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Review

DC-targeting lentivectors for cancer immunotherapy

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Summary

Lentivectors (LVs) induce sustained transgene expression and are attractive vaccine platforms for complex immune scenarios like cancer and persistent infections. This review summarises the literature on lentivectors with potential uses for *in vivo* immunotherapy, focussing on those targeting the most potent antigen-presenting cells: dendritic cells (DCs). There is a growing interest in myeloid-targeting therapies as, by influencing an early stage in the immune hierarchy, they can orchestrate a more diverse and complex targeted immune response. We dissect the nature of DC-targeting LVs and their induced immune responses to understand the state of the art, identify the knowledge gaps and guide efforts to maximise the generation of potent and effective immune responses. Lentivector-based vaccines provide several advantages over other vaccine platforms, such as directed tropism and limited vector immunogenicity, and have been shown to generate effective and sustained immune responses. Overall, DC-targeting lentivectors stand out as promising tools to be exploited in cancer immunotherapy, and new-generation LVs can further exploit the gained knowledge in the study of naturally occurring lentiviruses for a more directed and adjuvanted response.

Keywords: lentivectors, dendritic cells, lentivirus, vaccines, myeloid-targeting therapies

Abbrevations: APCs: antigen presenting cells; BMDCs: receptors; bone marrow DCs; cDCs: conventional DCs; DCs: dendritic cells; GALV: gibbon-ape leukaemia virus; HCV: hepatitis C virus; HIV-1: human immunodeficiency virus type 1; HSCs: haematopoietic stem cells; HSPGs: heparan sulphate proteoglycans; iDCs: inflammatory DCs; IRES: internal ribosome entry site; LDLR: low-density lipoprotein receptor; LTR: long terminal repeat; LVs: lentivectors; MMLV: Moloney murine leukaemia retrovirus; mo-DCs: monocyte-derived DCs; NILV: non-integrating LV; NY-ESO-1: New York esophageal squamous cell carcinoma-1; pDCs: plasmacytoid DCs; PRRs: pattern recognition; RRE: Rev responsive element; SIGN-Rs: DC-SIGN-related receptors; SIV: simian immunodeficiency virus; TAR: trans-activation responsive region; TfR: transferrin receptor; TIDCs: tumour-infiltrating DCs; TLS: tertiary lymphoid structures; TME: tumour microenvironment; VLPs: virus-like particles; VSVG: Vesicular stomatitis virus glycoprotein.

Introduction

Immunotherapy aims to tune the immune system to better face insults or fix inadequate immune responses. Vaccines are a key target tool in the development of immunotherapy and different vaccine vehicles have been optimised over the last 30 years. Recently, the development of mRNA vaccines against SARS-CoV-2 brought a new promising scenario, but vaccine vehicles that allow a sustained transgene expression and do not interfere with normal cell function may provide a solid alternative for more immunologically complex scenarios like cancer or persistent infections.

Among the different vaccine vehicles available, virus-based delivery methods have proven to be highly effective for the development of a specific immune response. Compared to other methods, virus-based platforms take advantage of mechanisms evolved by viruses themselves for targeting and delivering their cargo. In particular, lentiviral-based vaccines have been shown to induce potent and durable specific immune responses by providing sustained antigen expression with little vector immunogenicity.

Lentivector vaccine vehicles provide clear advantages over other delivery methods. Other promising platforms such as adenovirus-based vaccines offer some of these advantages, but lower cell target efficiency and pre-existing immune responses to their core proteins (e.g. capsid) [1] challenge their efficiency and safety. Overall, lentivectors (LVs) are not only proven to elicit potent adaptive immune responses but also (i) have high plasticity in terms of cargo and tropism, (ii) are highly efficient at transgene delivery and (iii) have a superior safety profile.

The first (i) advantage of LVs is that tropism, packaging and cargo can be easily manipulated for optimisation. Their composition can be finely tuned to achieve cell specificity, making LVs the most effective vehicle for specific delivery to the myeloid compartment without affecting their function [2]. In addition, LVs have a large cargo capacity and can encode sizeable transgenes, up to 8kb [3]. Second (ii), the transgene delivery is stable, efficient and sustained—whether it is integrative or not—providing constitutive expression of the gene of interest. Third (iii), LVs are safe and have little vector immunogenicity, providing strong potential for clinical feasibility. Nonetheless, the full picture of how LVs interact with the immune system, their adjuvant properties or the routes they use for antigen delivery is still under-characterised, leaving room for further improvement. This understanding may not only help to optimise the development of new-generation LVs but could potentially improve other vaccine vehicles.

The first gene delivery vehicles started to develop in the mid-1980s as a tool for gene transfer based on retroviral backbones. Retroviruses contain RNA as their genetic material, which is retrotranscribed into dsDNA and integrated into the genome of the infected cell. Highly oncogenic retroviruses were the first of

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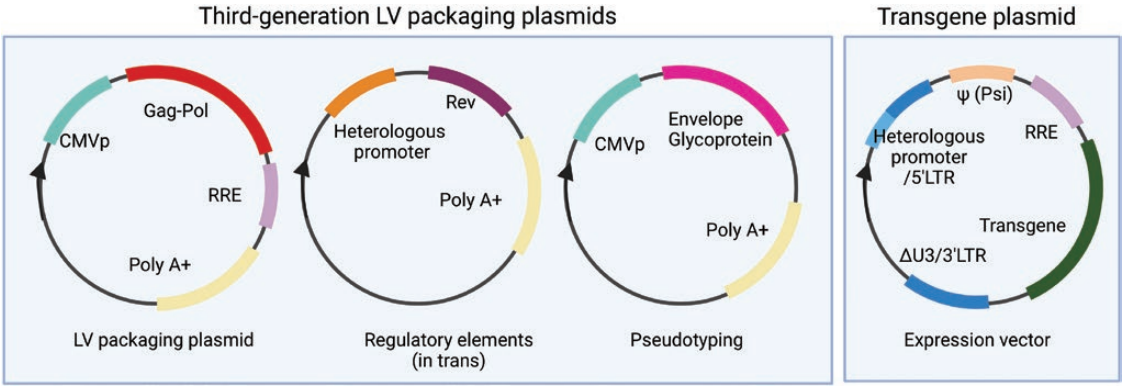

Figure 1. Schematic representation of a third-generation packaging and transgene encoding plasmids needed for lentiviral production. Some of the characteristics of each plasmid are shown. CMVp: cytomegalovirus promoter, LTR: long-terminal repeat, RRE: rev responsive element, Psi: packaging signal.

this kind to be used in the development of vaccine vectors. These first retroviral vector systems derived from C-type retroviruses [4, 5], which are particularly efficient at integrating into the host cell without causing major pathogenicity. These gene delivery vehicles provided sustained expression by integrating the transgene into the genome, but only in actively dividing cells. In the early 1990s the field made a substantial advance with modified lentiviruses, based on human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), which allowed the transduction of non-dividing cells.

Today, LVs have evolved to exhibit superior biosafety by limiting vector immunogenicity in what are called 'third-generation lentivectors'. Compared to first and second-generation LVs, third-generation mainly encode the regulatory elements required for efficient gene delivery, but most coding viral regions have been deleted [6]. Today's LVs contain *gag* to provide a structural protein, *pol* encoding the retroviral enzymes (integrase, polymerase and reverse transcriptase) together with a heterologous viral envelope glycoprotein. Third-generation LVs substantially increased the feasibility for clinical translation, as the structure minimises biological or pathological risks and limits immune responses to the vector. The structure and sequence of currently used LVs, especially in gene therapy, have been recently reviewed by Johnson *et al* [6].

Here, we approach the use of lentivectors targeting the DC compartment *in vivo* from the immunological perspective. Within the complexity of the immune system, targeting the myeloid compartment has indeed expanded the potential of immunotherapies and broadened the extent to which we can tune the immune response. By exploiting the master regulator of the immune response, the impact on the response is amplified, with the induction of a broad range of adaptive immune cell subsets. In the next sections, we review LV design, their immune interactions and the immune response that can be elicited when targeting professional antigen presenting cells (APCs), to understand LV impact and identify the knowledge gaps that need to be addressed for the generation of a long-lasting effective adaptive immune response.

Lentiviral structure, optimisation, and production

The development of HIV-based LVs substantially improved viral-based platforms for therapeutic potential by allowing the transduction of non-dividing cells. To reduce biosafety concerns, first-generation LVs were designed to be replicative deficient [6] by depleting *cis*-regulatory elements, truncating specific genes and using separate complementary plasmids acting in *trans* for safe particle assembly. For further improvement, second-generation LVs were deployed from viral proteins considered non-essential (Env, Vif, Vpr, Vpu, and Nef), and which had no impact on gene delivery, but maintained all the regulatory elements for sustained transgene expression [7].

Today's third-generation LVs have further altered the expression of the regulatory elements Tat and Rev for increased biosafety (Fig. 1), mainly maintaining *gag-pol* from the original backbone. In contrast, the introduction of a heterologous envelope glycoprotein, which can be from a wide range of viruses, directs the LV particle tropism through the mechanism of pseudotyping (discussed in the myeloid cell targeting section).

Tat and Rev are two virus-encoded proteins required for viral replication that act as trans-acting regulatory elements. Tat binds to the trans-activation responsive region (TAR) in the long terminal repeat (LTR) to initiate proviral transcription [8]. Rev is required for the transport of spliced and unspliced viral RNAs from the nucleus to the cytoplasm, facilitating the production of viral proteins [9]. In third-generation LVs, Tat in the 5′LTR has been substituted with a constitutively active heterologous promoter upstream of the gene of interest and Rev is delivered in *trans*, as a separate plasmid, to minimise the potential for replication-competent viruses [10] (Fig. 1). A fourth-generation LV that replaces the Rev responsive element (RRE) with heterologous viral sequences has also been developed, but to date, these have not succeeded, probably due to low viral titrs [11].

The lack of other viral-encoded proteins and the decoupling of the regulatory elements Tat and Rev provide LVs with the qualities of safe efficient gene delivery and long-term expression. These LVs are able to generate a potent immune response while reducing any possible anti-vector immunity that could divert that response, as well as minimising future reactions against these vaccine vehicles.

One potential concern is that of uncontrolled transgene integration into the host genome, which could cause a perturbation of host gene expression known as genotoxicity. A subgroup of lentiviral vectors called non-integrating LV (NILV)

are constructed with the depletion of the LV integrase enzyme to overcome this issue. NILVs avoid the integration of the viral DNA into the host genome [12, 13], and instead, the viral DNA exists in an episomal form, which also ensures sustained transgene expression [12]. NILVs have been shown to stimulate adaptive immune responses effective against tumours [14]. However, how these two different designs, integrative *vs.* non-integrative, may lead to differences in antigen expression and subsequent immunogenicity has not been investigated.

A relatively neglected field is the study of the molecular composition of the LV particle, which is usually acquired from the producer cell. Newly produced LV particles bud from the producer cell membrane, which dictates LV envelope composition by incorporating host molecules in it. Proteins, lipids and sugars associated with the membrane envelope of LVs, or even packaged within the LV particle, can play an important role in directing, adjuvanting or depleting the immune response through virus-host interactions. In this regard, Tremain *et al.* have recently reported the influence of glycosylation in inducing antigen-specific tolerance using an antigen conjugated to a synthetic glycosylated polymer [15]. Active recruitment of certain host molecules to the LV budding sites has been described for HIV fully encoding lentiviruses that bud from a particular area of the plasma membrane with a specific composition rich in lipid content [16]. Now that the field of lipidomics is developing, it will be of interest to understand the variety of lipid compositions in different viral vehicles, their effect on target cells and their influence on antigen delivery.

A few studies have characterised LVs composition and the stoichiometry of associated cellular proteins incorporated into or co-purified with the LV particles [17–19], but no comparison has been made of the impact of using different producer cell types. Moloney murine leukaemia retrovirus (MMLV)-derived LV particles produced in HEK293 cells were found to contain the tetraspanins CD81 and CD9—also present in HIV budding sites [20]—in addition to cell adhesion molecules and some late endosomal markers like CD63 and Lamp-2 [19]. In HIV-derived LVs, Wheeler *et al.* identified the presence of nuclear proteins, elongation factors as well as chaperone and heat shock proteins (like HSP70 and Histone 2A) [17]. Denard *et al.* found cellular proteins incorporated into or co-precipitated with virions. These included ALIX (an endosomal sorting cargo protein known to interact with p6 in HIV [21]), Cyclophilin A (found to interact with HIV capsid, increasing viral infectivity [22, 23]) and L-lactate dehydrogenase B chain (LBDH, catalyses pyruvate to lactate) [18].

There are limited data on how the presence and stoichiometry of these cell-associated molecules packaged into LV particles may impact the fate of the cargo and the efficiency of the induced immune response. One of the few known examples is cGAMP, which is packaged in HIV viral particles and extracellular vesicles during viral production [24], activating the cGAS-STING pathway in target or neighbouring cells and thereby increasing CD4+ and CD8+ T-cell responses [25]. Recently, HIV-1 capsid maturation has been discovered to require the packaging of the cellular metabolite inositol hexakisphosphate (IP6). With insufficient levels of IP6, the virus is no longer infectious due to capsid instability [26], with obvious implications for the field of LV production.

Thus, modulating the LV content and stoichiometry of cGAMP, other messengers, PAMPs, cytokines or other packaged host molecules could have an impact on the LV production and subsequent immune response. It would be of interest to have a complete picture of how different vaccine vehicles or producer cells promote packaging differences and translate into specific scenarios in immunotherapy.

In this regard, the structure of third-generation LVs has been manipulated, with the removal of several viral proteins, aiming for a high biosafety profile. One cannot dismiss the possibility that these deletions may also have an effect on the recruitment or packaging of specific host molecules to the plasma membrane or the particle itself, affecting LV composition and potentially their subsequent efficiency and performance in triggering a specific immune response. The loss of some proteins that are able to counteract host restriction factors may, at the same time, limit recognition by innate immune sensors, thereby affecting a wide range of processes like antigen processing and presentation.

LVs are usually produced in HEK293 or derivatives, like HEK293T—expressing SV40 T (simian vacuolating virus 40 large T antigen) [27], the fast-growing HEK293FT, or the recently described SJ293TS [28] that allow serum-free transductions; all of these render highly efficient LV production. HEK293 are human embryonic kidney cell lines that originated from healthy female foetus cells transformed with adenovirus 5 (Ad5), which allows for high levels of protein production under the CMV promoter [29]. Other cell lines used to produce LVs have human or monkey origin like COS-1, COS-7, HT1080, TE671, CV-1 and CHO [30]. However, other than the African green monkey kidney epithelial cell line COS-1, which is widely used for LV screens [31], the other cell lines seem to be less efficient at LV production [32]. How LVs vary in their specific composition based on the nature of the producer cells and whether this can dictate the quality of the generated response is underexplored. It would be of interest to compare and understand these differences and their implications for the immune response, especially with the monopoly provided by kidney-origin HEK293-derived cell lines for LV production.

To maximise the uses for LVs in immunotherapy, it will be important to generate a deep understanding of the cell biology in lentiviral assembly and production. Further studies are needed to evaluate the influence of different host co-factors packaged in the LV particles to tailor their production to specific immunotherapy requirements.

The importance of targeting the myeloid compartment in the context of cancer immunotherapies

Myeloid-targeting immunotherapies are an attractive way to manipulate the immune response from an early stage, giving it the power to influence the downstream response. DCs can govern the nature of the adaptive response, not only by their capacity to present antigens and delineate antigen specificity but also by their ability to shape it with other co-stimulatory signals. DCs have also the unique capacity to cross-present antigens to CD8+ T cells, which is intimately correlated with the generation of robust CTL responses [33, 34] and tissue-resident memory CD8+ T cells [35, 36], making them a desirable target against cancer.

DC-based immunotherapies have, in addition, the potential to reduce immunotherapy off-target effects and can be combined with other cancer treatments such as checkpoint inhibitors or adoptive T-cell therapy. Importantly, DC-based immunotherapies could provide long-lasting immune memory by priming memory T cells.

DCs are a heterogeneous population comprising circulating conventional DCs (cDCs) and plasmacytoid DCs (pDCs), as well as tissue-specific DCs such as Langerhans cells, monocyte-derived DCs (mo-DCs) and inflammatory DCs (iDCs). Each subset displays different intrinsic characteristics influencing their capacities to elicit and promote adaptive immune responses. The different DC subsets vary in their properties for migration, antigen presentation, activation cytokine production, and recruitment of other immune cell subsets.

In humans, immunotherapies have focused on the subsets that excel at antigen-presenting such cDCs. Circulating cDCs mainly subdivide into cDCs1 (CD141+), with a higher ability to cross-present antigens, and cDCs2 (CD1c+), mainly known for priming CD4+ T cells and coordinating cytotoxic and antibody-producing B cells. In a very thorough review on DCs, Cabeza-Cabrerizo *et al.* warn about sticking to the dogma and attributing these functions solely to specific subsets, as some conclusions may be based on the system compensating functions when one of the subsets is missing [37]. Other interesting DC subsets to be specifically targeted in immunotherapy are tumour-infiltrating DCs (TIDCs) and DC subsets associated with tumour stroma and tertiary lymphoid structures (TLS).

In cancer, cDCs are key in T-cell priming, both at the draining LNs and, together with mo-DCs, within the tumour microenvironment (TME) to promote antitumour responses. DCs can not only promote CD4+ and CD8+ responses to neoantigens but they can also break the tolerance against tumour antigens [38]. A very detailed review on the role of DCs in cancer by Pittet *et al* has recently been published [39].

Directly targeting cDC subsets can refine specific immune responses. To our knowledge, no virus-based vaccine has specifically targeted a single DC subset. In this review we have analysed those studies that have, so far, broadly targeted the DC compartment. This encourages future studies to aim for the specifically targeting of particular cDC subsets and to evaluate the power and influence they might have in sculpting a specific response.

Although the importance of targeting the DC compartment is obvious, there are still many concepts of the basic principles of cDC biology that are yet to be defined. It is essential to invest in understanding fundamental cDC processes related to subset activation, localisation and antigen presentation to help fine-tune successful immunotherapies.

Myeloid cell targeting

LV particles are usually complemented by pseudotyping with a heterologous viral envelope glycoprotein that is incorporated into the LV membrane and facilitates cellular entry. Vesicular stomatitis virus glycoprotein (VSVG) is the most commonly used glycoprotein to pseudotype LV particles, as it allows non-specific fusion into many cell types through an endocytosis mechanism. Other strategies for specific targeting also incorporate specific ligands or antibodies against surface molecules at the target cell.

The use of VSVG has considerably broadened the tropism from classical LVs that were limited to CD4-bearing cells by the requirements of the HIV envelope glycoprotein. VSVG binds to the low-density lipoprotein receptor (LDLR) present in most cells, which takes the LV into the endosomal pathway that will fuse with the cell membrane following acidification [40]. This universal mechanism for VSVG cell entry has allowed difficult stem cell or neuron transductions [41, 42], but its uses *in vivo* are limited, as VSVG-LVs appear to be partially inactivated by serum [43, 44]. Alternatives like cells that allow for serum-free LV production or the development of serum-resistant VSVG have been proposed to overcome this issue [45, 46].

VSVG-LVs broad tropism enhances transduction of difficult cell types, but its use can also be detrimental: indiscriminate transduction can cause an off-target effect and decrease its efficacy. In immunotherapy, the approach has been to perform *ex vivo* transduction to avoid this off-target effect but direct *in vivo* delivery of transgenes and antigens by LVs vaccination would make immunotherapy interventions more powerful and cost-effective [47].

Targeting LVs to a specific immune cell *in vivo* can directly reach antigen presenting pathways or genetically modify adaptive immune cells for a more potent and targeted immune response, increasing the efficiency and impact of immunotherapy. To achieve that, LVs are usually pseudotyped with a heterologous fusion/viral envelope protein that directs their tropism and increases transduction specificity to the desired target cells.

Different retroviral envelopes have narrowed and optimised LV tropism and have been used extensively [43]. Retroviral glycoproteins have proven efficient in transducing human CD34+ haematopoietic stem/progenitor cells, especially LVs with gibbon-ape leukaemia virus (GALV) or feline leukaemia virus (RD114) envelopes [48, 49]. Baboon endogenous retroviral envelope (BaEV) has allowed for transduction of human haematopoietic stem cells (HSCs) while maintaining their stem-ness [50] and some *Rhabdoviruses* can specifically target neural cells [51, 52]. For the purpose of this review, we focus on LVs directly targeting the DC compartment. Pseudotyping of LVs targeting different cell types has been extensively reviewed by Joglekar and Sandoval [43].

Ku *et al*. used a relatively unexplored DC-targeting system, consisting of coupling the LV platform with a β2-microglobulin promoter instead of the most commonly used CMV promoter. The use of a β2-microglobulin promoter does not avoid the off-target effect but utilises the fact that this promoter is highly active in immune cells, especially in DCs, to direct the expression of the gene of interest. Although the authors only tested the effect of their β2-microglobulin-based lentivector *ex vivo*, they highlighted its safety as compared to the CMV promoter, which contains enhancer elements prone to insertional mutagenesis [53].

Myeloid immunotherapies are and have been performed successfully *ex vivo*, re-injected after LV transduction [54], but specific *in vivo* targeting in humans is still under early development. *In vivo* APC-restricted LVs have been achieved using the dectin-2 gene promoter, which directed the LV expression to splenic and CD11c+ DCs in the draining LNs in mice [55]. Measles virus (MV) glycoprotein has been extensively used to

target lymphocytes and DCs through the SLAM and CD46 receptors [56–59], but the Sindbis virus envelope has proved highly specific for DC-specific LV delivery by targeting DC-SIGN (CD209) [60–63].

Sindbis glycoprotein binds to DC-SIGN, a C-type lectin expressed in DCs, which allows internalisation of the LV into clathrin-dependent coated pits [64, 65], with subsequent endo-lysosomal trafficking [66]. Yang *et al.* were among the first to use Sindbis pseudotyping for *in vivo* DC-targeting [62]. In direct comparison with VSVG-pseudotyped LVs, Sindbis pseudotyping showed higher infection of DCs and fewer off-target transductions [63]. Ma *et al.* increased the effect of DC transduction efficiency by incorporating LIGHT, a type II transmembrane protein, in their Sindbis-pseudotyped LVs. When expressed in DCs, LIGHT protein acts as a co-stimulatory molecule for T cells, boosting the effect of the transduction into a more potent immune response. This method enhanced the T-cell response and led to a rapid enlargement of the draining lymph nodes in mice, suggesting enhanced trafficking of DCs and increased T-cell proliferation [67].

Although Sindbis pseudotyping targets DCs specifically, it is not clear what DC subsets it targets more efficiently. The expression of DC-SIGN is not uniform across all DC subsets and it is not a characteristic marker of either cDC1 or cDC2, but can be upregulated during infection or inflammation [68]. DC-SIGN is mostly constitutively expressed in tissue-specific DCs like mucosal DCs [68]. The use of specific promoters like CD11c could be used to target cDCs [69], but this would be post-entry and may not represent the most efficient targeting for circulating cDCs. The ability to target specific DC subsets could represent a big step forward in the field.

Another important point to consider when assessing these murine *in vivo* studies is that the Sindbis glycoprotein might target DCs differently in mice from humans. Sindbis virus is an arbovirus primarily transmitted to vertebrate hosts, known to bind to DC-SIGN-Related Receptors (SIGN-Rs) and heparan sulphate proteoglycans (HSPGs) [70]. There are some differences between human DC-SIGN and the most similar mouse orthologue SIGNR5/CD209a regarding its expression [71] and their ligand binding preference [72]. While hDC-SIGN binds to mannose and fucose-terminated glycans similarly to mouse SIGNR3, SIGNR5 does not seem to bind mammalian glycans with high affinity [72]. There is no comparative study addressing how differently the mouse and human DC-SIGN bind to Sindbis glycoprotein. It would be interesting to explore how these different affinities translate to the targeting efficiency of different DC populations, as well as how the glycan composition in LVs can bias this effect. That said, Schetters *et al.* evaluated the expression and functionality of mDC-SIGN, finding it comparable to hDC-SIGN in its ability to endocytose antigen and induce CD4+ and CD8+ T-cell responses [71], but whether these are comparable at a systemic level is not known.

So far, myeloid targeting has proven quite efficient in *in vivo* models, especially using the Sindbis envelope glycoprotein. However, given the current knowledge gaps, there is a need for a deeper understanding of the Sindbis glycoprotein specificity in targeting human DCs, and to find other molecules that can efficiently target human DCs *in vivo*. The Sindbis envelope is an exogenous protein that has the potential to cause vector immunogenicity if used in repeated immunisations. Thus, a strategy using endogenous molecules to target LVs to DCs would be more beneficial. Moreover, the internalisation receptor deployed by the DCs could play an important role, as it might direct cargo antigens to a specific antigen processing route, thereby exerting a major influence on the subsequent immune response.

LV interactions with the innate immune system: sensing and adjuvanticity

Despite the fact that LVs are depleted of most viral proteins, they do lead to infection of the target cell and enter into the early viral life cycle (Fig. 2). Thus, LV infection can trigger similar innate defence mechanisms to those induced by a viral infection (Fig. 3). The effects of a LV particle in contact with, fusing with or entering the cell can induce recognition by pattern recognition receptors (PRRs) and initiate an immune response. These could either be detrimental or serve as an adjuvant for the LV as a vaccine vehicle.

As previously discussed, the origin and composition of the LV can dictate PRR recognition. In fact, LV transduction has been described as a trigger for DC maturation *in vitro* [73], and has proved to induce DC activation *in vivo* [74]. DC maturation initiates their migration to the draining LNs, enhancing and adjuvanting the immune response. But what are the mechanisms of DC activation upon the entry of the viral particle?

The fusion of the LV to the cell can itself disrupt membrane homeostasis and lead to sensing. In human primary macrophages, HIV fusion induces a TBK1-dependent interferon response independently of the containing envelope or the producer cell [75]. With a VSVG-pseudotyped LVs, fusion has been shown to induce PI3K-dependent immune stimulation in mouse bone marrow DCs (BMDCs) [76].

LV components, including different forms of nucleic acids, easily activate DCs (Fig. 3). Different TLRs can be triggered by LV transduction. For example, TLR3 has been shown to directly recognise LVs *in vivo* [77], through double-stranded RNA (dsRNA) recognition [78], resulting in interferon (IFN) production [73]. pDCs sense single-stranded RNA (ss-RNA) derived from HIV via TLR7 *in vitro*, which has also been proposed as a mechanism of LV recognition *in vivo* [77]. These TLR activations might not only influence DC maturation but also determine subsequent antigen processing and presentation [79].

On the other hand, Kim *et al.* found that the delivery of the LV viral genome activated DCs in a TLR-independent fashion through the cGAS-STING pathway [76]. The cGAS-STING pathway senses dsDNA in the cell cytosol and activates a type I IFN response, production of interferon-stimulated genes (ISGs) and can also activate NF-kB signalling [80, 81]. STING activation has been described to be important for cross-presentation, CD8+ T-cell priming, enhancing T-cell infiltration and promoting antitumour immunity [82].

The secondary messenger involved in the cGAS-STING pathway, Cyclic GMP-AMP (cGAMP), has been shown to be packaged in viral particles [24, 83] and to act as a powerful adjuvant [25, 84] with strong potential in the field of immunotherapy. The increased immunogenicity for vaccine vehicles co-packaging cGAMP appears to be more relevant *in vivo* than suggested by preliminary data *in vitro*. Kim et al. recently reported that while *in vitro* delivery of LV encoding

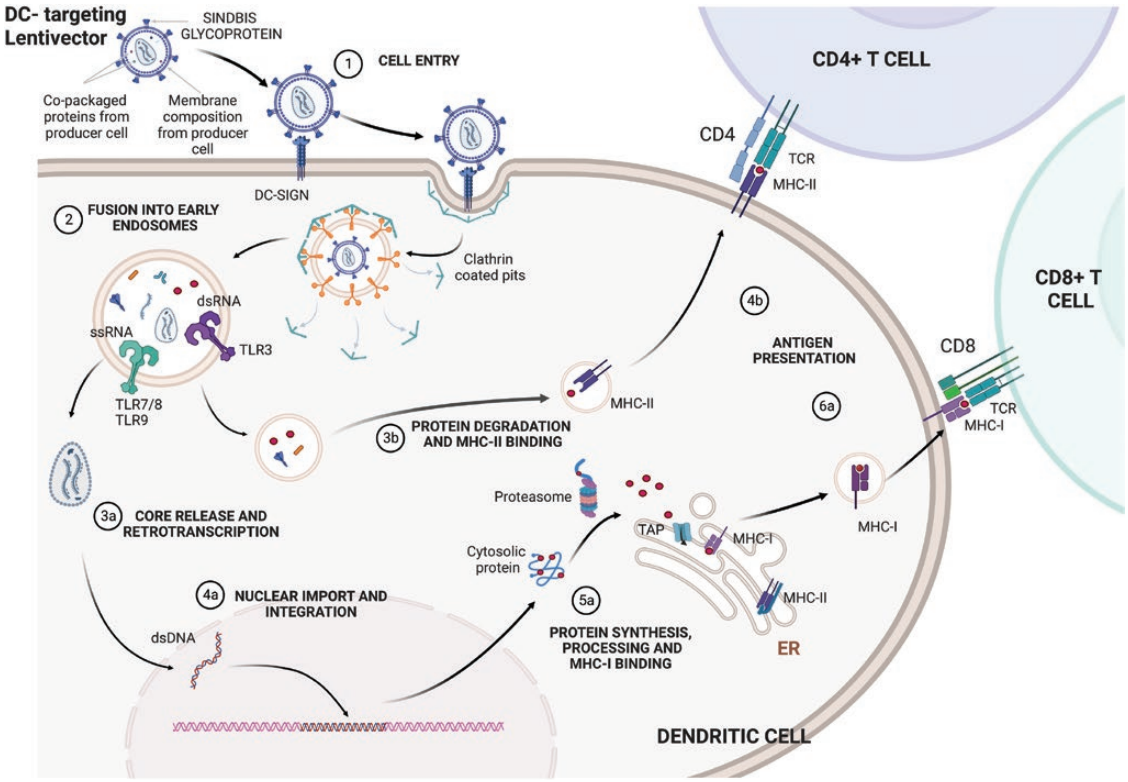

Figure 2. DC-targeting lentiviral life cycle in a dendritic cell. After attachment to the specific receptor (in this scheme Sindbis virus glycoprotein that would bind to DC-SIGN), the LV particle will fuse into early endosomes. From there, the LV packaged proteins or molecules will be degraded and processed for antigen presentation possibly to MHC-II. The capsid might be released to the cytoplasm and the RNA will retrotranscribe to DNA, which is then imported to the nucleus for integration. From there, the proteins encoded in the transgene can be synthesised and processed for antigen presentation, probably via MHC-I.

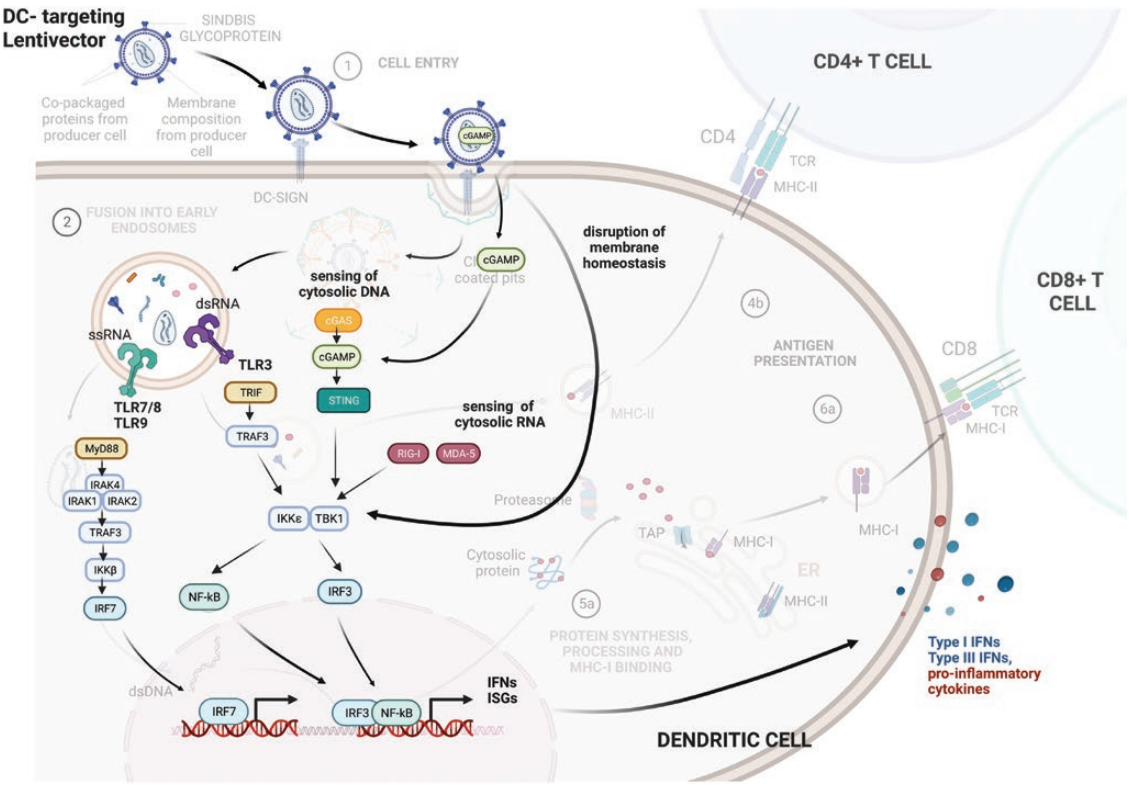

Figure 3. Proposed sensing mechanisms of a DC-targeting lentivirus in a Dendritic cell, with potential for an adjuvanting activity.

antigen triggered a trivial immune response, the magnitude was substantial *in vivo* [76]. cGAMP packaged into VLPs enhances CD4+ and CD8+ T-cell responses and increases antibody titres and T follicular helper cell responses in mice [25]. However, LV appear to induce the cGAMP-STING pathway without the need for packaging of cGAMP, due to cellular DNA packaged into the LV [25], demonstrating a redundant mechanism for cGAS-STING activation.

Other than the intrinsic lentiviral adjuvants and ligands for sensors, exogenous adjuvants can be delivered simultaneously or integrated into the lentiviral particle to enhance immunogenicity. A TLR3 agonist used as an adjuvant together with virus-like particles (VLPs) triggered humoral responses against HIV [85] and a TLR9 ligand augmented cellular responses in an SIV DNA-based vaccine [86]. In fact, a selective combination of TLR ligands used as adjuvants was shown to enhance protection in different vaccine strategies in mice [87]. TLR3/9 agonists delivered together with LV immunisation improved antitumor immunity by rescuing and enhancing the effector function of LV-transduced CD8+ tumour-infiltrating lymphocytes (TILs) [88, 89]. However, in an adenovirus-based vaccine in mice, TLR3 stimulation was found to be detrimental to a CD8+ T-cell-specific response, whereas TLR4 ligands increased it [90]. It might be, then, that different TLR agonists need to be evaluated as adjuvants for each specific vehicle and scenario to determine the power of their interactions and their potential to enhance a specific response.

Full genome lentiviruses can subvert immune recognition by expressing viral proteins able to evade this recognition or by depleting restriction factors [91]. Restriction factors are host proteins that inhibit different steps of the viral cycle. Through the co-evolution of viruses and the immune system, viruses have evolved to overcome these restriction factors through the expression of some viral proteins. It is therefore possible that LVs deployed from these SIV/HIV main or accessory proteins that participate in immune evasion are more susceptible to PRR detection and lead to a greater DC activation and maturation. More data comparing the outcomes with different adjuvanting molecules-LV combinations is needed to understand whether after the deletion of certain LV proteins this increased detection can serve as an adjuvant or if it would be detrimental to the generation of long-term immune responses.

Antigen expression, processing, and presentation after LV transduction

LV can create prolonged immunisation by providing sustained antigen expression and presentation. The fate of the LV components, once it has fused to the cell, may vary based on the endocytosis receptor used. Normally, the LV cargo will be delivered into early endosomes and the encapsidated transgene will travel to the nucleus while retrotranscribing to DNA (Fig. 2). This DNA will either integrate into the host genome or, if non-integrative, remain episomal. In the transgene, the presence of an internal ribosome entry site (IRES) after the gene promoter appears to correlate with increased immunogenicity [91]. There is also the possibility that any of the nucleotide forms of the transgene could leak into the cytoplasm, which would explain some of the observed sensing mechanisms.

While the LV-encoded transgene would be retrotranscribed to dsDNA, translated, expressed and later processed as an intracellular antigen, being presented via major histocompatibility complex class I (MHC-I), any antigen carried within the LV will be accessed exogenously. Although the external acquisition of antigens is biased to the MHC-II pathway of antigen presentation, some endocytosis receptors or co-stimulation by PRRs can divert the presentation through MHC-I (Fig. 2). For example, endocytosis of soluble antigens through the mannose receptor directs them to early endosomal compartments to be presented exclusively via MHC-I [92]. In DC-SIGN-mediated endocytosis, TLR4 stimulation has been shown to translocate antigens from early endosomal compartments in DCs to the cytosol, diverting from MHC-II presentation towards cross-presentation of external antigens by MHC-I [93]. Thus, protein subunit antigens carried by a LV can be prompted to follow the classic MHC-II presentation for extracellular antigens or be cross-presented through MHC-I, important for inducing effective anti-tumour immunity by priming CD8+ T cells [94]. It is also important to consider that the directed LV entry mechanism and the chosen receptor potentially have an influence on LV cargo antigenic presentation and subsequent T-cell activation.

It is expected that CD8+ T cells will be induced better via MHC-I by the LV system when the transgene is expressed in the cell. However, some LVs have been engineered to target the intracellular expression of their transgene to the MHC-II pathway to potentiate CD4+ T-cell and humoral responses. Rowe *et al*. fused either transferrin receptor (TfR) or invariant chain (Ii) to an OVA antigen so it could be targeted to the MHC-II pathway, which led to a superior immune response, with more CD4+ T-cell help and a more powerful humoral and CD8+ T-cell response than elicited simply by intracellular OVA expression in mice [74]. This Ii-LV stimulated the most cytokine secretion by CD4+ T helper cells and potently induced IFNγ secretion from CD4+ and CD8+ T-cells, sufficient to protect mice from a challenge with OVA-expressing tumour cells [74]. In other preclinical studies, antigens have been fused to the invariant chain to trigger a CD4+ T helper response. The first human trial of a strategy to direct antigens to MHC-II was recently reported, in a prime-boost regime using MVA and Adenovirus 3, that contained an Ii-fused antigen for hepatitis C virus (HCV). The MHC-II-targeted vaccine enhanced the magnitude and breadth of HCV-specific memory CTL responses [95].

Both LV targeting MHC-I and MHC-II have shown potent effects *in vivo* in inducing immune responses in animal models. It would be interesting to undertake a direct comparison between these two types of LV, to understand the differences regarding the long-term immune response, and memory and breadth of their immunogenicity.

LV-induced adaptive immune responses to tumour antigens

Cancer vaccines for immunotherapy direct efforts to enhance anti-tumour immune responses either by (1) delivering antigen to antigen-presenting cells to boost tumour-specific responses or (2) genetically manipulating cells from the immune system to induce or increase anti-tumour immunity.

LVs provide a platform that can direct the expression of the gene of interest into antigen-presenting cells, to further boost

and tailor the immune response against the antigen. But what do we know about the characteristics of this LV-elicited immune response? An ideal vaccination vehicle would not only elicit a potent and effective adaptive response but would also trigger the formation of the memory compartment to efficiently face rechallenges.

Prolonged and sustained antigen presentation will determine the potency and induce the persistence of the elicited immunity, as well as the breath and duration of strong T-cell memory. Sustained antigen presentation has been shown to stimulate T-cell memory and promote long-lived memory CD8+ and CD4+ T-cell responses [96–98]. After LV immunisation, the magnitude of antigen presentation is attained more than 3 weeks after immunisation as opposed to peaking at 5 days in other systems [78]. However, despite low anti-vector immunity, some have pointed out that subsequent immunisation with heterologous LV vaccines leads to a diminished secondary T-cell response. This was ascribed to LV cells being rapidly cleared by an effective CTL response, restricting the durability of antigen presentation after a LV boost with diminished T-cell memory formation [99].

In an *in vivo* mouse model for melanoma, immunisation with a non-targeted LV encoding a melanoma-associated antigen efficiently triggered a tumour-specific T-cell response stronger than a peptide vaccine carrying the same antigen and a TLR9 agonist. Importantly, these cells expressed the memory markers CD127 and CD62L and were still detectable 3 months after immunisation [100]. However, the tumour-rejection capacity of these cells was not assessed.

The induction of the immune response by targeting the DC compartment *in vivo* does direct anti-tumour immunity more efficiently. *Ex vivo* DC-targeting LV vaccination elicits greater CTL responses than *ex vivo* modified DCs with other antigen systems [78]. Induced T-cell responses after *ex vivo* lentiviral DC transduction have prevented the development of tumours in mice more successfully than other methods like peptide-pulsed DCs [78]. E*x vivo* transduced DCs that were subsequently injected induced a long-lasting CD8+ T-cell response with greater killing potential and secreted higher levels of IFNγ than simply protein- or peptide-pulsed DCs [101]. However, these previous experiments *ex vivo* had shown no difference between regular LV and DC-targeted LV in raising a T-cell response, probably because *ex vivo* transduction intrinsically removes the off-target effect and so does not reveal any advantage for directed cell targeting. E*x vivo* experiments do not perfectly simulate physiological conditions, such as cell-to-cell interactions and adjuvanting properties through innate immune activation in response to LV vaccination, so the immune response may appear less powerful. This might also be because fewer injected DCs migrate to draining lymph nodes when transduced *ex vivo* [102]. Indeed, *in vivo* experiments in mice have shown more robust CD4+ and CD8+ T-cell responses and decreased waning of the activation of antigen-specific CD8+ T cells when compared to other methods of vaccination including *ex vivo* DC transduction [67, 91]. The first *in vivo* DC-targeted LV, which used the Sindbis glycoprotein to target DC-SIGN, induced antigen-specific CD8+ T cells and a significant antibody response while inducing regression of established tumours in a mouse model [62].

A single immunisation with a modified Sindbis-based DC-targeted LV vaccine delivering breast cancer antigens (Lalba and ERBB2) inhibited tumour growth and amplified to 6-fold antigen-specific CD8+ T cells compared with naïve mice. This immunisation proved to be prophylactic but also therapeutic, by reducing tumour growth in mice with an established tumour. These results were performed in mice expressing a human breast cancer antigen, but when using transgenic mice expressing the same antigen as "self" the vaccine was able to delay tumour growth [103], meaning that even in a self-antigenic environment DC-targeted LV vaccines are effective. Importantly, immunisation with DC-targeted LV expressing breast cancer self-antigens prevented the development of tumours (from 75% to 29%) in a spontaneous tumour mouse model using the same transgenic mice [103].

The innate LV activation of DCs is thought to boost CD8+ T-cell responses, providing external signals that would favour the development of a powerful immune response [91]. After an *ex vivo* DC-targeted LV that used the β2-microglobulin promoter in mice, to enhance the transgene expression in immune cells, Ku *et al.* describe higher IL-2 production and a polyfunctional CD8+ T-cell response, paired with the development of higher-quality central memory T cells despite a comparable CD8+ T-cell response when using Ad5 [53]. Ma *et al.* found that using the previously mentioned type II transmembrane protein LIGHT in their Sindbis-pseudotyped DC-targeted LVs, those LV particles induced autophagy, which is essential for effector and memory CD8+ T-cell formation [104], and suggested that autophagy could control T-cell activation and proliferation through CDKN1B [67].

Regarding the kinetics of the T-cell response elicited by DCs-transduced by LVs, Esslinger *et al.* found that the T-cell response peaked at day 9 and 10-40% of the CD8+ cells were antigen-specific after *ex vivo* LV DC transduction [91]. T-cell responses were still greater 2 weeks after immunisation [67], and detectable after 3 months [100].

Targeted DC-LVs have been shown to produce a very powerful CD4+ and CD8+ T-cell response, able to provide protection and prophylaxis against tumour development in different studies in mice. Although the data point to superior memory development by LV when compared to other platforms, the full potential for DC-targeting LV to generate a memory compartment is still not fully characterised. More work is needed to quantify the efficiency of DC-targeting LV to generate central memory T cells and to understand the long-lasting effects needed for protection. Importantly, there is a need to understand the translatability of these results to humans and whether they will generate long-lasting memory responses in people.

Advantages and disadvantages of other vaccine vehicles

Vaccine platforms deliver antigens by providing peptides or encoding genetic material. Among the different options, LV offer a superior biosafety profile, an effective transduction rate, strong specificity and sustained antigen expression. Consequently, LV can elicit a coordinated, well-established, long-term memory immune response. To date, while other vaccine platforms may also induce strong immune responses, their memory potential appears to be more limited [105–107].

LVs are attractive vaccine candidates because they can deliver antigens to APCs *in situ* with high specificity, potentially

controlling antigen presentation for the life of the transduced cell. Moreover, the amount of antigen needed to generate an immune response is much less than free or untargeted antigen. The resulting immune response is focused and directed to the encoded antigen since LVs encode only the minimum of viral proteins. In this aspect, LVs are more similar to DNA or RNA vaccines, although they confer the advantage of more efficient antigen delivery that can be specifically targeted to DCs with sustained expression that can potentially lead to long-term memory responses.

Vaccine platforms targeting the myeloid compartment can better coordinate the development of an effective and durable adaptive immune response. LVs appear to be 2–10 times more effective at transduction than adenovirus immunisation [108], with greater specificity for both mature and immature DCs [109]. Previously discussed, enhanced anti-tumour protection was shown when using a LV with the human β2-microglobulin promoter to transduce DCs, which favoured a CD8+ T-cell central memory phenotype as compared to Ad5 that showed more of an effector phenotype [53]. Importantly, when targeting the myeloid compartment, other vaccine platforms have been shown to interfere with DC functions like the inflammatory responses or stimulation of T-cell immunity, while LVs do not [2, 101, 109].

DC-targeted LV vaccines have also shown that one immunisation might be sufficient to slow or prevent tumour growth, while DNA vaccines need multiple immunisations [103]. Although multiple immunisations can increase the level of immune response in all vaccine regimes, LVs generate limited vector immunity. This is particularly important in cancer immunotherapy, as a very powerful and directed response is needed to have an effect in an immunosuppressive tumour microenvironment [110].

In terms of biosafety, the use of LVs has shown potent immune responses without observing serious adverse events. Concerns such as the production of infectious lentiparticles or an oncogenic potential have not been observed [111, 112]. The first-in-class DC-targeting LV human clinical trials, using third-generation, nonreplicating, integration-deficient lentivirus-based vector expressing New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1) cancer testis antigen in DCs, induced strong T-cell responses with no serious adverse events [111]. Importantly, this treatment has been recently evaluated in a prime-boost regime in patients with advanced cancer and showed a safe delivery [113]. We have depicted the advantages of the use of LVs for DC-based immunotherapies as compared to the most common used virus-based vaccine platforms in Table 1.

Table 1. Main characteristics, advantages and disadvantages of the use of LVs for DC-based immunotherapies compared to the most common used virus-based vaccine platforms

	Lentiviruses	Adenoviruses	Retroviruses*	Vaccinia Virus
Long-term gene expression	High (Both for Integrative and non-integrative)	High (Both for replicative deficient and competent)	High	Short-lived compared to other systems Needs a strong promoter [114]
Packaging capacity (sizeable gene)	Up to 8Kb [3]	Up to 2Kb. 5–6Kb in ΔE1A viruses. +2Kb if ΔE3 gene [115].	Up to 8Kb [116]	25–30Kb [117]
Safety	LVs are encoded in different plasmids to avoid a replicative competent form Most viral proteins have been deleted	Existence of pre-existing immunity Risk of reversion to pathogenic form	Generation of replication-competent form	Modified Vaccinia Ankara (MVA) is used as an attenuated vector
Major disadvantages	Complex manufacturing	Pre-existing immunity Can affect the normal DC functions [2, 101, 109]	Target only actively dividing cells Potential of tumourigenesis [118] Risk of reversion to replication-competent virus	Pre-existing immunity Not suited for immunocompromised settings [117] Need of repeated vaccinations [114]
Major advantages	Easy manipulation and targeting of non-dividing cells Safety Strong and sustained immune responses High quality CD8+ T-cell responses [53] Do not interfere with DC functions [2, 101, 109]	High titers [116] Strong and sustained immune responses	Strong immune response	Easy manipulation Large gene inserts

*Retroviruses here exclude lentiviruses.

Prospective and future directions for lentiviral use in cancer immunotherapy

It is clear that DC-targeted LVs have great potential to become central tools for cancer immunotherapy. Non-replicating LVs can provide sustained antigenic expression that can enhance the durability of the response and break tolerogenesis. Importantly, the contents of the LV particle can trigger innate immune pathways, most likely adjuvanting and reinforcing the mounted response. This is important, as these adjuvanting properties could overcome the mechanisms of immunosuppression within the tumour microenvironment by providing strong activating signals to DCs. This immune reinforcement at the DC level could broadly improve T-cell functions within the immunosuppressive microenvironment. Moreover, the LV cargo and its endocytosis route can play an important role in the antigen presentation pathways and promote the development of a strong, polyfunctional and long-lasting immune response.

The route of vaccine administration adds another layer of complexity to the intricate mechanisms required to generate a good response, although this might be independent of the vaccine system used. Vaccination routes do affect clinical outcomes and this can be influenced by the presence of specific DC subsets or tissue-DCs at the site of injection that can lead to a more efficient uptake of the antigen. It would be interesting to see whether this effect is stronger when targeting DCs with a LV-based vaccine, how the site of injection can bias the subset DC activation and the type of immune response that develops.

Fundamental questions like the cell biology of the lentiviral assembly are important to tailor the immunogenicity of these particles. LV can also be used in combination with existing therapies. The use of DC-targeted LV together with immune checkpoint inhibitors, a pivotal treatment for cancer, has been recently associated with greater anti-tumour efficacy of anti-PD-1 in mice, but not with anti-PDL1 or anti-CTLA4 [119]. Thus, the potential of LVs to determine the fate of tumour responses is not only limited to their effects but can also influence other cancer immunotherapies. Overall, LVs are a powerful tool that can elicit a powerful immune response and provide the solid generation of the memory compartment.

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