use of the model antigen OVA led to a large increase in OVA-specific $CD8^+$ T cells, which correlates with an improved antitumor immunity in a tumor therapy model. An enhancement in the immune response was also seen with the coexpression of OVA with either vFLIP or the MKK6 protein, which stimulates the p38 pathway, using IDLVs (71).

APC-targeting LV

The ability of LVs to target and transduce specific cell types is an important feature for the advancement of LVs for clinical purposes, as it can help to avoid the negative effects of transgene expression in non-target cells. Many studies have focused on developing targeting LVs (96-98), and Froelich *et al*. (99) have also reviewed the strategies of targeting vectors to specific cell types by either LV surface modifications or utilizing transcriptional targeting through tissue-specific promoters. This second approach has been successful in restricting transgene expression in immune cells, such as APCs, T cells, B cells, hematopoietic stem

cells (HSCs), macrophages, etc. Since LVs are known to preferentially integrate into transcriptionally active sites, additional features, such as insulators, inducible expression, and the use of IDLVs may be necessary to prevent non-specific transcription activated from upstream genes.

The other strategy involves incorporating ligands, peptides, cytokines, or single-chain antibodies, or using molecular bridges such as ligand-receptors, avidin-biotin, chemical conjugations, and monoclonal antibodies, into the pseudotyping envelope of the LV. However, the modified LVs are limited by low transduction efficiencies, and a co-display of VSV-G with the targeting envelope raises the transduction efficiency but also removes the targeting specificity. Thus, a system that mimics the natural viruses that utilize an attachment protein and a fusion protein as two distinct molecules to separate the functions of binding and fusion appears to be an attractive strategy for engineering targeting LVs.

In a study using the γ-RV, Lin *et al*. (100) engineered a binding-defective, fusion-competent hemagglutinin (HA) protein, derived from the fowl plague virus, to serve as a fusion protein, and a chimeric glycoprotein with a specificity to the Flt-3 receptor as a binding protein. They demonstrated that the two proteins were able to work together to mediate the targeted transduction of Flt-3-expressing cells *in vitro*. Another study recently showed that the envelope glycoprotein of the measles virus (MV), which includes a protein capable of receptor recognition, hemagglutinin (H), and a fusion protein (F), could used to pseudotype LVs by truncating their cytoplasmic tails. These vectors could be further modified by fusing either a single-chain antibody against CD20 or an epidermal growth factor to the mutant H protein, resulting in a loss of native receptor recognition by the glycoprotein (101).

Another envelope glycoprotein that has been modified in such a way is the Sindbis virus glycoprotein. The native binding recognition sites were mutated so that the envelope protein only retained its fusion function (102). The co-incorporation of this mutant glycoprotein with a CD34 antibody on an LV surface resulted in the specific transduction of $CD34^+$ cells in a sample of non-purified human mobilized PBMCs (103). We incorporated this mutant Sindbis glycoprotein onto LVs as a fusion protein, along with membrane-bound anti-CD20 antibody to confer the binding specificity. This resulted in the preferred transduction of cells expressing CD20, a B-cell marker (104-105). The Sindbis virus fusogen molecule was further mutated to elevate the fusion functions in a pH-dependent manner (105). This system was also useful for targeting monospecific Ig-expressing B cells (106), $CD3^+$ T cells (107), and CD117-expressing HSCs (108). To better understand the interactions between the binding and fusion processes of the LVs and the target cells, the GFP-Vpr protein was incorporated into the LVs to label and track the vector particles by confocal microscopy. We concluded that the LVs specifically targeted the desired cell types and that the release of the viral core into the cytosol correlated with maturation of the endosomes and occurred following virus-endosome fusion in the early endosomes (109).

The ability to target APCs, especially DCs, is important for *in vivo* immunizations using LVs because it improves both the efficacy and safety of the procedure (110). Studies have examined the use of tissue-specific promoters, such as the dectin 2 gene promoter for use in DCs (64), and the MHC class II promoter, which is active in APCs (68), to drive the expression of tumor antigens and/or induce antitumor immune responses. In one study that utilized bone marrow chimeras, the use of the DC-STAMP gene as a promoter region allowed the transcriptional targeting of DCs by LVs, resulting in the induction of antigen-specific $CD4^+$ and $CD8^+$ T-cell tolerance *in vivo* (111). To target APCs, single-chain antibodies that recognized MHC class II molecules were fused to the amino terminal of the amphotropic murine leukemia virus envelope (MLV-A) protein, generating an increased level of transduction of MHC class II^+ cells and augmenting the antigen-specific immune

response. However, the overall transduction efficiency was low, due to the loss in functionality of the chimeric envelope protein after the modifications were made (112). To avoid these pitfalls of inserting molecules into the glycoprotein, we engineered the Sindbis virus envelope glycoprotein to be blind to its canonical binding target, heparin sulfate, but it retained its ability to bind to the DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (66). This mutant glycoprotein was termed SVGmu and was able to target both murine and human DCs expressing DC-SIGN with the pseudotyped LVs. The direct injection of SVGmu-pseudotyped LVs encoding the model antigen OVA resulted in the specific transduction of DCs, confirmed by the GFP reporter gene, and conferred both protective and therapeutic effects against tumors expressing OVA. This strategy benefits from a more efficient induction of antigen-specific responses, requiring a reduced load of vector injection. It also enjoys a built-in DC maturation effect resulting from the attachment of SVGmu to DC-SIGN and the TLR3/7-mediated DC activation provided by LV transduction (59).

Using IDLV for immunizations

The improvement of the safety profile of LV-based vaccinations remains a critical issue. The development of leukemia in children treated for X-linked severe combined immunodeficiency (X-SCID) by retrovirus-mediated gene therapy (16) has called for a reexamination of the consequences of transgene integration, once believed to be an advantage of using retroviral vectors in the gene therapy of HSCs (113). It was determined that γ-RVs preferred to integrate into the 5′ flanking regions of the gene, near the transcription start sites, while LVs were more prone to integrate across the entire transcriptional unit (114-118). Montini *et al*. (9) utilized a tumor-prone mouse model to determine that SIN LV integration resulted in low level of genotoxicity. In another study (119), a murine line was used to observe the frequency of IL-3-independent mutants and insertional mutagenesis by LVs was also seen. It has been shown recently that transcriptionally active LTRs play a major role in the determination of the likelihood of genotoxicity. LVs containing chimeric γ-retroviral LTRs carried a significantly lower risk of genotoxicity than unaltered γ-RVs, which is an observation that is supported by the intrinsic targeting of cancer genes by γ-RVs (10). The comparison of LVs and γ-RVs in the same setting provides valuable information to optimize the safety profile of LVs to avoid the adverse effects seen in γ-RV-mediated gene therapy (16,120-123). Although some developments in LV technology may reduce the risk of insertional mutagenesis, such as the use of SIN LVs, tissue-specific promoters, envelope modifications, the addition of genetic insulators, and an inducible expression system, the most effective solution is to utilize IDLVs. In these vectors, the integrase function and/or the attachment site in the LTRs have been disabled to greatly reduce the risk of insertional mutagenesis. IDLVs have been studied for use in vaccinations, homologous recombination, and site-specific integration and transportation. Several reviews have described the progress and application of IDLVs in detail (17-22).

Normally, retroviral or retroviral vector RNA is converted into linear double-stranded DNA by reverse transcription after infection occurs and the proviral integration of the viral DNA into the host cell genome is mediated by integrase (124-125). Meanwhile, nuclear proteins assist in the formation of circular episomes of viral DNA (126-129). The integrated provirus then functions as the template for viral gene expression, while the circular virus episomes exhibit very low transcriptional activity (130-131). Although levels of HIV-1 2-LTR circular episomes have been found to be stable after infection (132-134), these are quickly diluted by cell division and slowly degraded *in vivo* (135). The processes responsible for the circulization of the episomal DNA, such those for 1-LTR and 2-LTR circles, have been thoroughly discussed in a previous review (18), and quantification of the different episomes

have been conducted through quantitative PCR (136) and a novel shuttle-vector assay (137). As IDLVs lack an integration capability, they mainly produce episomal viral DNA following reverse transcription, and first-generation vectors were only able to achieve low to negligible gene expression (7,138-140). After the development of SIN LVs, IDLVs have demonstrated efficient gene expression in several studies (141-143). In one recent study, an extension of the LTR U3 deletion region that included the *cis* negative regulatory element significantly increased gene expression, suggesting that the SIN factor was able to enhance episomal expression (137). Thus, although gene expression by LV episomes is lower than that of the integrated viral DNA, efficient expression can be achieved, allowing for the use of IDLVs in gene delivery. Several studies have now demonstrated effective transduction by IDLVs in tissues such as brain, ocular muscle, and liver (137,141,144-145).

IDLVs pose minimal risk for insertional mutagenesis and can also be applied to LV immunizations due to their ability to maintain durable gene expression in non-dividing cells. DCs are slowly proliferative and can be transduced to prime adoptive immune responses. In one study, human APCs, including monocyte-derived DCs and macrophages, were transduced by IDLVs encoding the influenza matrix M1 protein. This resulted in an expansion of M1-primed $CD8^+$ T cells *in vitro* and induced multiple cytokine secretion by antigen-specific $CD8^+$ T cells (146). Therefore, although antigen expression by IDLVs is relatively low, this vector system remained effective for both antigen presentation and the induction of antigen-specific responses. IDLVs encoding various antigens, such as the codon-optimized HIV-1_{JR-FL} gp120 sequence (70,74), a secreted form of the West Nile virus (WNV) envelope (79), the model antigens GFP (75) and OVA (71,147), and a secreted HBV surface antigen (71), have also been directly injected with promising results. For example, Negri *et al*. (70) determined that a single IM injection of HIV-1_{JR-FL} gp120 was sufficient to elicit both specific and long-lasting $CD8^+$ T-cell and antibody responses for at least 90 days. The vector 2-LTR episome remained detectable in the injection site even 3 months post-immunization (70). Additional tests using a low dose of integrating LVs *in vivo* were conducted to determine whether the immune effects were a product of residual integrating vectors; this resulted in no measureable immune response, suggesting that the immune responses were generated by the non-integrated vectors. In another study, a single IP injection of IDLVs expressing the WNV envelope induced specific immune responses, capable of protecting the immunized mice against a challenge with a lethal dose of WNV (79). SIV-1-based IDLVs have also been successfully used for immunizations using the model antigen GFP (75). To test the model antigen OVA, Karwacz *et al*. (71) compared various IDLV-producing mutants, including combining mutations in the integrating function and the attachment site within the LTR, which may further improve vector safety. They reported persistent antigen presentation and effective antitumor immunity post-IDLV immunization. They also showed that the IDLV was effective in an immunization against the HBV surface antigen. However, all of the studies have shown that IDLV required higher doses for an optimal immune response, as compared to the doses required by its integrating counterpart.

We have also demonstrated that IDLVs are capable of transducing mouse BMDCs efficiently and can stimulate OT1 cells *in vitro*. In addition, a single footpad injection of IDLVs was sufficient to induce strong and long-term immune responses *in vivo* (147). IDLVs pseudotyped by SVGmu can also preferentially transduce DCs through DC-SIGN, and a higher dose of the targeting IDLV could generate the same level of persistent immune responses as its integrating counterpart (148). We believe that this DC-targeting IDLV is eventually cleared from the host, due to the short lifespan of the DCs, and that this may provide additional safety in terms of vector recombination and mobilization. Further studies are required to determine the off-target effects and the frequency of residual integration of the antigen in transduced cells.

LV as a tumor vaccine

Antigen-specific CD8+ T-cell responses

Many studies have examined and improved our understanding of the molecular and cellular basis of T-cell-mediated antitumor responses (149-150). Importantly, $CD8^+$ T cells have emerged as potent effectors of the adaptive antitumor immune response, and tumor-reactive T cells that are activated and expanded *ex vivo* can be adoptively transferred back to the patient, resulting in potent clinical antitumor responses (151). In a mouse study, the adoptive transfer of gp100-specific pmel-1 transgenic T cells, along with antigen-specific immunization and the co-administration of a T-cell activation factor, resulted in the eradication of large, established, poorly immunogenic, unmanipulated solid B16 tumors (152). These studies suggest that tumor-specific $CD8^+$ T cells are critical for the success of tumor vaccines. Potent cytotoxic $CD8^+$ T cells that are capable of recognizing specific or diversified epitopes are induced when LVs encoding various tumor antigens are directly injected; these cells can efficiently kill peptide-pulsed target cells or antigen-expressing tumor cells both *in vitro* and *in vivo*, and can also secrete IFN-γ either alone or together with IL-2 and TNF-α after re-stimulation by the peptide (Table 1).

One difficulty in establishing effective tumor immunotherapy is due to the fact that most tumor antigens are shared by the healthy tissues and are thus also self-antigens (82,153). To combat this, peptide ligands were created in one study with specific point mutations to increase their binding affinity to MHC class I molecules and thus experienced increased antigen presentation of multiple epitopes of a non-immunogenic melanoma tissue self-antigen TRP-1 (154). Also, Asn-Gln mutations were included to alter the glycosylation pathway and increase antigen processing. Immunizations of mice with the mutated TRP-1 vaccine resulted in the induction of cross-reactive $CD8^+$ T-cell responses against many non-mutated epitopes and melanoma cells. In addition, the immunized mice were effectively protected against a B16 melanoma challenge, with the prolonged survival of mice receiving 4 consecutive immunizations post-tumor challenge. The success of this strategy prompted the design of LVs encoding the mutated TRP-1, resulting in potent and long-lasting $CD8^+$ T-cell responses against multiple TRP-1 epitopes (155); the activated $CD8^+$ T cells were also able to recognize the wildtype TRP-1 epitope. As many as 10% of the $CD8^+$ T cells were effector cells against the TRP-1 antigen, able to kill the wildtype TRP-1 peptide-pulsed target cells *in vivo* and also produce IFN-γ following *ex vivo* stimulation. Immunized mice were protected from a B16 tumor challenge, and tumor-bearing mice exhibited $CD8^+$ T-cell responses as a result of the LV immunization. Tumors of immunized mice experienced dramatically higher numbers of T cells that were functional and produced IFN-γ; in particular, the numbers of $CD8^+$ T cells were greatly boosted. The level of the response generated was sufficient to eradicate small, 3-day subcutaneous B16 tumors and inhibit the growth of 5-day tumors.

Antigen-specific CD4+ T-cell responses

Another critical component to a successful immunization for antitumor immunity is the $CD4^+$ helper T cell (149,156). These cells are needed to help primary $CD8^+$ T cells *in vivo* (157) and to reactivate memory CTLs to kill tumors (158). $CD4^+$ T cells are able to help $CD8^+$ T cells through a mechanism called DC licensing (159). This involves the capture of antigens from necrotic or apoptotic cells by DCs, processing of the antigen for MHC class II-restricted presentation to $CD4^+$ T cells, which then upregulate CD40L expression and interact with CD40 to activate the DCs, which in turn present the processed MHC class I-restricted antigen to naive $CD8^+$ T cells. Also, tumor-specific $CD4^+$ T cells are capable of lysing MHC class II^+ tumor cells after stimulation with melanoma antigen peptides (160-161). Helen *et al*. (53) studied the stimulation of antigen-specific $CD4^+$ T cells, which

are important for developing protective immunity against tumors and infectious diseases. LVs were produced to express membrane-bound OVA antigen through fusion to either the invariant chain (Ii-OVA) or the transferrin receptor (TfR-OVA) to target the MHC class II presentation pathway. DCs transduced by either vector *in vitro* were able to stimulate OT-II ($CD4^+$, OVA TCR transgenic) T cells, and adoptively transferred OT-II T cells could be activated *in vivo* by direct immunization with either vector. The fact that the Ii-OVA vector more potently induced IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells and that it also afforded complete protection against the OVA-expressing tumor suggested the importance of antigen-specific $CD4^+$ T cells in developing antitumor and antiviral immunotherapy. Later studies also confirmed the induction of antigen-specific $CD4^+$ T-cell responses through LV immunization. In one study, LVs expressing the OVA antigen were able to activate the OVA-specific $CD4^+$ T cells necessary for an adequate primary and memory CTL response (80). In another study, an LV encoding the human melanoma antigen, NY-ESO-1, stimulated a $CD4^+$ T-cell response against a newly identified NY-ESO-$1_{86\text{-}99}$ epitope that was presented by H2 I-Ab (64). Furthermore, depletion of the $CD4^+$ T cells resulted in the complete abrogation of B-cell and $CD8^+$ T-cell responses against NY-ESO-1 (162). Thus, vaccines that are able to stimulate both $CD4^+$ and $CD8^+$ T-cell responses will result in better overall immune responses.

Memory $CD8^+$ T cells

Although tumor-specific effector T cells are responsible for attacking tumor cells and initiating tumor regression, their subsequent decline and differentiation into memory T cells suggests that long-term protection against tumors depends on both the quantity and quality of the memory T cells generated (83). Memory T cells are able to migrate to non-lymphoid tissues and mucosal sites to aid in the surveillance of the immune system. They can also undergo rapid expansion with secondary antigen exposure, express IFN-γ and cytotoxic molecules, and survive over a long period of time under homeostatic cell division. Thus, the generation of tumor-specific memory T cells after an immunization is highly desirable, and responses conferred by LV immunizations have been observed through successful boosting of the $CD8^+$ T-cell response (69,80) and the direct characterization of antigen-specific $CD8^+$ T-cell phenotype by tetramer staining (81). In one study, memory $CD8^+$ T cells were not only quickly recalled 150 days after the priming immunization but also retained potent cytotoxicity against the tumor cells (80). Another study examined memory $CD8^+$ T cells and the effect of an *in vivo* immunization with LVs encoding the melanoma-associated antigen Melan-$A_{26\text{-}35}$, which contains a mutation to improve the affinity of the resulting peptide analogue (81). It was discovered that the Melan-$A_{26\text{-}35}$ $tetramer^+$ $CD8^+$ T cells expressed the memory precursor marker CD127 at a high level when the immune response was at its peak (~14 days). After determining that the majority of the T cells expressed the memory markers CD44, Ly-6C, and CD62L, along with CD127 after the priming immunization, they concluded that the cells were antigen-specific memory T cells. A recall vaccination using the peptide in adjuvant at day 130 boosted the Melan-$A_{26\text{-}35}$ $tetramer^+$ $CD8^+$ T cells and further confirmed the presence of the memory T cells.

LVs as vaccines against infectious diseases

Viral vector vaccines have been shown to induce strong cellular immune responses, making them promising vehicles for targeting infectious diseases (3). In an early study, recombinant vaccinia virus was used to express the HBV surface antigen; this resulted in the generation of strong and rapid antibody responses, granting protective immunity against HBV infection in both rabbits and chimpanzees (1-2). Subsequent studies have focused on the development of more effective vectors with improved safety features. An effective vaccine induces antiviral immunity that is capable of clearing both viruses and infected cells, and includes neutralizing antibodies and T cell-mediated immunity (169-172). Antibody responses

against viral infections play a key role in conferring immunity; however, $CD4^+$ T cells also play an important role for antibody production, while $CD8^+$ T cells contribute to protection against the infection (173-174). On the other hand, $CD8^+$ T cells play a major role in the control of virus infection, evidenced by the ability of adoptively-transferred virus-specific $CD8^+$ T cells to eliminate a chronic LCMV infection (175). CTLs are able to kill infected cells by recognizing viral proteins loaded on the target cell membrane, while they can also secrete antiviral cytokines to limit virus replication (176). A long-term antibody response and the retention of memory B and T cells results in long-lasting immunity against re-infection (175,177-179). However, for some highly complicated pathogens, such as HIV, conventional vaccine methods are insufficient and novel vaccine strategies must be explored (3,169,171-172).

It has been previously shown that the direct injection of antigen-expressing LVs has been very effective in generating neutralizing antibodies against WNV, and also antigen-specific cellular immunity against both SIV and HIV. In one study, both conventional LVs and IDLVs were used to carry a secreted form of the WNV envelope protein. A single IP immunization was sufficient to induce strong antibody responses and protected the immunized mice against a lethal dose of WNV (78-79). Also, the neutralizing antibody response was found to be long-lasting and effective in protecting the mice against WNV infections up to 3 months post-immunization. The VIRxSYS Corporation has reported an LV vaccine candidate that expresses the HIV-1 gag, pol, and rev antigens under the control of the native LTR promoter. Vaccination with this LV resulted in antigen-specific $CD4^+$ and $CD8^+$ T cells, and an anti-HIV immunoglobulin G response (87).

Heterologous lentiviral/adenoviral (Ad) vectors have also been shown to provide immunizations that improve the frequency and polyfunctionality of HIV-specific $CD4^+$ and $CD8^+$ T cells in a mouse model. Thus, a prime/boost regimen may be an efficient method to increase the anti-HIV immune response. We have demonstrated that an immunization using DC-targeting LVs encoding HIV-1 gag was efficient in generating strong gag-specific $CD8^+$ T cells (76). Also, a homologous prime/boost regimen using LVs resulted in a broader functionality and epitope recognition of the gag-specific T cells, and also enhanced the gag-specific antibody response compared to a DNA prime/Ad boost or a DNA prime/LV boost strategy.

LVs encoding either the full-length HIV-1 HXB2 Rev/Env sequence or the codon-optimized $HIV\text{-}1_{JR\text{-}FL}$ gp120 sequence were able to elicit antigen-specific CTL responses, which reacted to *in vitro* re-stimulation with peptides and lysed peptide-pulsed cells by secreting IFN-γ (72). Immunization with $HIV\text{-}1_{JR\text{-}FL}$ gp120-expressing LVs also generated an anti-gp120 antibody response, but no neutralizing antibodies were detected. A following study showed that LVs that encoded the HIV-1 polyepitope could potently induce a strong, diversified, and durable CTL response in mouse models (77).

Although mouse models are useful for studying biological phenomena *in vivo*, they are insufficient for determining the safety and effects of treatments for humans. Thus, it is more useful and relevant to evaluate the efficiency of LVs as a vaccine strategy against SIV in a non-human primate model. LVs encoding a codon-optimized SIV gag gene were also able to efficiently induce both cellular and humoral immune responses (73). Beignon *et al*. (84) performed the first proof-of-concept study for a prime/boost vaccination strategy in non-human primates using LVs pseudotyped with one of two VSV-G proteins derived from non-cross-reactive serotypes. The LVs were able to generate robust and broad cellular immune responses against SIV gag in cynomolgus macaques and conferred a strong protection against a high-dose challenge with SIVmac251. A reduction in viremia was observed at the

peak of acute infection, and memory $CD4^+$ T cells were preserved during the acute phase, suggesting a potent level of protection against pathogenesis (84).

Conclusions

Over the years, several key developments have been made to the LV system to improve its safety and efficacy. The third-generation LVs include a SIN design to enhance its safety by reducing the risk of replication-competent recombination (4) and the potential genotoxicity arising from transgene integration (10). The development of IDLVs has further improved the safety profile of LVs by greatly reducing the risk of insertional mutagenesis (17-22). Recently, LVs have been engineered to target specific cell types by either modification of the envelope protein or through the use of tissue-specific promoters, which may also aid in reducing the risk of activating genes that are downstream of the integration sites. These features improve the desirability of using LVs for vaccine purposes. However, some safety issues still require solutions before LVs can be used in clinical vaccines. Concerns remain regarding the risk of replication-competent recombination during vector preparation and following transduction (180), vector mobilization from the full-length transcript of the integrated provirus (32) and viral episomes (181), and the low level of reverse transcription fidelity (182). Also, the use of IDLVs for gene transfer into dividing cells is dependent on enhancing the replication of episomes (18).

In terms of immunizations, the interaction between the immune system and disease pathogenesis must be better understood to enhance and direct immune responses against tumor cells and viral pathogens, not only by delivered antigens. Tumor immunotherapy faces many challenges, including the naturally immunosuppressive tumor microenvironment (82), poor immunogenicity of tumor/self antigens (153), and possibility of ‘tumor escape’ (183). A mutated form of TRP-1 (155) has been shown to successfully increase the immunogenicity of the tumor antigen. One factor affecting the immunosuppressive tumor microenvironment are Treg cells (184), and it has been found that a combination of an IDO-inhibitor drug, which converts Tregs to the Th17 phenotype, and an LV vaccine resulted in enhanced $CD8^+$ T-cell activation and improved antitumor efficacy (185). Thus, encouraging progress has been made, but further research into methods to break tumor tolerance needs to be conducted.

For the use of LV vaccines against infectious diseases, most studies have focused on HIV-1, which is one of the most challenging and dangerous human pathogens. As of yet, there are no successful HIV vaccines, and the failure of previous clinical trials has discouraged the development of AIDS vaccines (169). Many features of HIV make creating vaccines particularly challenging: there is enormous sequence diversity, infection occurs in critical immune cells, the virus is adept at avoiding the usual immunological defenses, and the virus has a period of latency. Due to the error-prone nature of reverse transcriptase, HIV can easily mutate and adapt, resulting a large spread in sequence diversity. A better understanding of the correlation between the immune system and HIV protection may be necessary for the development of an effective HIV vaccine candidate.

Lastly, because tumors and HIV are intrinsically geared towards and highly adept at immune avoidance, a vaccination alone may be insufficient in treating these complex diseases. Thus, the use of a vaccine in conjunction with another therapy, such as RNAi therapy (186) or TCR immunotherapy (151), may be beneficial for the overall treatment of the disease.

Acknowledgments

We thank our collaborators at California Institute of Technology (Prof. David Baltimore, and Dr. Lili Yang) for their contributions to LV-related research. We also thank our group members for their discussion and critical

reading of this manuscript. Research in our laboratory is supported by the National Institute of Health, Bill & Malinda Gates Foundation, Keck Foundation.

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Table 1

Lentiviral vectors as tumor vaccines by *ex vivo* transduction of antigen-presenting cells or direct *in vivo* injection

Tumor antigen	Vector type	Vaccine details	Immunity	Tumor suppression	Ref.
Hepatoma mTAAs (Sca-2, GP38, RABP1)	HIV-1 SIN LV/2 VSV-G	Twice S.C. injection of LV-transduced BMDCs in BALB/c weekly	CTL, secreting IFN-γ	Yes	(163)
Prostate cancer murine erbB2tr	HIV-1 SIN LV /2 VSV-G	Twice I.P. injection of LV-transduced BMDCs in C57BL/6 at 2 week interval	erbB2tr-specific Th1 cytokine and antibody	Yes	(164)
Melanoma human MAGE-A3	HIV-1 SIN LV /2 VSV-G	Human MoDCs transduced by LV	CTL (new epitope EGDCAPEEK)		(49,60,165)
Melanoma murine TRP2	HIV-1 LV/1 VSV-G	S.C. injection of LV-transduced BMDCs in C57BL/6	T-cell response	Yes	(51,166)
Melanoma murine TRP2	HIV-1 SIN LV/3 VSV-G	Twice I.V. injections of LV in C57BL/6, 1 week apart	Ag-specific $CD8^+$ T cells, secreting IFN-γ	Yes	(68)
Melanoma mTRP2 Breast tumor NeuED	HIV-1 SIN LV /2 VSV-G	S.C. injection of LV in C57BL/6 or BALB/c-Neu	Ag-specific $CD8^+$ T cells, secreting IFN-γ	Yes	(63)
Melanoma human Melan-A	HIV-1 SIN LV/2 VSV-G	Human MoDCs transduced by LV	Antigen specific $CD8^+$ T cells, secreting IFN-γ	N/A	(167)
Melanoma hMART1, mTRP2	HIV-1 SIN LV/3 VSV-G	S.C. injection of BM cells cotransduced by LV- GM-CSF/IL-4 + LV-hMART 1/mTRP2 in C57BL/6	Antigen specific $CD8^+$ T cells; CTL, secreting IFN-γ	Yes	(61)
Melanoma human tyrosinase	HIV-1 SIN LV/2 VSV-G	Human APCs transduced by LV	Activation of Ag-specific TILs	N/A	(44)
Melanoma polyepitope	HIV-1 LV/1 VSV-G	S.C., I.V., or I.P. injection of LV in HHD mice	vigorous and multi-specific long term CTLs	N/A	(47)
Melanoma human NY-ESO-1	HIV-1 SIN LV/3 VSV-G	S.C. injection of LV in HLA-A2/H-$2K^b$ mice	Ag-specific T and B-cell responses	N/A	(162)
Melanoma human NY-ESO-1	HIV-1 SIN LV/2 VSV-G	S.C. or I.V. injection of LV in HHD mice; or boost with VV-ESO	Ag-specific $CD4^+$ and $CD8^+$ T cells; CTLs	N/A	(64,69)
Melanoma human Melan-A/$ELA_{26–35}$	HIV-1 SIN LV/3 VSV-G	S.C. injection of LV in HLA-A2/H-$2K^b$ mice; or boost with the same LV	Ag-specific $CD8^+$ T cells	N/A	(85,168)
Melanoma human Melan-A_{26-35}	HIV-1 SIN LV/3 VSV-G	S.C. injection of LV in HLA-A2/H-$2K^b$ mice; or IL-7 as adjuvant	Ag-specific $CD8^+$ memory T cells; CTLs	N/A	(81,93)
Melanoma murine mutated TRP1	HIV-1 SIN LV/3 VSV-G	Footpad injection of LV in C57BL/6	$CD8^+$ T cells recognizing multi-epitopes; CTL cytokines; TILs	Yes	(155)
Human telomerase reverse transcriptase	HIV-1 SIN LV/2 VSV-G	S.C. injection of LV in HHD mice; or boost with peptide/IFA	potent and diversified CTLs, secreting IFN-γ	Yes	(86)