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## Targeting lentiviral vectors for cancer immunotherapy

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### Abstract

Delivery of tumour-associated antigens (TAA) in a way that induces effective, specific immunity is a challenge in anti-cancer vaccine design. Circumventing tumour-induced tolerogenic mechanisms *in vivo* is also critical for effective immunotherapy. Effective immune responses are induced by professional antigen presenting cells, in particular dendritic cells (DC). This requires presentation of the antigen to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the context of strong co-stimulatory signals. Lentiviral vectors have been tested as vehicles, for both *ex vivo* and *in vivo* delivery of TAA and/or activation signals to DC, and have been demonstrated to induce potent T cell mediated immune responses that can control tumour growth. This review will focus on the use of lentiviral vectors for *in vivo* gene delivery to DC, introducing strategies to target DC, either targeting cell entry or gene expression to improve safety of the lentiviral vaccine or targeting dendritic cell activation pathways to enhance performance of the lentiviral vaccine. In conclusion, this review highlights the potential of lentiviral vectors as a generally applicable 'off-the-shelf' anti-cancer immunotherapeutic.

### Keywords

dendritic cell; lentiviral vector; cancer; immunotherapy

## 1. General introduction

The identification of tumour-associated antigens (TAA) (Boon 1996), which are selectively or preferentially expressed by tumour cells, has led to the design of various TAA-based anti-tumour vaccines (Mocellin, Mandruzzato et al. 2004). These vaccines are designed to initiate or re-stimulate anti-tumour immune responses. Although the precise immune effector mechanisms involved in tumour eradication are not well understood yet, it is known that cytotoxic CD8<sup>+</sup> T cells (CTL) play a major role (Boon and van der Bruggen 1996). In addition, the activation of CD4<sup>+</sup> T helper type 1 cells (T<sub>H</sub>1) is recognized to be important to initiate the correct immune response and to sustain immune effector mechanisms *in vivo*, contributing to tumour rejection (Bonehill, Heirman et al. 2004; Bonehill, Heirman et al. 2005). In contrast, other immunological mechanisms can inhibit anti-tumour immunity. These mechanisms are mediated by regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC), and tumour cells themselves (Emens 2003; Whiteside 2008). Thus, anti-cancer vaccines should ideally elicit strong T<sub>H</sub>1 cellular immune responses which are refractory to inhibitory mechanisms.

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In the immune system, antigen presenting cells, particularly dendritic cells (DC), regulate immune responses against pathogens and aberrant cells. DC are a heterogeneous cell population, which exhibits extremely efficient antigen uptake, processing and presenting properties. Upon encounter with pathogens, they undergo a complex maturation programme characterised by their up-regulation of co-stimulatory molecules, pro-inflammatory cytokines and importantly the chemokine receptor CCR7. DC then migrate to lymphoid organs where they stimulate naive and antigen experienced T cells through the presentation of MHC-peptide complexes to the T cell receptor (TCR) (signal 1) and through strong co-stimulation. This stimulation (signal 2) is provided by co-stimulatory molecules such as CD80/CD86 and inflammatory cytokines such as interleukin (IL) -6, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-12 (Steinman 2007). Therefore, DC have been extensively studied as a cellular anti-cancer vaccine.

Different strategies for the generation of large numbers of clinical grade DC have been developed, reviewed by Tuyaerts *et al* (Tuyaerts, Aerts et al. 2007). Furthermore, several viral and non-viral systems have been successfully developed for the genetic modification of DC (Breckpot, Heirman et al. 2004; Mossoba and Medin 2006; Van Tendeloo, Ponsaerts et al. 2007). One of these strategies is the use of HIV-1 (human immunodeficiency virus 1)-based lentiviral vectors (Breckpot, Aerts et al. 2007; Breckpot, Emeagi et al. 2008). HIV-1 is a member of the family *Retroviridae*, differing from oncoretroviruses, their retroviral cousins, by their ability to integrate their cargo into the host genome independently of its replication status (Lewis and Emerman 1994). This is an important asset in view of DC modification, since human DC are generally differentiated from blood-derived quiescent CD14<sup>+</sup> monocytes or from mitotically hypoactive CD34<sup>+</sup> cells. The biology of lentiviruses and the development of lentivirus-based gene transfer systems has been reviewed in depth elsewhere (Breckpot, Aerts et al. 2007; He, Munn et al. 2007; Breckpot, Emeagi et al. 2008; Spirin, Vil'gelm et al. 2008).

The first successful transduction of human monocyte-derived DC was described by Unutmaz *et al* in 1999 (Unutmaz, KewalRamani et al. 1999). Since then, several research groups have reported on the successful transduction of monocyte-(Schroers, Sinha et al. 2000; Dyllal, Latouche et al. 2001; Firat, Zennou et al. 2002; Breckpot, Dullaers et al. 2003; Breckpot, Corthals et al. 2004; Lizée, Gonzales et al. 2004) and CD34<sup>+</sup>-derived human DC (Salmon, Kindler et al. 2000; Oki, Ando et al. 2001; Cui, Golob et al. 2002; Sumimoto, Tsuji et al. 2002), and bone marrow-derived mouse DC (Metharom, Ellem et al. 2001; Zarei, Leuba et al. 2002; Esslinger, Chapatte et al. 2003) with varying efficiencies. Subsequently, it was shown that TAA delivered to DC by lentiviral transduction were processed and presented to established T cell lines, demonstrating the presentation of epitopes derived from various TAA (MAGE-3, Melan-A, tyrosinase and ovalbumin, TRP-2, in the human and mouse system, respectively) (Metharom, Ellem et al. 2001; Firat, Zennou et al. 2002; Breckpot, Dullaers et al. 2003; Lizée, Gonzales et al. 2004). More importantly, *in vitro* priming of naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells against weak immunogenic TAA such as MAGE-3, was reported (Breckpot, Dullaers et al. 2003), resulting in the generation of T cell clones and the description of a novel HLA-Cw7-restricted MAGE-3 peptide (Breckpot, Heirman et al. 2004). Similarly, Firat *et al* primed bulk CTL following *in vitro* stimulation with DC transduced with lentiviral vectors encoding multiple melanoma antigen-derived epitopes (Metharom, Ellem et al. 2001). Furthermore, lentivirally transduced DC were evaluated in several mouse models as a therapeutic against cancer. Immunization with bone marrow-derived DC transduced with OVA-encoding lentivectors (Breckpot, Dullaers et al. 2003) or tumour antigens such as TRP-2 (Metharom, Ellem et al. 2001) or erbB2 (mouse analogue of human Her-2/neu) (Mossoba, Walia et al. 2008), induced strong CTL responses, decreased tumour growth and tumour protection. Moreover, Wang *et al* (Wang, He et al. 2006) extended these data in a mouse hepatoma model, immunizing with lentivector

transduced DC expressing three hepatoma-associated antigens, self-antigens highly over-expressed in tumour cells. CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses against all three TAA were demonstrated, resulting in regression of established tumours. Delivery of multiple TAA might overcome the problem of tumour escape due to antigen loss (Dullaers, Van Meirvenne et al. 2006). Importantly, several groups demonstrated that lentivector-modified DC elicited stronger, longer-lasting anti-tumour T cell responses compared with peptide-pulsed or mRNA electroporated DC, both clinically approved DC-based vaccines (Dullaers, Breckpot et al. 2004; He, Zhang et al. 2005; Metharom, Ellem et al. 2005). These studies suggest that *ex vivo* lentivirally transduced DC are effective in therapeutic treatment of melanoma and other tumours. However, this strategy has important drawbacks. Because the vaccine is patient-specific it requires specialized personnel and facilities for vaccine production. As a consequence, there is the high cost and considerable time required for vaccine production and quality control. It is for that reason that direct lentivector administration *in vivo* has gained substantial interest. Selective *in vivo* lentivector targeting to DC or restricting transgene expression in DC will further improve selectivity, safety and efficacy. These topics will be further discussed in the following sections.

## 2. Lentiviral vectors as an off-the-shelf therapeutic

Encouraged by the first successes of recombinant retro- and lentivirus-mediated gene therapy, lentiviral vectors have been explored to deliver TAA *in vivo* to induce strong anti-tumour immune responses. This requires that lentiviral vectors transduce antigen presenting cells, preferentially DC, delivering both the tumour antigen and signals for DC activation.

### 2.1. Broad tropism lentiviral vectors transduce dendritic cells *in situ*

Broad tropism lentiviral vectors transduce DC *in vivo*. Dullaers *et al* (Dullaers, Van Meirvenne et al. 2006) used a PCR-based method to demonstrate the presence of transgene<sup>+</sup> cells in the draining lymph node, at day 2 and 10, but not day 25 post administration of lentiviral vectors in the footpad. These data were confirmed in flow cytometry, demonstrating that the PCR signal correlated with a small percentage (less than 1%) of transduced CD11c<sup>+</sup> cells (unpublished data Dullaers *et al*). Esslinger *et al* (Esslinger, Chapatte et al. 2003) performed immunohistochemical analysis of frozen lymph node sections and found that the majority of the lentivirally transduced cells were CD11c<sup>+</sup>. More recently, He *et al* (He and Faló 2006) demonstrated that the green fluorescent protein<sup>+</sup> (GFP<sup>+</sup>) DC found in the lymph node after footpad injection, originated from locally transduced migratory skin DC. Intravenous administration of lentiviral vectors leads to transduction of antigen-presenting cells in the spleen (VandenDriessche, Thorrez et al. 2002; Palmowski, Lopes et al. 2004).

These studies indicate that the first pre-requisite for the success of TAA-encoding lentiviral vectors as a therapeutic, i.e. the *in situ* transduction of DC and their localization in lymphoid organs has been met.

### 2.2. Immune responses and inhibition of tumour growth after direct administration of tumour antigen-encoding broad tropism lentiviral vectors

A second pre-requisite for immunisation is that the transduced DC process the lentivirally delivered TAA and subsequently present TAA-derived epitopes in the context of MHC molecules and strong co-stimulation in order to induce strong effector T cell responses. In this case, the degree of TAA-specific CTL induction can be considered as a reliable measure for the value of direct administration of TAA-encoding lentiviral vectors in tumour immunology. Antigen-specific CTL responses could be generated upon direct administration of lentiviral vectors using HLA-Cw3 as a model antigen (Esslinger, Chapatte et al. 2003).

Comparison of the immune response generated upon direct administration of lentiviral vectors with that generated upon vaccination with *ex vivo* lentivirally transduced DC, demonstrated that superior immune responses were generated by the former both in terms of strength and longevity. Similar results were obtained in HLA-A\*0201 transgenic mice using a lentivirus encoding a minigene containing the dominant MART-1/Melan-A HLA-A\*0201 epitope (Chapatte, Colombetti et al. 2006). Using ovalbumin as an antigen, it was confirmed that direct administration of lentiviral vectors is superior to vaccination with *ex vivo* transduced DC, both in terms of the number of IFN- $\gamma$  producing CTL as determined *in vitro* by ELISPOT and the lytic capacity of CTL as determined by an *in vivo* CTL assay (Dullaers, Van Meirvenne et al. 2006). Moreover, memory CTL responses were significantly stronger with direct lentiviral vector administration. Other studies performed with relevant tumour antigens such as NY-ESO (Palmowski, Lopes et al. 2004), TRP-2 (Kim, Majumder et al. 2005), TRP-1 (Liu, Peng et al. 2009) and carcinoembryonic antigen (CEA) (Loisel-Meyer, Felizardo et al. 2009), have also shown potent immune responses upon *in vivo* administration of lentiviral vectors. Chapatte *et al* (Chapatte, Colombetti et al. 2006) compared direct administration of lentiviral vectors encoding the human MART-1/Melan-A antigen with the clinically approved peptide-adjuvant vaccination strategy in a model of HLA-A\*0201 transgenic mice. They demonstrated that the anti-MART-1/Melan-A immune response was higher when immunization was performed with lentiviral vectors when compared to peptide-adjuvant vaccination. Although the generation of a specific CTL response is a convenient read-out for the success of a vaccination strategy, there are many examples of discrepancies between immune responses and anti-tumour responses (Rosenberg, Sherry et al. 2005). Therefore, it is of paramount importance to evaluate the induction of TAA-specific T cells and its influence on tumour growth. Rowe *et al* (Rowe, Lopes et al. 2006) showed significantly improved protection of direct administration of an ovalbumin-encoding lentiviral vector against a subsequent tumour challenge. More significantly, Dullaers *et al* (Dullaers, Van Meirvenne et al. 2006) showed that direct administration of lentiviral vectors offers increased protection to a subsequent tumour challenge compared to DC vaccination and a significantly improved survival of tumour bearing mice. Other studies using TRP-2 (Kim, Majumder et al. 2005), TRP-1 (Liu, Peng et al. 2009) or CEA (Loisel-Meyer, Felizardo et al. 2009) as TAA, demonstrated improved survival of tumour bearing mice receiving lentivirus-encoding the TAA. Moreover, Liu *et al* demonstrated that this type of immunization was able to result in complete regression of small subcutaneous tumours, which correlated with enhanced numbers of CD4<sup>+</sup> and functional CD8<sup>+</sup> T cells in the tumour environment (Liu, Peng et al. 2009). Therefore, there is substantial evidence for the induction of specific immune responses by TAA-encoding lentiviral vector administration *in vivo*, which can induce protective and therapeutic immunity.

### 2.3. Immunogenicity of lentiviral vectors

Although lentiviral vectors do not express viral proteins, administration of lentivectors elicits significant immune responses against transgene-encoded proteins. This suggests immunogenicity of lentiviral vector particles or components present in lentiviral preparations, leading to activation of innate viral-sensing pathways and strong adaptive immune responses.

It has been shown that wild-type HIV-1, from which most recombinant lentiviruses are derived, induces cell- and antibody-mediated responses in humans (Liu, Roberts et al. 1997; McMichael and Phillips 1997). Moreover, HIV-1 activates plasmacytoid DC, a subset of DC specialized in virus recognition, through engagement of toll like receptor (TLR) 7, leading to type I IFN production (Fonteneau, Larsson et al. 2004; Beignon, McKenna et al. 2005). Type I IFN are potent anti-viral cytokines that induce the maturation of plasmacytoid DC, as

well as bystander maturation of other DC subsets (Fonteneau, Larsson et al. 2004), which than can initiate adaptive immune responses. As recombinant lentiviral vectors are HIV-derived and contain single stranded RNA (a ligand for TLR7), they may also trigger a similar innate immune response. Interaction of lentiviral vector particles with TLR7 has been demonstrated in a cell line reporter assay (Breckpot, Emeagi et al. 2007). Furthermore, Brown *et al.* (Brown, Sitia et al. 2006) demonstrated that administration of lentiviral vectors to mice triggers a rapid and transient type I IFN response. The observed effect was independent of the pseudotype, but dependent on functional vector particles, suggesting the necessity of cell entry. Furthermore, as reverse transcription of the viral genome generates double stranded DNA, it has been suggested that TLR9 may be triggered by these lentiviral vectors. Recently, Pichlmair *et al.* (Pichlmair, Diebold et al. 2007) demonstrated that vesicular stomatitis virus glycoprotein (VSV.G) pseudotyped lentivirus preparations are contaminated with tubulovesicular structures of cellular origin, which carry nucleic acids, including plasmid DNA. These structures triggered TLR9 in plasmacytoid DC, hence inducing type I IFN production. However, lentiviral vectors with a gammaretroviral envelope do not trigger TLR9 in plasmacytoid DC but still immunise effectively (Lopes, Dewannieux et al. 2008), suggesting that this particular mechanism is not necessary for potent immune stimulation. Thus all of these studies report activation of plasmacytoid DC by lentiviral vectors, although different mechanisms have been proposed.

In contrast, the effect of lentiviral vectors on conventional DC remains controversial. Some studies with human monocyte-derived DC have been performed. Gruber *et al.* (Gruber, Kan-Mitchell et al. 2000) reported that transduction of immature conventional DC at low multiplicity of infection (MOI) did not result in phenotypical or functional maturation, whereas Tan *et al.* (Tan, Beutelspacher et al. 2005) described that transduction of these DC with a MOI of 500 results in up-regulation of adhesion, co-stimulatory and human leukocyte antigen (HLA) molecules. Furthermore, these DC displayed enhanced allo-stimulatory capacities and an altered cytokine secretion pattern. Breckpot *et al.* (Breckpot, Emeagi et al. 2007) demonstrated that transduction of DC at low MOI results in considerable transgene delivery, without activation, whereas transduction at higher MOI (15–150) indeed leads to phenotypical and functional maturation. It was demonstrated in the latter studies that protein kinase R (PKR), a cytosolic receptor, which interacts with double stranded RNA, an intermediate in the lentiviral replication, is phosphorylated upon transduction at high MOI (Tan, Beutelspacher et al. 2005; Breckpot, Emeagi et al. 2007). This PKR phosphorylation leads to the degradation of I $\kappa$ B and subsequent activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor associated with DC maturation (Taylor, Haste et al. 2005). However, the activation of conventional DC, the DC subset believed to orchestrate the immune response, *in vivo* has - to our knowledge - not been studied.

It is important to note that induction of a strong anti-tumour response is not only dependent on antigen-recognition by T cells and co-stimulation provided by the DC, but is crucially dependent on an inflammatory environment, in order to overcome tolerance and active inhibitory mechanisms. Such an inflammatory environment can be achieved by strong activation of the innate arm of the immune system, in particular through the engagement of TLR. Two studies, one by Yang *et al.* (Yang, Huang et al. 2004) and the other by Lang *et al.* (Lang, Recher et al. 2005), demonstrate that tolerance of antigen-specific CTL could be broken by persistent TLR ligation. Furthermore, it has been recently described that signalling through certain combinations of TLR on DC not only provided a synergy with respect to the production of cytokines such as IL-12, which is essential for skewing CD4<sup>+</sup> T cells toward a T<sub>H</sub>1 phenotype (Gautier, Humbert et al. 2005; Napolitani, Rinaldi et al. 2005), but also offered protection from inhibitory Treg that quench the anti-tumour immune response (Warger, Osterloh et al. 2006).

In addition to breaking tolerance, a productive CD4<sup>+</sup> T cell response is required for the induction of a strong and sustained CTL response. In this regard, it has been recently demonstrated with *ex vivo* lentivirally transduced DC, cultured in foetal calf serum (FCS) free medium, that transduction with high titer lentiviral vectors, generated in the presence of FCS, results in the transfer of FCS components into MHC class II molecules, as such stimulating FCS-specific CD4<sup>+</sup> T cells and providing antigen a-specific CD4<sup>+</sup> T cell help (Bao, Guo et al. 2009). Whether the presence of FCS results in the induction of FCS-mediated CD4<sup>+</sup> T cell help *in vivo* remains to be seen. With regard to direct administration of lentiviral vectors, several groups have shown that both a CTL response and an antigen-specific CD4<sup>+</sup> T cell response can be induced (Esslinger, Chapatte et al. 2003; Dullaers, Van Meirvenne et al. 2006; Rowe, Lopes et al. 2006). However, not much data is available on the role of CD4<sup>+</sup> T cell help in the induction of CTL upon lentiviral immunization. Esslinger *et al* (Esslinger, Chapatte et al. 2003) showed that CD4 depletion reduces the primary CTL response upon direct administration of lentiviral vectors. Similarly, Dullaers *et al* (Dullaers, Van Meirvenne et al. 2006) showed that although there was a larger requirement for CD4<sup>+</sup> T cell help during the primary response in case of immunization with *ex vivo* transduced DC compared to direct administration of lentiviral vectors, CD4<sup>+</sup> T cell depletion strongly reduced the capacity to mount a recall CTL response in both cases. Interestingly, Marzo *et al* (Marzo, Vezys et al. 2004) showed that in the case of a VSV infection, a functional CD8<sup>+</sup> T cell memory response can be generated in the absence of CD4<sup>+</sup> T cells, this in contrast to an infection with *Listeria monocytogenes*. These authors suggest that the difference might be due to the fact that VSV can directly infect DC whereas *Listeria monocytogenes* needs to be cross-presented. Since, the currently applied lentiviral vectors are pseudotyped with the envelope of VSV, it needs to be further examined to what extent the CTL response is CD4<sup>+</sup> T cell dependent. Overall, these studies indicate that lentiviral vectors induce DC activation through TLR signalling and other mechanisms, explaining their potency as an anti-tumour vaccine.

### 3. Towards *in vivo* targeting of dendritic cells by lentiviral vectors

Although direct *in vivo* administration of lentivectors leads to transduction of DC, the broad tropism of the vectors used in most of the studies so far also implies that other cell types are transduced. The antigen expressed from cells other than DC could be cross-presented by neighboring DC and might further improve the processing and presentation of the antigen (Schulz, Diebold et al. 2005). However, specific transduction of DC could improve the safety and efficacy of the vaccination. Several strategies have been used for this which can be divided in two categories: transductional and transcriptional targeting.

#### 3.1. Transductional targeting

The lentivector tropism is determined by the glycoproteins incorporated in the viral envelope, which interact with specific receptors on the membrane of the target cells. Since lentivectors are usually pseudotyped with heterologous glycoproteins, the natural tropism of the envelope used for pseudotyping can restrict the vector entry to specific cells or tissues.

There is an ever-growing list of glycoproteins that have been successfully used for pseudotyping of lentivectors. Examples are glycoproteins from *retroviridae*, *rhabdoviridae*, *arenaviridae*, *flaviviridae*, *paramyxoviridae*, *baculoviridae*, and *filoviridae* (Bouard, Alazard-Dany et al. 2009). Although each of these glycoproteins preferentially interacts with specific cell types, finding a natural envelope for DC-specific targeting has been unsuccessful. Therefore, envelopes have been engineered in several ways to re-direct their tropism towards specific cell types.

One way of doing this is by genetically modifying the envelope glycoprotein to ligands that bind to receptors present in the cells to be transduced. However, ligand-fused glycoproteins often results in poor infectivity due to inability of the retargeted envelope to induce membrane fusion and sequestration of the viral particles to some cell surface molecules (Frecha, Szecsi et al. 2008). Another alternative is the modification of the existing envelopes to re-direct receptor attachment without hampering membrane fusion. Yang et al (Yang, Bailey et al. 2006) showed that by introducing a mutation to the envelope of Sindbis virus, its affinity for an ubiquitous receptor (heparan sulphate) was ablated while preserving the capacity to bind to DC-SIGN, a lectin-type receptor present on some DC subsets. OVA-encoding lentivectors pseudotyped with this modified envelope specifically transduced DC in vivo, inducing efficient immune responses against OVA-expressing tumors. However, engineering of re-targeted envelope proteins by fusion to natural ligands has proven to be difficult, and these strategies were at first applied with limited success (Waehler, Russell et al. 2007).

The viral-packaging system can also be exploited to modify the viral envelope. As the virus is generated from a producer cell line, on budding it incorporates part of the cell membrane in its envelope. When a specific receptor is overexpressed on the cell membrane of a producer cell line, it can also be displayed on the viral envelope. When overexpressing stem cell factor (SCF) on the cell membrane of an ecotropic producer cell line, Chandrashekran et al. (Chandrashekran, Blood, 2004 and J Gene Med, 2004) showed that retrovirus thus produced was able to preferentially transduce c-kit expressing human stem cells. Recently, Yang et al. (Yang, PNAS, 2006) demonstrated that this approach can also be applied for lentiviruses. This method could thus be modified to generate lentiviral vectors that can specifically target DC (or other cell types) and still yield high expression of the transgene. Receptors that could be used include CD40 ligand, which interacts with CD40, and CTLA-4, which interacts with CD80 and CD86. As many of these receptors are expressed on other cell types than DC, specificity will still be limited.

Single-chain antibodies (scFv) specific against surface proteins present on the target cell have been used for this purpose (Fig. 1). Specific targeting of DC has been achieved with antibodies directed against C-type lectins, such as DEC-205 (Bonifaz, J Exp Med, 2002 and 2004) and DC-SIGN (Dzionic, J Exp Med, 2001). Display of scFv in the context of envelope glycoproteins of measles virus seems to be a promising approach since measles virus enters cells through direct fusion at the cell membranes. Target versus non-target cell discrimination has been shown in vitro using this system (Funke, Maisner et al. 2008).

As molecular cloning of classic antibodies or fragments thereof offers serious challenges, alternatives have been explored. One of them is the use of antibodies of members of the family of camilidae (i.e., dromedaries, camels, llamas), which produce a unique class of antibodies composed of two identical heavy chains as opposed to the conventional (four-chain) antibody repertoire (Hamers, Nature, 1993). The antigen-binding part of the molecule is composed of only one single variable region (termed VHH, or nanobody). These antigen-specific antibody fragments offer many advantages: (1) they are highly soluble, (2) they can refold after denaturation whilst retaining their binding capacity, (3) cloning and selection of antigen-specific nanobodies obviate the need for construction and screening of large libraries, (4) as nanobodies can be fused to other proteins, it should be possible to present them on the cell membrane of a producer cell line, such as HEK 293T, thus, generating lentiviral particles that incorporated a DC-specific nanobody in their envelope during budding as described above.

### 3. 2. Transcriptional targeting

Transgene expression can be targeted to specific cell types by the use of cell or tissue-specific promoters. Several promoters have been studied with this purpose (reviewed in (Frecha, Szecsi et al. 2008)). In the specific case of DC, different promoters can offer the opportunity to target expression to specific DC subtypes. For example, the CD11c can limit antigen expression to myeloid DC (Noti, Reinemann et al. 1996). BDCA-2 and Langerin promoter results in exclusive expression in pDC (Dzionic, Sohma et al. 2001; Takahara, Omatsu et al. 2002) and Langerhans cells (Takahara, Omatsu et al. 2002) (Takahara, Omatsu et al. 2002), respectively.

Using lentivectors, Gorski *et al.* (Gorski, Shin et al. 2003) identified the promoters of B7-DC and CCL17 as active in bone marrow-derived DC but not in macrophages. More recently, Lopes *et al.* (Lopes, Dewannieux et al. 2008) evaluated the use of the mouse dectin-2 promoter to drive GFP expression in mouse BMDC cultures and in human skin-derived Langerhans and dermal DC. When these lentiviral vectors were injected intravenously, GFP expression was detected in splenic dectin-2<sup>+</sup> cells, whereas subcutaneous injection resulted in transduced CD11c<sup>+</sup> DC in the draining lymph node. Immunization with dectin-2 lentiviruses encoding NY-ESO-1 resulted in an antigen-specific CD8<sup>+</sup> T cell response in HLA-A2 transgenic mice and further stimulated a CD4<sup>+</sup> T cell response to a newly identified NY-ESO-1 epitope presented by H2 I-Ab. Importantly, it was demonstrated that immunization with dectin-2 lentiviruses was similar to that with lentiviruses containing a strong constitutive viral promoter, demonstrating that targeting antigen expression to DC results in an effective vaccine. These studies demonstrate that the transgene expression limited to DC is a promising and safer strategy in lentivector immunization.

## 4. TARGETING DENDRITIC CELL DIFFERENTIATION AND MATURATION

Another interesting approach to enhance a lentivector-based vaccine is to increase its intrinsic immunogenicity. Untargeted and DC-targeted lentivector vaccines can elicit potent CD4 and CD8 T cell responses in several infection and tumour models. In addition, the immunogenic potential of lentivectors can be manipulated by expression of molecules that enhance DC maturation or prolong antigen presentation. Tumour cells arise endogenously and most TAA are self-antigens. Therefore, T cells recognizing many of the potential tumour-specific T cells have already been eliminated by central or peripheral tolerance (Walker and Abbas 2002). Additionally, tumour cells have acquired several T cells suppressive mechanisms including antigen presentation in the absence of costimulation, expression of inhibitory/death inducing signals (e.g. PD-L1, FasL), expression of immunomodulatory enzymes (e.g. indoleamine-2,3-deoxygenase) and secretion of suppressive cytokines and chemokines such as IL-10 TGF- $\beta$  (Zitvogel, Tesniere et al. 2006). Moreover, these immunosuppressive mechanisms promote the differentiation of immune cells suppressive cells such as regulatory DC and T cells, and myeloid-derived suppressor cells (Emens 2003) (Lizee, Radvanyi et al. 2006).

A productive T cell immune response requires specific recognition of an MHC/peptide complex by the TCR (signal 1) together with signaling through co-stimulatory molecules (signal 2). In addition, to establish strong antitumour responses, an inflammatory environment (signal 3) is required. All these requirements can be achieved by strong activation of the innate arm of the immune system, in particular through TLR signalling (van Duin, Medzhitov et al. 2006) (Iwasaki and Medzhitov 2004) (Pasare and Medzhitov 2005) (Rakoff-Nahoum and Medzhitov 2009). In fact, TLR signaling controls DC maturation, including their capacity to migrate, up-regulate co-stimulatory molecules such as CD40, CD80, CD86 and CD70 and produce pro-inflammatory cytokines such as IL-1, IL-6, TNF- $\alpha$



and IL-12 (Iwasaki and Medzhitov 2004; Pasare and Medzhitov 2004; van Duin, Medzhitov et al. 2006). Accordingly, persistent TLR ligation may be necessary to break tumour-induced tolerance in the context of antigen-loaded mature DC (Yang, Huang et al. 2004).

Ideally, a lentivector vaccine could be engineered so that it would include DC activators of differentiation and maturation, and providing at the same time expression of TAAs. This approach would directly target DC activation and tumour antigen presentation, and at the same time prevent the actions of tolerogenic mechanisms over transduced DCs.

#### 4.1. Mechanisms of NF- $\kappa$ B and MAPK activation in dendritic cells

Understanding the mechanisms of TLR signalling and their exploitation is key to enhance lentivector-based vaccines. Binding of TLR to their ligands trigger a complex network of signalling molecules and adaptor proteins that will modulate DC responses in innate and adaptive immunity (Caparros, Munoz et al. 2006). All these networks integrate and converge into a few pathways, such as that of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPK) (Luft, Rodionova et al. 2006) (Fig. 2). NF- $\kappa$ B is a transcription factor that binds to a 10 base pair consensus DNA element (5'-GGGAATTTCC-3') and its many variations (Sen and Baltimore 1986). The NF- $\kappa$ B family of proteins is central to immunity and inflammation (Ghosh and Karin 2002). Their members exist as homo- or heterodimeric complexes formed by combinations of five subunits, p65/RelA, RelB, c-Rel, p50 and p52. Of these, RelA, RelB and c-Rel are synthesized in their mature forms and contain a transactivation domain. NF- $\kappa$ B p50 and p52 are synthesized as precursors containing carboxy-terminal ankyrin repeats, which are processed by the proteasome leading to mature proteins. Both p50 and p52 contain a DNA binding domain, but lack a transactivation domain. In particular, RelB is implicated in DC differentiation and maturation (Weih, Carrasco et al. 1995) (Burkly, Hession et al. 1995). All NF- $\kappa$ B proteins present a conserved amino-terminal 300 amino acid Rel homology domain, responsible for dimerization and DNA binding. Furthermore, this domain binds to inhibitory regulatory factors, the inhibitors of  $\kappa$ B (I $\kappa$ B) proteins. The binding of I $\kappa$ B keeps cytosolic NF- $\kappa$ B dimers in an inactive state. NF- $\kappa$ B is activated by pro-inflammatory cytokines (such as TNF- $\alpha$ , IL-1), T cell delivered signalling (CD40L), bacteria, viruses and cellular stress leading to DC maturation (Rothwarf and Karin 1999). NF- $\kappa$ B pathways share adaptor molecules with MAPK pathways, and can be broadly divided into a classical (canonical) and non-classical pathway (Edwards, Bartlett et al. 2009) (Lee, Jeon et al. 2007) (Akira, Uematsu et al. 2006) (Wang, Miyahara et al. 2008). Both pathways result in activation of I $\kappa$ B kinases (IKK) which will phosphorylate I $\kappa$ B proteins leading to their ubiquitination and degradation. This releases active NF- $\kappa$ B dimers. I $\kappa$ B kinase (IKK) is a multi-subunit protein complex consisting of two catalytic subunits, IKK $\alpha$  and IKK $\beta$  and a regulatory subunit, IKK $\gamma$  (also called NEMO, NF- $\kappa$ B essential modulator). Phosphorylation of I $\kappa$ B at two critical serine residues (Ser32/Ser36 in I $\kappa$ B $\alpha$  and Ser19/Ser23 in I $\kappa$ B $\beta$ ) by the IKK complex targets them for proteasomal degradation (Dawson, Hastings et al. 1997). In the classical pathway, it has been shown that IKK $\beta$ , but not IKK $\alpha$ , is important in NF- $\kappa$ B activation. Furthermore, it has been demonstrated that these two kinases have distinct rather than overlapping functions (Hu, Wang et al. 1999) (Li, Van Antwerp et al. 1999) (Takeda, Takeuchi et al. 1999) (Li, Chu et al. 1999). The classical pathway includes signalling from TLR/IL-1R family members, intracellular pattern recognition receptors including retinoic acid inducible gene (RIG-I), melanoma differentiation associated factor-5 (MDA-5) and protein kinase R (PKR), as well as TNFR signalling (Hacker and Karin 2006). Mediators such as lymphotoxin- $\beta$ , CD40L and receptor activator of NF- $\kappa$ B ligand (RANKL) activate the non-classical pathway, which involves IKK $\alpha$  phosphorylation, processing of the p52 precursor p100 and nuclear translocation of the heterodimer p52/RelB (Senftleben, Cao et al. 2001) (Lawrence and Bebiec 2007).

NF- $\kappa$ B targets genes associated with DC maturation, such as cytokines (e.g. IL-6, IL-12, TNF- $\alpha$ ), chemokines (e.g. MIP-1 $\alpha$ , MCP1), adhesion molecules (e.g. ICAM-1), inducible effector enzymes (e.g. COX-2) and apoptosis regulators (e.g. c-IAP, XIAP, Bcl-xL). Consequently, the NF- $\kappa$ B pathway is tightly regulated by negative feedback mechanisms that prevent excess inflammation and autoimmunity. Several feedback molecules have been described, including I $\kappa$ B, A20 (or TNF- $\alpha$  inducible protein 3, TNFAIP3), tripartite-motif protein (TRIM) 30 $\alpha$  and phosphatase PP2A, to name a few (Breckpot, Aerts-Toegaert et al. 2009) (Kawai and Akira 2007) (Coornaert, Carpentier et al. 2009) (Barisic, Strozyk et al. 2008; Bowie 2008; Shi, Deng et al. 2008). The A20 negative regulator has been recently studied and targeted to enhance tumour antigen-specific immune responses. A20 is under the immediate control of NF- $\kappa$ B and it is induced in many cell types, amongst which mouse and human DC (Dixit, Green et al. 1990) (Breckpot, Aerts-Toegaert et al. 2009) (Song, Evel-Kabler et al. 2008) (Beyaert, Heyninck et al. 2000). A20 is an ubiquitin-editing enzyme with an amino-terminal de-ubiquitinase activity and ubiquitinase activity in the C-terminus zinc finger domain, important for modulating NF- $\kappa$ B signaling by interaction with proteins of the TNF-, IL-1/TLR-signaling pathways (Heyninck and Beyaert 1999; Beyaert, Heyninck et al. 2000; Zhang, Kovalenko et al. 2000; Boone, Turer et al. 2004; Wang, Li et al. 2004; Wertz, O'Rourke et al. 2004; Saitoh, Yamamoto et al. 2005).

MAPK are a diverse group of intracellular serine/threonine kinases which are phylogenetically conserved and regulate a wide range of cellular processes, including immune responses (Ardeshtna, Pizzey et al. 2000). Three groups of MAPK have been identified: the extracellular signal-regulated protein kinases (ERK) (Boulton, Nye et al. 1991) (Boulton and Cobb 1991), the c-Jun N-terminal kinases (JNK) (Derijard, Hibi et al. 1994) (Kyriakis, Banerjee et al. 1994) and the p38 stress-activated protein kinases (p38) (Lee, Laydon et al. 1994) (Han, Lee et al. 1994). MAPK pathways consist on a three-modular cascade involving activating phosphorylations of downstream kinases by upstream kinases. In this way, MAPK kinase kinases (MAPKKK) are firstly activated by phosphorylation after their recruitment to TLR cytoplasmic domains. These phosphorylated MAPKKK subsequently phosphorylate MAPK kinases (MAPKK), which in turn will phosphorylate MAPK. The TLR cytoplasmic tail contains the Toll/IL-1 receptor (TIR) domain, essential for signal transduction (Beutler 2009), and mainly two TIR domain adaptor molecules, MyD88 (myeloid differentiation factor 88) and TRIF (Toll/IL-1 receptor domain-containing adaptor-inducing IFN- $\beta$ ) are recruited to the TIR domain. Then, MAPKKK are activated through recruitment of various protein kinases and scaffold proteins, such as ubiquitin ligases TNFR-associated factor 6 (TRAF6) and TRAF3 (Hacker, Redecke et al. 2006). MAPK activation has been shown to depend on MyD88-TRAF6-mediated recruitment of a complex containing MAPKKK TAK1 (transforming growth factor- $\beta$ -activated kinase-1) (Sato, Sanjo et al. 2005), but also in an alternative pathway involving TRIF-TRAF3 (Hacker, Redecke et al. 2006).

In DC, ERK is involved in cell survival (Rescigno, Martino et al. 1998), regulation of inflammation and immune suppression (Agrawal, Dillon et al. 2006) (Escors, Lopes et al. 2008). MAPK p38 comprises at least four splice isoforms from which p38 $\alpha$  and p38 $\beta$  are ubiquitously expressed. In the context of TLR activation, p38 phosphorylation is completely dependent on TAK1 (Sato, Sanjo et al. 2005), which phosphorylates MKK3 and MKK6, the two main p38 MAPKK (Ninomiya-Tsuji, Kishimoto et al. 1999) (Moriguchi, Kuroyanagi et al. 1996). Classical p38 targets range from kinases such as MK2 and transcription factors such as ATF, p53, c/EBP and NFAT just to mention a few (Zarubin and Han 2005). MAPK p38 plays an important part in DC maturation and secretion of pro-inflammatory cytokines. MAPK p38 is involved in up-regulation of DC co-stimulatory molecules and maturation markers such as CD40, CD80, CD86, CD83 and MHC class II (Arrighi, Rebsamen et al.

2001), and secretion of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-12 (Arrighi, Rebsamen et al. 2001) (Yu, Kovacs et al. 2004) (Lu, Yang et al. 1999). MAPK c-Jun N-terminal kinase (JNK) proteins are encoded by 3 genes with 10 or more alternative splice forms (Gupta, Barrett et al. 1996), and they are activated by UV irradiation, environmental and chemical stress, pro-inflammatory cytokines and TLR signalling (Hacker, Redecke et al. 2006) (Wang, Deng et al. 2001) (Ninomiya-Tsuji, Kishimoto et al. 1999) (Gupta, Barrett et al. 1996). JNK is activated by dual phosphorylation by MAPKK, MKK4 and MKK7. The classical JNK targets are c-Jun, from which it receives its name, ATF2, p53, Elk-1 and c-Myc amongst others (Junttila, Li et al. 2008) (Morton, Davis et al. 2004) (Gupta, Barrett et al. 1996) (Morton, Davis et al. 2003). ATF2 and c-Jun are components of AP-1, a transcription factor that transactivates many proinflammatory genes. Consequently, JNK is involved in inflammatory responses and inhibitors are being evaluated in clinical trials for the treatment of autoimmune diseases such as rheumatoid arthritis as well as asthma, inflammatory diseases and some types of leukaemia (Roberts and Der 2007). In the context of TLR signalling, TAK1 is involved in JNK activation through MKK3 and MKK7 phosphorylation (Sato, Sanjo et al. 2005) (Wang, Deng et al. 2001) (Ninomiya-Tsuji, Kishimoto et al. 1999) (Hammaker, Boyle et al. 2007). In general, JNK activity enhances DC maturation and proinflammatory cytokine secretion, although at lower levels compared to p38 (Escors, Lopes et al. 2008) (Nakahara, Uchi et al. 2004). Therefore, the specific delivery of TAAs with modulators of MAPK pathways to DC could enhance tumour-specific immune responses.

#### 4.2. Lentivector-modification of DC to mimic persistent TLR activation

In recent years, strategies to deliver activation signals to DCs simultaneously to expressing TAAs have been developed. One of these strategies aims to mimic persistent TLR activation to drive DC maturation. DC express TLR4 which binds LPS resulting in DC maturation (da Silva Correia, Soldau et al. 2001) (Ardehna, Pizzey et al. 2000) (Arrighi, Rebsamen et al. 2001). LPS-mediated activation remarkably enhances stimulation of DC-mediated immune responses *in vitro*, and could overcome suppression by regulatory T cells, a critical factor in anti-tumour immunology (Pasare and Medzhitov 2003). However, its clinical use is prohibited due to cytotoxicity. Therefore, a constitutive active TLR4 (caTLR4) for DC maturation has been evaluated (Abdel-Wahab, Cisco et al. 2005) (Bonehill, Tuyaerts et al. 2008) (Cisco, Abdel-Wahab et al. 2004) (Xu, Darcy et al. 2007). This was achieved by truncating positions M620 to P621 and linkage to the nerve growth factor or the LAMP1 leader sequences (Abdel-Wahab, Cisco et al. 2005) (Cisco, Abdel-Wahab et al. 2004) (Bonehill, Tuyaerts et al. 2008) [187, 189][188]. Alternatively, TLR4 cytoplasmic domain was linked to the extracellular single-chain immunoglobulin anti-erbB2 (Xu, Darcy et al. 2007). Delivery of these active TLR4 constructs resulted in NF- $\kappa$ B activation leading to DC maturation, and moreover, these DC were stimulated MELAN-A and Trp2-specific CTL (Abdel-Wahab, Cisco et al. 2005) (Bonehill, Tuyaerts et al. 2008) (Cisco, Abdel-Wahab et al. 2004).

Another strategy consisted on the introduction of the major TLR adaptor molecules MyD88, TRIF or IRAK-1, which were shown to stimulate downstream signals in the absence of TLR stimuli. Akazawa *et al.* expressed MyD88 and TRIF in mouse DC using lentiviral vectors, resulting in DC with different properties. MyD88-modified DC produced IL-6 and IL-12p40, but no up-regulation of phenotypic markers, whereas TICAM-1 expression stimulated interferon IFN- $\beta$  production and increased levels of CD86. Both MyD88 and TRIF increased the allo-stimulatory capacity of modified DC, and tumour outgrowth was delayed after immunization with these modified DC (Akazawa, Shingai et al. 2007). Xu *et al.* generated retroviral vectors encoding chimeric proteins consisting of the extracellular single-chain immunoglobulin anti-erbB2 linked to either MyD88 or IRAK-1. These

experiments were performed in an immortalized DC line, JAWS II, and only the IRAK-1 chimera mediated IL-12 and TNF-alpha secretion. The latter demonstrated enhanced OVA-specific OT-II CD4 T cell responses (Xu, Darcy et al. 2007).

#### 4.3. Lentivector-targeted activation of NF- $\kappa$ B in dendritic cells

NF- $\kappa$ B has been one of the first pathways to be targeted in DC as an adjuvant by overexpressing NF- $\kappa$ B inducing kinase (NIK) using adenovirus vectors. This led to increased DC maturation and increased Th1 GFP-specific immune responses, although the relevance of this strategy in anti-viral or anti-tumour immunity was not assessed (Andreacos, Williams et al. 2006). Sustained NF- $\kappa$ B activation in DC using lentiviral vectors has been achieved by expressing Kaposi's sarcoma associated human herpes virus (KSHV) vFLIP (Rowe, Lopes et al. 2009). In this case, DC maturation was enhanced by up-regulation of MHC I and II, co-stimulatory molecules CD80, CD86, CD40 and ICAM-I, and increased secretion of TNF-alpha and IL-12. vFLIP-modified DC significantly increased antigen-specific CD8+ T cell responses resulting in enhanced anti-tumour and anti-parasite immunity (Rowe, Lopes et al. 2009) (Karwacz, Mukherjee et al. 2009).

Another effective approach leading to sustained NF- $\kappa$ B activation consists of down-regulation of negative feed-back mechanisms. As mentioned above, A20 is one of the feedback regulators of NF- $\kappa$ B. A20 deactivates several adaptor molecules of the TNFR, IL-1/TLR signaling pathways by ubiquitination/de-ubiquitination activities. In this way, A20 controls IKK and thus the degradation of I $\kappa$ B $\alpha$ . Therefore, A20 down-regulation could result in prolonged NF- $\kappa$ B activation, mimicking persistent TLR ligation and resulting in DC with enhanced stimulatory capacity. Lentivirally delivered A20-targeted shRNA and direct introduction of siRNA were applied to downregulate A20 (Breckpot, Aerts-Toegaert et al. 2009) (Song, Evel-Kabler et al. 2008). These approaches showed that A20 controls DC maturation, cytokine production and immunostimulatory potency. Human DC with down-regulated A20 expression increase NF- $\kappa$ B activity and show enhanced and sustained IL-10 and IL-12 secretion. These DC were more potent in stimulating MelanA-specific CD8+ T cells (Breckpot, Aerts-Toegaert et al. 2009). Mouse DC with down-regulated A20 expression showed enhanced expression of co-stimulatory molecules and pro-inflammatory cytokines and they were refractory to regulatory T cell inhibition, leading to activated tumour-infiltrating CTL and T helper cells (Song, Evel-Kabler et al. 2008). Therefore, A20 is an ideal target for anti-tumour immunotherapy, since it enables DC to induce strong effector T cell responses and inhibit regulatory T cells.

#### 4.4. Lentivector-targeted activation of MAPK pathways in dendritic cells

Lentivectors have been used to increase DC immunogenicity by introducing specific genes that modulate intracellular MAPK pathways. ERK and p38 were activated by expressing MEK1 and MKK6 mutants containing glutamate and aspartate residues in their activation loop, mimicking activating phosphorylated serine or threonine residues (Raignaud, Whitmarsh et al. 1996). A fusion protein between MKK7 and JNK1 was expressed to achieve constitutive JNK1 phosphorylation (Escors, Lopes et al. 2008). In addition, expression of constitutive activators prevents inactivation by phosphatase-dependent negative feedback mechanisms, which may be important to counteract tolerogenic mechanisms in anti-tumour immunity. In the absence of TLR stimulation, p38 activation resulted in a DC maturation phenotype different from full maturation as achieved by LPS treatment (Escors, Lopes et al. 2008). Particularly, there was specific up-regulation of co-stimulatory molecules CD80, CD40 and ICAM-I, and absence of significant secretion of pro-inflammatory cytokines (Escors, Lopes et al. 2008). This is in contrast to studies using p38 inhibitors after TLR stimulation (Arrighi, Rebsamen et al. 2001) (Lu, Yang et al. 1999; Yu, Kovacs et al. 2004). Consequently, p38 activation may not be directly involved in

transcriptional up-regulation of pro-inflammatory cytokine genes (Saccani, Pantano et al. 2002). Interestingly, co-expression of OVA with the p38 activator in DC significantly increased antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses leading to increased anti-tumour immunity in a OVA-expressing lymphoma model (Escors, Lopes et al. 2008) (Karwacz, Mukherjee et al. 2009). Additionally, MAPK p38 constitutive activation also increased CD8<sup>+</sup> T cell responses to human tumour antigens NY-ESO in a humanized HLA-A2 mouse model and MelanA/MART-1 in a human DC-T cell culture (Escors, Lopes et al. 2008). Specific activation of JNK1 in DC showed only a moderate up-regulation of CD80 and ICAM-I and no significant secretion of pro-inflammatory cytokines, confirming previous studies which suggested that JNK marginally control DC maturation (Escors, Lopes et al. 2008) (Nakahara, Uchi et al. 2004). On the other hand, increased antigen-specific CD8<sup>+</sup> T cell expansion was achieved in mice after subcutaneous vaccination with LV expressing MKK7-JNK1, suggesting that JNK1 may play a subtle but important role in DC *in vivo* (Escors, Lopes et al. 2008).

## 5. POTENTIAL CLINICAL USE OF LENTIVIRUS VECTORS FOR CANCER IMMUNOTHERAPY

### 5.1. Lentivector persistence in DC

The duration of antigen presentation is clearly a relevant issue in the setting of LVs as potential genetic vaccines, since these vectors permanently integrate their genome into the host cell. Consequently, it would be expected that APCs targeted by LVs would produce sustained expression of the antigen for the life time of the cells. In the post-vaccination scenario with lentivectors, there is no clear picture of the factors that could influence the effects of a sustained antigen presentation.

Antigen persistence has been shown in the bone marrow, liver and spleen of mice injected systemically *in vivo* with lentivectors (Pan, Gunther et al. 2002; Kimura, Koya et al. 2007). We have recently shown that this route of immunization not only results in the transduction of lymphocytes, macrophages and different DC subsets, but also in transduction of a DC precursor within the spleen (Arce, Rowe et al. 2009). As a result, the percentages of GFP<sup>+</sup> DC increased over time and GFP<sup>+</sup> DC were still detectable two months after immunization. Presentation of OVA decreased over time, however it was still detectable after 2 months.

Although there is evidence that suggests that protracted antigen presentation can result in tolerance and impairment of the immune response (Zinkernagel and Hengartner 2004), a recent study showed that prolonged antigen expression promotes immunization even in the absence of a dendritic cell activation signal (Obst, van Santen et al. 2007). In fact prolongation of antigen presentation, achieved by inhibiting apoptosis of DC, has been shown to be more effective in vaccination (Kim, Hung et al. 2003). In some way, lentiviral vector immunization seems to mimic persistent viral infection, which can result in high level CD8<sup>+</sup> T cell responses, generated both from memory cells and naïve CD8<sup>+</sup> T-cell recruitment (Snyder, Cho et al. 2008). For cancer treatment, this is an important characteristic to help prevent tumour recurrence. Whether persistent memory plays a role in this is not completely clear, but there is no evidence of tolerance to the transgene in the long term.

### 5.2. Clinical trials

In spite of their extensive pre-clinical use, translation of lentiviral vectors into the clinical scenario is still in its early days. Results from the use of autologous CD4<sup>+</sup> T cells modified with a conditionally-replicating lentivector expressing an antisense gene against the HIV envelope showed sustained gene transfer and no evidence of insertional mutagenesis after

21-36 months. There was self-limiting mobilization of the vector and improvement of the immune function in four out of five subjects (Levine, Humeau et al. 2006). These results are promising in the sense that they show efficacy and safety of the vector. To our knowledge, no clinical trials using lentivectors for active immunization have yet been begun. We would suggest that the potential benefits of tumour therapy outweigh the risks of lentiviral vector immunisation in patients with advanced stage cancer. However, the potential for lentiviral vectors, even when targeted, to transduce dividing cells such as DC precursors imparts a theoretical risk of insertional mutagenesis. Therefore the future development of non-integrating lentiviral vectors, which could be sufficiently safe even for prophylactic vaccination, is essential. Such vectors were first used pre-clinically to treat a mouse model of inherited retinopathy (Yanez-Munoz, Balagga et al. 2006), and have also been used for gene delivery to non-dividing tissues such as muscle (Apolonia, Waddington et al. 2007). Recently we and others have shown immunisation with non-integrating lentiviral vectors encoding OVA, HBV (Karwacz, Mukherjee et al. 2009), HIV (Negri, Michelini et al. 2007), SIV (Michelini, Negri et al. 2009), or West Nile virus (Coutant, Frenkiel et al. 2008) antigens. It will now be of importance to examine the duration and quality of the immune response and the effectiveness of tumour therapy in comparison to integrating lentiviral vectors.

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## Abbreviations

<b>CEA</b>	carcinoembryonic antigen
<b>CTL</b>	cytotoxic T lymphocyte
<b>DC</b>	dendritic cell
<b>FCS</b>	foetal calf serum
<b>GFP</b>	green fluorescent protein
<b>HIV</b>	human immunodeficiency virus
<b>HLA</b>	human leukocyte antigen
<b>IFN</b>	interferon
<b>IL</b>	interleukin
<b>MDSC</b>	myeloid-derived suppressor cells
<b>MHC</b>	major histocompatibility complex
<b>MOI</b>	multiplicity of infection
<b>PKR</b>	protein kinase R
<b>TAA</b>	tumour-associated antigen
<b>TCR</b>	T cell receptor
<b>T<sub>H</sub></b>	T helper cell
<b>TLR</b>	toll like receptor
<b>TNF-<math>\alpha</math></b>	tumour necrosis factor- $\alpha$
<b>Treg</b>	regulatory T cell

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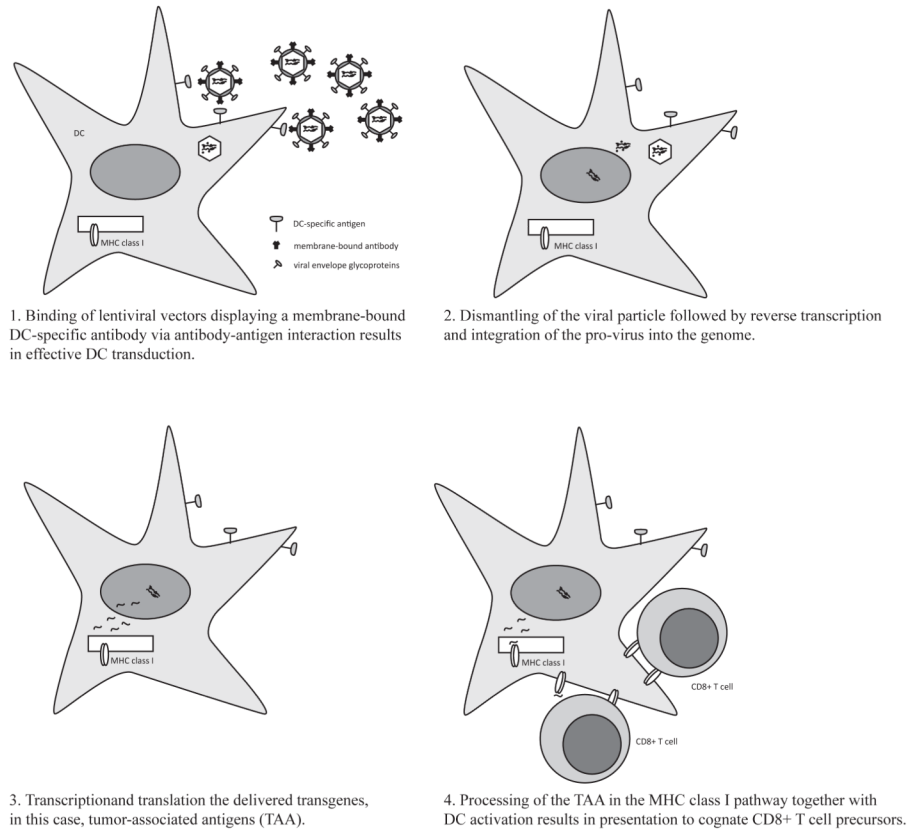
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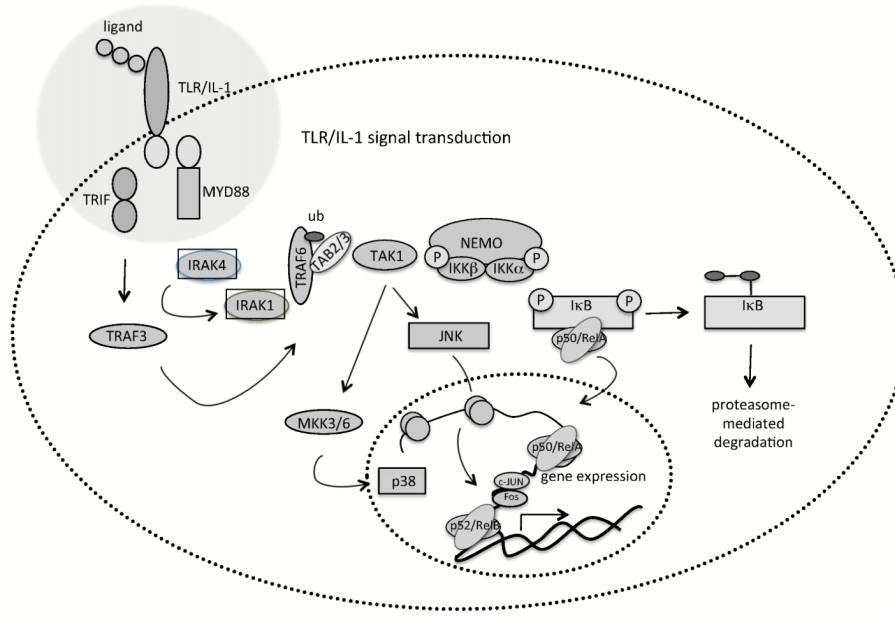
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**Fig. (1). Transductional targeting of DCs with lentiviral vector**

In this scheme, DC transduction by specific surface targeting is shown as an example. Lentiviral particles are pseudotyped with a fusion protein together with an antibody that is specific for a particular surface protein present only in DC. These lentivectors can then specifically bind to the DC surface when administered *in vivo* (1). After binding, lentivectors fuse with the DC membrane and the core is incorporated and dismantled in the DC cytoplasm. The genome RNA is reversed-transcribed into a cDNA copy that integrates into the DC genome (2). From the integrated lentivector, transcription takes place leading to synthesis of TAAs, which are subsequently degraded (2). TAAs are processed into peptides and loaded in the MHC I compartment, together with DC activation by the transduction process itself. MHC I-peptide complexes are displayed on the DC surface and presented to TAA-specific CD8 T cells that differentiate in effector anti-tumour CTLs.



**Fig. (2). Representation of MAPK and NF-κB pathways involved in DC maturation**  
 MAPK and NF-κB pathways are involved in DC maturation and can be triggered by engagement of TLRs or other stimuli (not shown). This results in a cascade of enzymatic reactions involving various adaptor and intermediate proteins and finishes with downstream activation of molecules that regulate transcription. MAPK p38 aids the binding of NF-κB to its consensus sequence. Both MAPK JNK and NF-κB control the expression of factors involved in antigen presentation, costimulatory and adhesion molecules, and pro-inflammatory cytokines.