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Dendritic Cells and Lentiviral Vectors: Mapping the Way to Successful Immunotherapy

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1. Introduction

Professional antigen presenting cells, in particular dendritic cells (DCs) are central players in the immune response (Steinman & Banchereau 2007). Their function is dual; on the one hand DCs evoke strong immune responses against antigens that are considered hazardous, on the other hand DCs induce tolerance against self-antigens. To that end, DCs need to present antigen-derived peptides in the context of MHC class I or class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively. It is the context in which these peptides are presented that determines the outcome of the immune response, immune activation *versus* tolerance. Consequently, DCs have become targets for immunotherapy against not only cancer and infectious disease, but also autoimmune diseases and transplantation rejection (Palucka *et al.*).

Key to successful DC-based immunotherapy is the delivery of the antigen of interest, be it cancer, viral or auto-antigens, to DCs, as well as the delivery of molecules that dictate the immune stimulatory capacity of the DCs. Therefore, it is not surprising that much effort has been put in the development of vectors for genetic modification of DCs (Breckpot *et al.* 2004c). Of these lentiviral vectors (LVs), often derived from human immunodeficiency virus type 1 (HIV-1) are amongst the most efficient gene delivery vehicles, for both *in vitro* and *in vivo* modification of DCs (Escors & Breckpot ; Breckpot *et al.* 2007a). In addition, these LVs were demonstrated to activate the innate immune system through interaction with amongst others Toll-like receptors (TLRs), a characteristic that makes LVs even better suited for immunotherapeutic approaches against cancer and infectious diseases (Breckpot *et al.* ; Brown *et al.* 2006a). As immune activation of DCs is critical for the induction of antigen-specific immunity, several strategies have been developed to further strengthen the immune response by introduction of immune modulating molecules or by modulation of well-known activation pathways such as the nuclear factor-kappaB (NF-κB), mitogen activated protein kinase (MAPK) p38 and MAPK c-Jun N-terminal kinases (JNK) pathways (Breckpot & Escors 2009a). Although LVs inherently activate DCs, they have also been evaluated for their ability to switch of the stimulatory capacity of DCs, thus to generate tolerogenic DCs. The strategies exploited therefore are similar to the strategies employed to activate DCs and include introduction of single inhibitory molecules and modulation of pathways that

regulate the tolerogenic potential of DCs, such as the MAPK extracellular signal-regulated kinase (ERK) pathway (Arce *et al.* ; Gould & Favorov 2003).
In the remainder of this chapter, we will give a comprehensive overview on how DCs have evolved to the cell type of choice for manipulation of the immune system, why LVs are successful for the genetic modification of DCs and which developments have led to the use of LVs to generate stimulatory or tolerogenic DCs. We will further touch upon the concerns that are raised in terms of translating the use of LVs to the clinic, *i.e.* the biosafety of LVs, summarizing strategies to avoid off-target transduction, and linked herewith insertional mutagenesis. Finally, we will conclude this chapter with our view on the future perspectives for the use of LVs to manipulate DCs, hence the immune system.

2. Dendritic cells

Dendritic cells (DCs) as we know them today were described during the mid 1970s by Ralph Steinman and co-workers as a rare subset of accessory cells, which are characterized by stellate cytoplasmatic protrusions. It was this tree-like morphology that led to their name (Dendron is Greek for tree) (Steinman & Cohn 1973). DCs are a heterogeneous population for which individual DC subtypes have been described. These DC subtypes differ in tissue distribution, surface marker expression and their capacity to stimulate T cells (Palucka *et al.*). Moreover, DCs have a remarkable functional plasticity. On the one hand DCs can induce immune responses against invading pathogens (non-self). On the other hand DCs can induce T cell anergy/depletion, and regulatory T cells (Treg) to avoid unwanted immune reactions against auto-antigens (self) (Fig. 1). This Janus-like functional behaviour is correlated with complex decision-making processes, triggered by the presence or absence of so called danger signals, hence resulting in the expression of stimulatory and/or inhibitory molecules, respectively (Coquerelle & Moser). Although, we are still deciphering the DC system in its complexity, DCs have entered the clinic as a cellular therapeutic.

2.1 Dendritic cell subsets

Originally it was thought that DCs were of myeloid origin. Studies demonstrating *in vitro* generation of DCs from monocytes (Sallusto & Lanzavecchia 1994), and *in vivo* studies demonstrating the differentiation of phagocytic monocytes to DCs (Randolph *et al.* 1999) supported this idea. Later on, the existence of lymphoid DCs was evidenced (Wu *et al.* 1996; Wu *et al.* 1998). These $CD11c^+$ MHC class II^+ myeloid and lymphoid DC subtypes were afterwards termed $CD8^-$ DCs or $CD8^+$ DCs, respectively. Together they are called conventional DCs (cDCs). Importantly, it was demonstrated in several *in vivo* studies that both $CD8^-$ and $CD8^+$ DC subsets could be generated from either lymphoid (Martin *et al.* 2000; Traver *et al.* 2000), or myeloid progenitors (Traver *et al.* 2000), mounting the question whether these subsets are really distinct or represent different developmental states. cDCs can be found within lymph nodes, spleen, and thymus, but not in bone marrow (Steinman & Cohn 1973). They are believed to cross-present antigens to T cells (den Haan & Bevan 2002), as such stimulate a T helper type 2 (T_H2, humoral) and type 1 (T_H1, cellular) immune response (Maldonado-Lopez *et al.* 1999). In humans, DCs expressing BDCA-1 (CD1c) and BDCA-3 (CD141) are considered the counterparts of mouse cDCs. However, these human DC subsets are often termed myeloid instead of conventional. Human myeloid DCs are characterized by their ability to produce high amounts of interleukin (IL)-12 in response to several stimuli (van Duin *et al.* 2006), thus to induce cellular immunity.

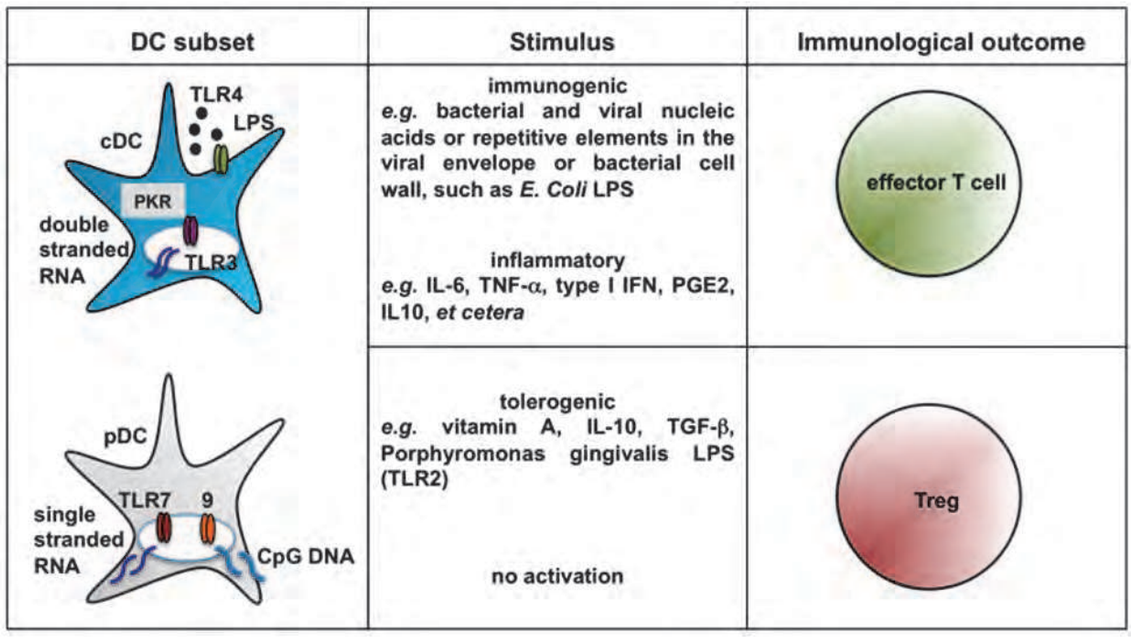

Fig. 1. **Schematic representation of DC subsets and their functional plasticity.** Generally DC subsets are divided into cDCs (blue) and pDCs (grey). These DC subsets differ in tissue distribution, expression of PRRs, hence their ability to sense pathogens and subsequently stimulate appropriate T cell responses. Dependent on the stimuli these DCs encounter they will become tolerogenic or immunogenic, hence induce tolerance (Treg, red) or immunity (effector T cells, green), respectively.

Another DC subset, the plasmacytoid DCs (pDCs) were described a decade ago (Siegal *et al.* 1999). Whereas mouse pDCs express CD11c, human pDCs express low to undetectable levels of CD11c. Instead, human pDCs are characterized by the expression of CD4, CD45RA, as well as the expression of high levels of CD123 (IL-3 receptor) and the c-type lectin receptor BDCA2. Recently, it was described that pDCs can be further divided into subclasses based on the expression of CD2 (Matsui *et al.* 2009). pDCs reside in the same tissues as cDCs, and can moreover be found in bone marrow (Nakano *et al.* 2001), where they are believed to be a precursor for cDCs (Soumelis & Liu 2006; Segura *et al.* 2009). Nonetheless, pDCs, isolated from mice and humans, are functionally distinct from cDCs. In their resting state, pDCs play an important role in the induction of tolerance (Martin *et al.* 2002). However, pDCs are best known for the ability to produce high amounts of type I interferon (IFN) in response to viral infection (O'Keeffe *et al.* 2002; Fitzgerald-Bocarsly *et al.* 2008). In fact, pDCs control the progress of a virus infection at various levels: (i) through non-specific blockade of viral replication by type I IFN, (ii) by promoting the maturation of pDCs as well as other DC subsets (Fonteneau *et al.* 2004), and (iii) through the specific stimulation of adaptive anti-viral $CD8^+$ T cell responses (Di Pucchio *et al.* 2008).

The last DC subset to be discussed, the epidermal Langerhans' cells (LCs), was in fact described in 1868 by Paul Langerhans (Merad & Manz 2009), almost a century before the description of cDCs (Steinman & Cohn 1973). LCs are characterized by the expression of Langerin and Birbeck granules. Furthermore, they are characterized by their long life span (weeks) when compared to other DC types (3-10 days) (Kamath *et al.* 2002). Upon activation LCs migrate through the dermis into regional lymph nodes to present antigens to T cells

(Romani *et al.* 2003). Because of this migration they are categorized as migratory DCs, a category, which also comprises other non-lymphoid tissue residing DCs, amongst which dermal DCs. LCs and dermal DCs are often grouped as skin DCs. It is generally accepted that these have a potent T cell stimulatory capacity (Romani *et al.* 2003; Larregina & Falo 2005; He *et al.* 2006). Nevertheless, as for pDCs and cDCs, a possible tolerogenic capacity has been reported for skin DCs (Grabbe *et al.* 1995; Kaplan *et al.* 2005).

2.2 Dendritic cells and the regulation of immune responses

In addition to subsets with functional specialization, DCs are endowed with a remarkable functional plasticity. The hypothesis is that distinct DC activation stages play a role in the induction of tolerance *versus* immunity. This is correlated with the two-signal model of T cell stimulation, in which it is proposed that a productive T cell response requires recognition of MHC/peptide complexes by the T cell receptor (TCR) (signal 1) along with signalling through co-stimulatory molecules (signal 2). Indeed, steady-state cDCs and pDCs, have been described to induce T cell tolerance (Jonuleit *et al.* 2001; Mahnke *et al.* 2002; Martin *et al.* 2002), whereas both activated DC types have been shown to induce immunity (Salio *et al.* 2003; Cerundolo *et al.* 2004; Salio *et al.* 2004).

Immature DCs efficiently take up pathogens, apoptotic cells and particulate antigens from the environment by phagocytosis, macropinocytosis, or endocytosis; process these but are considered inefficient in presenting these to T cells (Wilson *et al.* 2004). Hence, immature DCs are believed to induce tolerance (Steinbrink *et al.* 1997; Lutz & Schuler 2002). Indeed, in steady-state conditions DCs remain immature and tissue-resident, express small amounts of MHC and co-stimulatory molecules hence induce T cell anergy instead of T cell activation upon DC-T cell interaction (Hawiger *et al.* 2001). Furthermore, injection of immature DCs in humans induces tolerance (Dhodapkar *et al.* 2001; Jonuleit *et al.* 2001; Dhodapkar & Steinman 2002). In contrast, activated DCs are considered to be immunogenic. Maturation of DCs can be induced by a variety of signals, such as microorganisms (Rescigno *et al.* 2000; Beyer *et al.* 2001), cytokines (Jonuleit *et al.* 1997), interaction with $CD4^+$ T_H cells (Caux *et al.* 1994; Mackey *et al.* 1998a; Mackey *et al.* 1998b) and chemicals like haptens (Aiba *et al.* 1997; Aiba & Tagami 1998; Aiba & Tagami 1999). DC maturation is associated with several coordinated events, including: (i) loss of endocytic and phagocytic receptors; (ii) changes in morphology; (iii) up-regulation of MHC and co-stimulatory molecules, such as CD40, CD80 and CD86, adhesion molecules and chemokine receptors, such as CCR7 (Tuyaerts *et al.* 2007a). The latter is one of the first changes and enables DCs to migrate from the periphery to the T cell areas of draining lymphoid organs (Forster *et al.* 2008). Here DCs present antigenic peptides in the context of MHC molecules to T cells. The phenotypic changes, high expression of antigen presenting, co-stimulatory and adhesion molecules, make mature DCs potent inducers of T cell immunity. However, the view that immature DCs induce tolerance and mature DCs induce immunity is simplified. It has been demonstrated that mature DCs can contribute to T cell tolerance through the induction of Treg (Verhasselt *et al.* 2004). It was suggested that the maturation trigger dictates the T cell polarizing or tolerating immune functions of the DCs. Some stimuli have been demonstrated to promote induction of T_H1 responses, hence cellular immunity, whereas others hamper full DC maturation and cytokine production, hence promote tolerance.

2.3 Stimulatory dendritic cells

The immune system is constantly faced with choices. When confronted with a microbe, it must first decide whether to respond or not. If it chooses to respond, then it must decide

what kind of response to launch. A hallmark of the mammalian immune system is its ability to launch qualitatively distinct types of responses against different pathogens (Pulendran *et al.* 2008). Immune responses against T cell-dependent antigens can be divided in (i) $CD4^+$ T_H1 responses, which are characterized by high secretion of IFN-γ, and induced against intracellular microbes, (ii) $CD4^+$ T_H2 responses, which are characterized by the secretion of cytokines, such as IL-5 and IL-13, and induced against extracellular pathogens, (iii) $CD4^+$ T_H17 responses, characterized by the secretion of IL-17, and induced against fungi (Bettelli *et al.* 2007; Reiner 2007), and finally (iv) Treg responses, with suppressive activity, and which are essential to maintain tolerance to host antigens (Wing & Sakaguchi). Treg can also be induced by some microbial stimuli (Belkaid 2007), and are abundantly present in the blood, lymphoid organs and tumours of cancer patients (Vence *et al.* 2007; Ahmadzadeh *et al.* 2008), as such Treg enable evasion from the immune system.

For the treatment of cancer and infectious diseases, a $CD4^+$ T_H1 response is required to induce a strong $CD8^+$ cytotoxic T cell (CTL) response (Breckpot & Escors 2009a). These CTLs will then kill the target cells. To activate T cells, at least two signals are required (i) antigen recognition and (ii) co-stimulation. In the presence of tolerogenic mechanisms, as it is the case in cancer, an additional third signal is required. This is obtained by triggering of innate sensing pathways (Breckpot & Escors 2009a). As co-stimulatory molecules are not expressed by immature DCs, it goes without saying that DC activation (maturation) is a key event that determines the T cell stimulatory potential of DCs.

Differentiation of immature to mature DCs requires pathogen recognition. Groups of pathogens express similar structures such as bacterial and viral nucleic acids or repetitive elements in the bacterial cell wall or within the viral envelope, enabling the recognition of a wide variety of pathogens. These structures are called pathogen-associated molecular patterns (PAMPs) and are recognized on DCs by pathogen recognition receptors (PRRs). The best-studied PRRs are the TLRs, although other PRRs, such as Nod-like receptors, RIG-I-like receptors, as well as some members of the C-type lectin family, are described. Distinct pathogens express different PAMPs and this combination works as a fingerprint that triggers a specific set of PRRs (Akira *et al.* 2006; Barton & Kagan 2009; Geijtenbeek & Gringhuis 2009). As such complex signalling networks are activated. These cooperate, integrate and finally converge into a few pathways, of which the NF-κB and the MAPK pathways are examples (Rescigno *et al.* 1998; Sato *et al.* 1999; Re & Strominger 2001; Caparros *et al.* 2006; Kawai & Akira 2008). These are described in detail elsewhere (Breckpot & Escors 2009c).

The concept of co-stimulation was first introduced by Kevin Lafferty and co-workers (Lafferty *et al.* 1979). In the last decades, a large number of co-stimulatory molecules have been identified, which can be divided in members of the (i) B7 and (ii) tumour necrosis factor (TNF) type family. B7.1 (CD80) and B7.2 (CD86) are textbook examples of the B7 family. These transmit strong co-stimulatory signals to T cells through interactions with CD28 on T cells (Greenwald *et al.* 2005). Recently, this group was expanded with a number of new members including ICOS ligand, PD-L1 (B7-H1), PD-L2 (B7-DC), B7-H3 and B7-H4. All these molecules are expressed on DCs. The corresponding CD28 members that are inducible expressed on T cells are ICOS, PD-1 and BTLA. It is important to mention that not all B7 family members are co-stimulatory. In fact, many of these new members have been linked to induction of tolerance hence they are discussed in the next section. The TNF type family of co-stimulatory molecules, includes CD70, OX40L, GITRL and 4-1BBL, which are expressed on DCs and their corresponding receptors CD27, OX40 (CD134), GITR and 4-1BB (CD137), expressed on T cells (Watts 2005). Some co-stimulatory molecules exemplified by

CD83, which is expressed on mature DCs, but also on T cells, can't be classified in either group. So far, no receptor has been identified for this molecule (Hirano *et al.* 2006; Aerts-Toegaert *et al.* 2007; Prechtel *et al.* 2007).

Initial activation of naive T cells generally occurs through interactions with CD28, after which they differentiate into effector T cells, and up-regulate other co-stimulatory molecules. Depending on the stimulus, expression of co-stimulatory ligands on DCs will vary. Their relative expression will ultimately determine the quality of the T cell stimulation hence the T cell function. It has to be mentioned that the importance of co-stimulatory molecules is not limited to the stimulation of effector T cells, but can moreover involve modulation of Treg, as described for GITRL and OX40L. It has been shown that triggering of GITR results in alleviation of Treg suppression of effector T cells. Although Treg constitutively express GITR, the effect of GITR-GITRL interactions is not mediated through functional impairment of Treg, but rather protection of effector T cells against Treg (Stephens *et al.* 2004). However, whether these observations in mice can be translated to a human setting remains unclear (Tuyaerts *et al.* 2007b). Direct inhibitory effects on Treg suppression have been shown for OX40L (Valzasina *et al.* 2005), which upon binding with OX40 on Treg mediates down-regulation of Foxp3 and the Tregs' suppressive capacity (Vu *et al.* 2007). For other co-stimulatory molecules (CD70 and 4-1BBL), of which its receptors are also expressed on Treg, no unequivocal effect on Treg function has been described.

2.4 Tolerogenic dendritic cells

In physiological conditions, the organism is in direct contact with millions of innocuous antigens of different origins. Many of them are bacterial antigens, such as those present within the gut. Others vary from pollen, yeast, dust mites and chemicals of all sorts. Until recently, it was thought that the immune system was constantly and restlessly fighting potentially dangerous organisms and antigens. This view has certainly become obsolete, and it is not inaccurate to consider the immune system in a kind of steady-state in which tolerance is the default outcome and has to be maintained at all costs, except when a real threat arises. Therefore, several tolerogenic mechanisms are constantly in place.

One of the first mechanisms to be described is central tolerance, in which auto-reactive T cells are eliminated in the thymus by clonal deletion (Griesemer *et al.* 2010). Although this mechanism is essential to eliminate most auto-reactive T cells, it can't explain the aetiology of autoimmune disorders in which self-antigens are clearly recognised. Even though clonal deletion is efficient, it does not eliminate all auto-reactive T cells. Interestingly, many auto-reactive T cells that survive clonal deletion further differentiate into natural $Foxp3^+$ $CD4^+$ Treg (Sakaguchi *et al.* 2008; Griesemer *et al.* 2010). These are strong and intrinsic immunosuppressive, and are part of the central tolerance. Research on Treg has recently exploded, although ample experimental evidence of their existence was provided during the 1970s (Rich & Pierce 1973; Ha *et al.* 1974; Taussig 1974). However, early studies were abandoned partly by the inexistence of specific cell markers associated with suppressive T cells. Nevertheless, Sakaguchi and colleagues demonstrated that high expression of CD25 and Foxp3 was characteristic for natural Treg (Hori *et al.* 2003; Sakaguchi 2003), which re-awakened research into this fundamental T cell type. Importantly, many of the early studies drew similar conclusions to more recent studies on Treg (Basten *et al.* 1974; Kirchner *et al.* 1974; Polak & Turk 1974). Even so, clonal deletion and natural Treg activity can't explain tolerance towards many other auto- and foreign antigens, which are not present in the thymus. Theoretically strong immune responses should constantly arise towards a wide

variety of antigens. However, this is not the case, and the organism still remains tolerant towards most antigens. Differentiation of inducible Treg specific for peripheral antigens can partially fill this experimental and conceptual gap. Inducible Treg derive from naïve $CD4^+$ T cells and can be broadly classified into Tr1 ($CD4^+$ $CD25^+$ IL-10^+ or TGF-β^+) and T_H3 ($CD4^+$ $CD25^+$ $Foxp3^+$) cells (Mahnke *et al.* 2003; O'Garra *et al.* 2004; Peng *et al.* 2004; Arce *et al.* 2011). For differentiation of inducible Treg to occur, antigens have to be captured, processed and presented in a tolerogenic context. DCs, which induce Treg differentiation and inhibition of effector T cell expansion, are termed tolerogenic DCs.

There is no compelling evidence demonstrating that tolerogenic DCs are a truly specialised cell type that is exclusively devoted to suppress immune responses. In fact, tolerogenic DCs encompass a wide range of DCs which acquire immune suppressive activities in particular circumstances. Firstly, it is well known that steady state DCs can capture antigens in peripheral tissues and migrate to secondary lymphoid organs. Antigen presentation by immature DCs leads to T cell inactivation (anergy), apoptosis and Treg differentiation (Dhodapkar *et al.* 2001; Hawiger *et al.* 2001; Bonifaz *et al.* 2002; Kretschmer *et al.* 2005). Secondly, DCs located in certain anatomical parts such as the mucosa and gut, are intrinsically tolerogenic. Interestingly, retinoic-acid (vitamin A) metabolising enzymes are critical in their suppressive functions. Mucosal DCs in contact with many microbial-derived antigens are potently immunosuppressive, particularly after TLR2 signal transduction (Dillon *et al.* 2006; Ilarregui *et al.* 2009; Manicassamy *et al.* 2009). Treatment of DCs with lectin ligands such as galectin 1 or potent immunosuppressive cytokines also renders them tolerogenic (Corinti *et al.* 2001; Ghiringhelli *et al.* 2005; Dillon *et al.* 2006; Rutella *et al.* 2006; Ilarregui *et al.* 2009; Arce *et al.* 2011). Importantly, certain types of specialised myeloid-derived cells with very potent intrinsic immunosuppressive capacities have been found in recent years. These cells are known as myeloid-derived suppressor cells and inhibit T cell proliferation through a variety of mechanisms (Li *et al.* 2009; Srivastava *et al.* 2010). In addition, certain types of monocytes are immunosuppressive cells, and are involved in establishing tolerance after organ transplantation (Garcia *et al.* 2010). According to the expression of surface molecules, tolerogenic DCs are generally considered to be immature. This is characterised by low levels of MHC and co-stimulatory molecules (Rutella *et al.* 2006; Escors *et al.* 2008; Breckpot & Escors 2009b; Arce *et al.* 2010). It is believed that because of their immature phenotype antigen presentation is inefficient, and expansion of effector T cells is hampered. However, phenotypical mature DCs can also be potently tolerogenic. These mature tolerogenic DCs exert their suppressive activities through secretion of high levels of immunosuppressive cytokines (Rutella *et al.* 2006).

The mechanisms by which tolerogenic DCs exert their activity are certainly varied in nature, and it is likely that several of these take place simultaneously. As mentioned, tolerogenic immature DCs are thought to lead to inefficient antigen presentation to naive T cells. Therefore, expansion of effector T cells is, if not prevented, at least severely reduced. However, there is evidence that these DCs do present antigens, although the interaction between immature DCs and T cells is transient and leads to T cell anergy, apoptosis or Treg differentiation (Rothoeft *et al.* 2006). An important characteristic that seems to be common in all tolerogenic DCs is the secretion of potent immunosuppressive cytokines during antigen presentation (Ghiringhelli *et al.* 2005; Dillon *et al.* 2006; Escors *et al.* 2008; Ilarregui *et al.* 2009; Arce *et al.* 2010; Saraiva & O'Garra 2010). In fact, at least in the presence of TGF-β, strong TCR stimulation during antigen presentation greatly enhances $Foxp3^+$ Treg differentiation. Tolerogenic DCs can also secrete high amounts of IL-10, a potent immunosuppressive

cytokine, resulting in differentiation of mainly Tr1 cells (Kuhn *et al.* 1993; Saraiva & O'Garra 2010). In addition IL-10 autocrine and paracrine activities keep DCs in an immature stage (Takayama *et al.* 1998; Corinti *et al.* 2001). DCs can also up-regulate surface expression of molecules with T cell inhibitory activities. This is the case for some members of the B7 family. One of the most extensively studied immunosuppressive members is PD-L1, the ligand of the T cell inhibitory receptor PD-1. Binding of PD-L1 to PD-1 on T cells, especially in the case of chronic antigen exposure, renders T cells inactive (exhausted) (Sakuishi *et al.* 2010). This is a critical mechanism by which many tumour cells exert their immunosuppressive activities towards effector $CD8^+$ T cells. PD-L1 is expressed ubiquitously, but it is likely that its expression on DCs and other professional antigen presenting cells has a more specific role. Importantly, binding of PD-L1 expressed on DCs to CD80 on T cells has been shown to be required for TGF-β dependent antigen-specific Treg differentiation (Wang *et al.* 2008). A second PD-1 ligand was described that is specifically expressed on DCs and macrophages, termed PD-L2. However, it is unclear whether PD-L2 is truly immunosuppressive (Radhakrishnan *et al.* 2009). Recently, other B7 family members have also been shown to exert immunosuppressive activities (Sica *et al.* 2003). Finally, another interesting mechanism is up-regulation of amino acid-metabolising enzymes. Intriguingly, many of these are triggered by TGF-β (Belladonna *et al.* 2009). It has been known for some time that increased arginase activity in DCs suppresses immune responses (Munder 2009; Norian *et al.* 2009). Indoleamine 2,3-dioxygenase up-regulation, a tryptophan-metabolising enzyme, suppresses immune responses (Fallarino *et al.* 2002; Mellor & Munn 2004). Interestingly, Tregs can induce DCs to up-regulate several of these metabolic enzymes, resulting in infectious tolerance that amplifies the suppressive capacities of regulatory DCs and T cells (Cobbold *et al.* 2009).

3. Lentiviral vectors

Viruses are excellent candidates for the development of efficient gene delivery systems. As intracellular obligate parasites, they are specialized in the delivery of their genome to cells. Therefore it is no surprise that viruses have always been of interest for gene therapeutic applications. Nowadays a large number of viruses have been evaluated, *e.g.* adenovirus, adeno-associated virus, herpes virus, poxvirus, retrovirus, lentivirus, *et cetera* (Escors & Breckpot 2010). The first human gene therapy trial was performed in the 1970s and applied an arginase encoding Shope papilloma virus to treat hyperargininemia (Friedmann & Roblin 1972). By 1985, gene transfer with viral vectors to mammalian cells was performed routinely and the use of the γ-retroviral Murine leukaemia virus for gene delivery was introduced (Mann *et al.* 1983). This seemed promising as these viruses integrate their cargo into the host genome. It was Brenner *et al* (Brenner *et al.* 1993) who demonstrated the proof-of-principle of γ-retroviral gene transfer in hematopoietic stem cells (HSCs). The first clinical trial using γ-retroviral vectors (RVs) was carried out by Anderson and colleagues to correct severe combined immunodeficiency (SCID) in 1991 (Anderson *et al.* 1990). The majority of clinical gene therapy trials today use γ-RVs, despite their relative low stability, the low titers and their inability to transduce quiescent cells. More importantly, worrisome incidents of RV induced insertional mutagenesis were reported (Pincha *et al.* 2010). Lentiviruses, which as γ-retroviruses are members of the *Retroviridae*, were suggested to be an attractive alternative, since they are capable of transducing both dividing and non-dividing cells (Bukrinsky *et al.* 1993; Lewis & Emerman 1994). Moreover, their integration into the host genome is, in contrast to γ-retroviruses, associated with lower genotoxicity (Montini *et al.* 2006).

At the end of the 1990s, the use of recombinant LVs was boosted, especially for transduction of non-dividing cells (Akkina *et al.* 1996; Naldini *et al.* 1996a; Naldini *et al.* 1996b; Reiser *et al.* 1996). Another 10 years later the first clinical trial using LV modified cells for the treatment of HIV infection was completed (Lu *et al.* 2004).

3.1 Development of recombinant lentiviral vectors

Lentiviruses are characterized by a diploid 7-12 kb single stranded RNA genome with positive polarity that is reverse transcribed to double stranded DNA upon host cell entry (Coffin 1997). Diploidy permits genetic recombination, which accounts partially for their success as procreators of the acquired immunodeficiency syndrome, a disease that develops by slowly affecting the immune systems' function (lenti meaning slow). Lentiviruses include primate and non-primate retroviruses, *e.g*. HIV and simian immunodeficiency virus, and caprine arthritis-encephalitis virus, equine infectious anaemia virus, Maedi-visna virus, feline immunodeficiency virus and bovine immunodeficiency virus, respectively (Breckpot *et al.* 2008; Escors & Breckpot 2010). The spherical virion measures about 80-120 nm in diameter, has a mass of 2.5 x 10^5 kDA and a density of 1.16 g/ml in sucrose density gradient. Its envelope consists of a plasma membrane derived phospholipid bilayer loaded with surface (SU) and transmembranary (TM) glycoproteins and is supported on the inside by the non-glycosylated structural matrix (MA) proteins (Fig. 2). Within the envelope the nucleocapsid, comprised of capsid (CA) proteins, surrounds the viral genome, which is packaged together with nucleocapsid (NC) proteins and a few copies of the enzymes reverse transcriptase (RT), integrase (IN) and protease (PR).

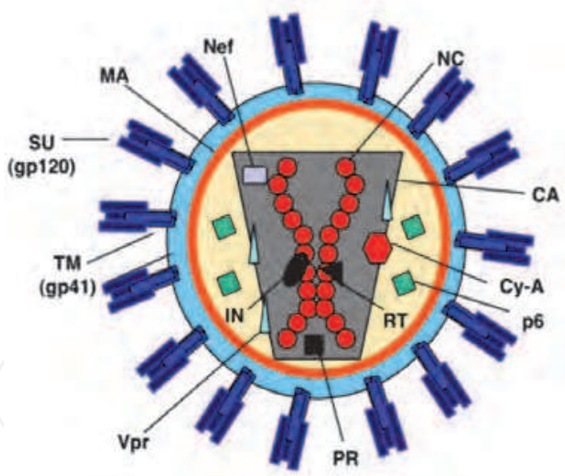

Fig. 2**. Schematic representation of a retroviral particle.** The viral envelope consists of a lipid bilayer loaded with viral proteins. These are composed of TM and SU components, linked *via* a disulphide bridge and encoded by de *env genes*. Internal non-glycosylated proteins are coded by the *gag* region of the viral genome and comprise MA, CA and NC proteins. The products of the *pol* – coding region are the RT and IN, while the PR is coded by the *pro* gene that resides between the *gag* and the *pol* gene.

The replication cycle of lentiviruses starts with the attachment of the viral envelope proteins to specific receptors on the host cell surface (Flint S.J. 2009). This interaction defines the tropism and results in conformational changes of SU and TM, which allows the hydrophobic fusion

peptide of TM to insert in the cellular membrane, as such allowing the release of the nucleocapsid complex in the cytoplasm (Schaffer *et al.* 2008). The reverse transcription process sets off by primer binding of a viral tRNA and produces successively the negative and positive linear DNA strands till the DNA molecule called provirus is formed. Important are its *cis*-acting ends called long terminal repeats (LTRs) that are shown juxtaposed in preparation for integration (Fig. 3). As a unique characteristic of lentiviruses, viral DNA and IN gain access to the nucleus by formation of a pre-integration complex. Therefore, the lentiviral genome contains not only a 3' polypurine tract (PPT), as other retroviruses, but also a central PPT (Fig. 3) (Charneau & Clavel 1991). The latter sequence together with a central termination sequence (CTS) controls the formation of a stable 99-nucleotide long plus strand overlap in the linear DNA molecule in *cis,* which enables active pro-viral nuclear import (Follenzi *et al.* 2000; Zennou *et al.* 2000). The subsequent integrative recombination is catalyzed by the IN, which uses the outer *att* sequences on the LTRs to grab the pro-viral DNA and results in random insertion of the viral DNA in the host genome. Transcription of the integrated provirus is mediated by cellular RNA polymerase II and results in different subsets of RNA molecules namely: mRNA molecules and new viral single stranded RNA genomes. The most important viral genes encoding mRNA molecules are: (i) the *env* (*envelope*) gene which encodes SU and TM; (ii) the *gag* (*group specific antigen*) gene which encodes the internal structural proteins MA, CA and NC; (iii) the *pol* (*polymerase*) gene which codes for the enzymes IN and RT including a DNA polymerase as well as its associated RNase H activity and finally (iv) the *pro* (*protease*) gene which encodes PR. Based on genomic organization retroviruses can be divided in simple and complex viruses. The simple viral genomes only encode the three genes, *gag, pol,* and *env*, common to all retroviruses, such as α-, β-, and γ-retroviruses. In contrast, complex viruses have a genome that encodes several accessory genes that affect viral gene expression and/or pathogenesis. Lentiviruses encode not only two extra regulatory genes *tat* and *rev* but also several accessory genes such as *vpr, vpu, vif* and *nef.* After transcription of the unspliced full-length viral RNA and translation of the mRNA encoding viral proteins, everything is transported to the cytoplasm where virion maturation occurs. The *cis*-acting packaging signal, ψ (psi), is required to ensure viral genome packaging and subsequent budding of the particle from the cell to give rise to infectious virions.

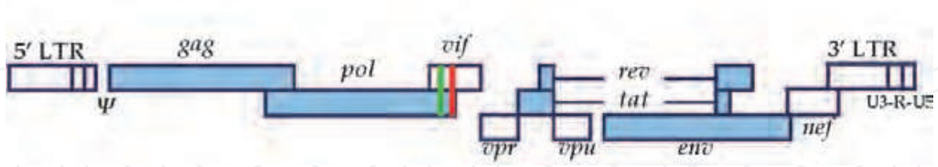

Fig. 3. **Schematic representation of the pro-viral HIV-1 genome.** All genes of the provirus (*gag, pol, pro, vif, vpr, vpu, rev, tat, env* and *nef*) are flanked on either side by identical 'long terminal repeats' (LTRs) that consist of a U3, R and U5 fragment. The pro-viral transcriptional control elements like the actual promoter and enhancer sequences can be found in the U3 regions. Ψ represents the encapsidation signal. Unlike most retroviruses, HIV and other LVs have two copies of the PPT, one at the border of the 3'LTR (3' polypurine tract) and the other located within the *pol-coding* region (central polypurine tract [red line] together with a central termination sequence [green line]).

Given the fact that lentiviruses are pathogenic, it is crucial to develop a recombinant LV that is replication deficient, safe and efficient in transduction of target cells. Therefore, LVs have been

vigorously modified, which has contributed to their widespread use as a gene delivery shuttle. A safety concern is the possible generation of replication-competent LVs (RCLs) as a consequence of genetic recombination (Wu *et al.* 2000). Therefore, LVs are produced by transient transfection of producer cells (HEK 293T) with at least three plasmids. This allows separation of *cis*- and *trans*-acting sequences. Generally the following plasmids are used: (i) a packaging, (ii) a transfer, and (iii) an envelope plasmid. All plasmids have been scrutinized to enhance their performance in terms of safety and efficacy (Breckpot *et al.* 2007a).

The packaging plasmid provides all viral structural and enzymatic sequences, encoded by *gag* and *pol,* to make an infective virion in *trans.* Based on the packaging plasmid recombinant LVs can be divided into different generations. The first generation is represented by a plasmid encoding the entire *gag* and *pol* sequence in *trans* to enable packaging of the transfer construct together with the viral regulatory genes *tat* and *rev* and the virulence genes *vif, vpr, vpu* and *nef*. The second generation plasmid is multiply attenuated by removal of the four virulence genes without any negative effect on transduction efficacy as these genes seemed dispensable for the efficient generation of HIV-derived LVs (Zufferey *et al.* 1997). In the third generation, the *rev* gene is expressed from a separate plasmid (Dull *et al.* 1998). Furthermore also the *tat* sequence could be removed by insertion of a strong constitutional promoter in the 5′ LTR of the transfer vector. More recently the importance of the development of non-integrating LVs (NILVs) has been brought to the attention because of the oncogene transactivation incidents in some clinical trials with γ-RVs. Although the LV integration profile seems more favourable than that of γ-RVs (Montini *et al.* 2009), several groups tested the transduction efficacy of NILVs by mutating the catalytic triad within the IN gene of the packaging plasmid (Wanisch & Yanez-Munoz 2009). Improved safety without major reduction in efficacy was demonstrated (Negri *et al.* 2007; Karwacz *et al.* 2009; Wanisch & Yanez-Munoz 2009). Downsides are the lower titers, higher doses needed and the fact that there still is a chance for integration of about 0.1 to 2.3% (Apolonia *et al.* 2007). An alternative to IN deficiency is site-specific integration into a safe harbour sequence of the target cell. Several strategies have been described, *e.g.* Cre-loxP carrying LVs, use of the zinc finger nuclease or meganuclease technology, *et cetera* (Matrai *et al.* 2010; Michel *et al.* 2010; Silva *et al.* 2011). Furthermore, the discovery that the LEDGF/p75 protein controls the site of integration of HIV-derived LVs presents new possibilities to control mutagenesis (Ciuffi 2008; Silvers *et al.* 2010).

The transfer plasmid is the only plasmid derived from the viral genome where all viral coding regions are replaced by the expression cassette. An important improvement for safety was the development of so called self-inactivating (SIN) vectors (Zufferey *et al.* 1998). These rely on the introduction of a deletion in the U3 region of the 3′ LTR. This deletion will be transferred to the 5′ LTR of the pro-viral DNA during reverse transcription, which abolishes production of full-length vector RNA in transduced cells. This has several advantages: (i) it minimizes the risk for emerging RCLs, (ii) it reduces the chance that cellular coding sequences located adjacent to the integrated pro-viral sequence will be aberrantly expressed due to the promoter activity in the 3′ LTR and (iii) it prevents transcriptional interference between the LTRs and an internal promoter. As the expression of genes delivered by LVs is often inefficient, several strategies were developed to ameliorate this. Firstly, the promoter within the expression cassette can be varied. Instead of using strong constitutive promoters, such as the promoter of cytomegalovirus or spleen focus forming virus, one can choose a cell-specific promoter as these are less sensitive to promoter inactivation and less likely to activate the host-cell defence machinery (Liu *et al.*

2004). Secondly, the incorporation of the cPPT and CTS into the transfer plasmid not only improved LV yields, but also provided enhanced transgene expression by mediating active nuclear import of the provirus (Follenzi *et al.* 2000; Sirven *et al.* 2000). Addition of posttranscriptional regulatory elements such as the Woodchuck hepatitis virus regulatory element (WPRE) has also been explored (Zufferey *et al.* 1999). Although some groups demonstrated improved gene expression by modification of polyadenylation, RNA export and/or translation, others reported only a negligible benefit (Breckpot *et al.* 2003). Another issue is epigenetic silencing and heterochromatin formation nearby the inserted provirus, which hampers its transcription. This silencing process can be avoided by the insertion of insulators or with vectors containing an enhancer-less ubiquitously acting chromatin-opening element (Zhang *et al.* 2007; Nielsen *et al.* 2009). To improve safety, the incorporation of a suicide-gene has been proposed to eliminate cells that are transformed as a consequence of LV integration (Tseng *et al.* 2009). Finally the discovery of RNA interference opens novel possibilities for LVs in terms of stable gene silencing (Gu *et al.* ; Arrighi *et al.* 2004), as well as for LV de-targeting strategies (Brown *et al.* 2006b).

Last but not least also the envelope plasmid is variable. Since wild-type HIV-1 glycoproteins have a restricted tropism and do not allow production of high titer LV preparations, heterologous glycoproteins are used for LV production. This process is termed pseudotyping and most commonly the envelope of the vesicular stomatitis virus (VSV.G) is used. This rhabdovirus envelope glycoprotein interacts with an ubiquitous receptor and subsequently confers a broad host-cell range and high vector particle stability (Burns *et al.* 1993; Marsac *et al.* 2002; Schaffer *et al.* 2008).

3.2 Lentiviral vectors for the *in vitro* modification of dendritic cells

Efficient transduction of human DCs with transgenic vectors has been challenging for several reasons. Human DCs are usually generated from peripheral blood-derived quiescent $CD14^+$ progenitors or from mitotically hypoactive primitive $CD34^+$-derived progenitor cells. Therefore, the capacity of LVs to transduce quiescent and non-dividing cells turned out to be an important asset for DC transduction. The first successful transduction of human monocyte-derived DCs with LVs was described by Unutmaz *et al* (Unutmaz *et al.* 1999) in 1999. Since then, several research groups have reported successful transduction of human monocyte-derived (Gruber *et al.* 2000; Schroers *et al.* 2000; Dyall *et al.* 2001; Firat *et al.* 2002; Breckpot *et al.* 2003; Lizee *et al.* 2004), human $CD34^+$-derived DCs (Salmon *et al.* 2000; Oki *et al.* 2001; Sumimoto *et al.* 2002) and mouse bone marrow-derived DCs (Metharom *et al.* 2001; Breckpot *et al.* 2003) with varying efficiencies. Transgene expression was found to be stable in monocyte-derived DCs (Gruber *et al.* 2000; Breckpot *et al.* 2003) and $CD34^+$-derived DCs (Oki *et al.* 2001). However, for mouse DCs, the kinetics are somewhat more complicated, due to a process which is called pseudotransduction (Dullaers *et al.* 2004), and which results in a wrong estimation of the transduction efficiency when analyzed early after transduction. Nevertheless, DCs can be modified at high efficiency (Breckpot *et al.* 2003). Importantly, there is quite some variability in transduction efficiency among different reports. This most likely reflects the diversity in DC sources, techniques and vectors used for transduction (Breckpot *et al.* 2004a).

3.3 Lentiviral vectors for the *in vivo* modification of dendritic cells

As broad-tropism LVs efficiently transduced mouse and human DCs *in vitro*, it was next questioned whether these LVs can be used to transduce DCs *in vivo*, as such circumvent the

labour-intensive, time- and money-consuming procedure of generating DCs *ex vivo*. Dullaers *et al* (Dullaers *et al.* 2006) used a PCR-based method to demonstrate the presence of the LV delivered transgene in the draining lymph node at days 2 and 10, but not day 25 post administration of LVs 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$^+$ cells (unpublished data Dullaers *et al*). Using the same injection route, Esslinger *et al* (Esslinger *et al.* 2003) showed transduction of CD11c$^+$ cells in the lymph node by immunohistochemical analysis, whereas He *et al* (He & Falo 2006) were able to demonstrate by flow cytometry that transduced DCs present in the lymph node after footpad injection of GFP encoding LVs originated from locally transduced migratory skin DC. More recently, a new imaging technique, *in vivo* bioluminescence imaging, was used to visualize cells transduced *in situ* with Firefly luciferase encoding LVs upon footpad injection (Breckpot *et al.*). Intravenous administration of LVs also leads to transduction of DCs in the spleen (VandenDriessche *et al.* 2002; Palmowski *et al.* 2004; Arce *et al.* 2009). These studies indicate that LVs, independent of the route of administration transduce DCs *in situ* and have instigated the exploration of LVs as an off-the-shelf vaccine.

4. Exploitation of lentivirally transduced dendritic cells in anti-cancer immunotherapy

Active anti-tumour immunotherapy is based on the delivery of tumour antigens (Boon & van der Bruggen 1996) in a way that induces therapeutic immunity. As several tumour-induced tolerogenic mechanisms are in place it is not sufficient to stimulate effector T cells, it is moreover critical to circumvent suppressive immune cells, such as Treg. Such anti-tumour immunity can only be induced by professional antigen presenting cells, in particular DCs, and requires presentation of the tumour antigen-derived peptides to both CD4$^+$ and CD8$^+$ T cells in the context of strong co-stimulation. As mentioned previously LVs have been tested as vehicles, for *ex vivo* and *in vivo* antigen delivery to DCs. In the following section we will discus the induction of potent T cell mediated immune responses that can control tumour growth by *ex vivo*, as well as *in situ* LV transduced DCs. Finally, we will discuss some strategies that have been explored to enhance the performance of LV-based vaccines.

4.1 Evaluation of *ex vivo* lentiviral vector transduced dendritic cells as a cellular anti-tumour vaccine

Since the beginning of the millennium, several reports on the use of tumour antigen encoding LVs for the *ex vivo* modification of DCs have been published. As it is of paramount importance that tumour antigen-derived peptides are efficiently processed and presented on the DC surface in order to efficiently prime and activate tumour antigen-specific T cells, it was first evaluated whether LV transduced DCs activate established T cell lines. Note, strategies in which the tumour antigen encoding genetic sequence is fused to the sequence encoding class II targeting signals, such as the first 80 amino acids of the invariant chain (Ii80), were employed to obtain not only presentation of antigenic peptides in MHC class I, but furthermore in MHC class II in order to activate CD8$^+$ and CD4$^+$ T cells, respectively (Bonehill *et al.* 2005). As mentioned before, the activation of IFN-γ producing CD4$^+$ T_H1 cells supports priming and maintenance of CTLs, moreover in anti-tumour immunotherapy, it has been shown that these CD4$^+$ T_H1 cells mediate tumour rejection

(Bonehill *et al.* 2003). Both human and mouse LV transduced DCs were able to activate established $CD8^+$ and $CD4^+$ T cell lines specific for epitopes derived from various relevant tumour antigens, such as MAGE-3 (Breckpot *et al.* 2003), Melan-A (Firat *et al.* 2002; Sumimoto *et al.* 2002) and tyrosinase (Lizee *et al.* 2004) in the human system and for the surrogate antigen ovalbumin (OVA) (Breckpot *et al.* 2003; He *et al.* 2005), as well as for TRP-2 (Metharom *et al.* 2001) in the murine system.

Moreover, several groups reported on the *in vitro* priming of naive T cells against tumour antigens using LV transduced human DCs. Firat *et al* (Firat *et al.* 2002) stimulated $CD8^+$ T cells in bulk with monocyte-derived DCs that were transduced with LVs encoding a melanoma poly-epitope and demonstrated expansion of $tetramer^+$ $CD8^+$ T cells, which could specifically lyse gp100 and Melan-A peptide-pulsed targets. We showed priming of both $CD8^+$ and $CD4^+$ T cells against the poorly immunogenic melanoma antigen MAGE-A3 after *in vitro* stimulation with DCs transduced with LVs encoding the fusion protein Ii80MAGEA3 (Breckpot *et al.* 2003). The primed $CD8^+$ T cells were further cloned and characterized enabling the identification of a novel HLA-Cw7 restricted MAGE-A3 peptide (Breckpot *et al.* 2004b). A number of groups evaluated the potential of mouse DCs transduced with LVs as a cellular anti-tumour vaccine *in vivo*. Herein, the induced immune response was characterized and tested for protection against tumour growth. We showed that immunization with DCs transduced with OVA encoding LVs induced a strong CTL response, resulting in specific lyses of OVA-expressing tumour cells after *in vitro* restimulation (Breckpot *et al.* 2003) or *in vivo* upon delivery of autologous OVA peptide-pulsed spleen cells (Dullaers *et al.* 2006). He *et al* (He *et al.* 2005) confirmed these data. It was moreover demonstrated that these CTL responses were protective against a subsequent challenge with a lethal dose of OVA-expressing B16 melanoma cells and slowed down the outgrowth of pre-existing tumours (He *et al.* 2005; Dullaers *et al.* 2006). Later on, it was shown with endogenous tumour antigens that the results obtained with the strong immunogenic OVA were not an overestimation of the potential of LV transduced DCs as a cellular therapeutic. Tumour antigens such as TRP-2 (Metharom *et al.* 2001) and erbB2 (mouse analogue of human Her-2/neu) (Mossoba *et al.* 2008) were used to demonstrate induction of strong CTL responses and decreased tumour growth. Importantly, Wang *et al.* (Wang *et al.* 2006) extended these data in a mouse hepatoma model, immunized with LV transduced DCs expressing three hepatoma-associated antigens, which are self-antigens that are highly expressed in tumour cells, demonstrating $CD4^+$ and $CD8^+$ T cell responses against all three hepatoma antigens, as well as regression of established tumours. Delivery of multiple tumour antigens might overcome the problem of tumour escape due to antigen loss (Dullaers *et al.* 2006). Finally, it has to be noted that several groups compared DCs transduced with LVs to DCs pulsed with (tumour) antigen-derived peptides (He *et al.* 2005; Metharom *et al.* 2005) or electroporated with mRNA (Dullaers *et al.* 2004), two strategies that were approved in the clinic, demonstrating that LV modified DCs elicited stronger and longer-lasting anti-tumour T cell responses.

These studies suggest that *ex vivo* LV transduced DCs are effective in therapeutic treatment of cancer. However, this strategy has important drawbacks common to all DC-based vaccination approaches. Because the vaccine is patient-specific it requires specialized personnel and facilities for vaccine production. Consequently, there is a high cost and considerable time required for vaccine production and quality control. It is for that reason that direct administration of LVs *in vivo* has gained substantial interest.

4.2 Evaluation of lentiviral vectors as an off-the-shelf anti-tumour vaccine

For LVs to be an effective vaccine they have to transduce DCs *in situ*, a pre-requisite that is fulfilled. Furthermore, the *in vivo* transduced DCs need to process the transgene, have to be activated so they migrate to lymphoid organs, where they subsequently present transgene-derived epitopes in the context of MHC molecules and strong co-stimulation to T cells. Hence, the degree of tumour antigen-specific CTL induction can be considered as a reliable measure for the value of direct administration of tumour antigen encoding LVs in tumour immunology. Antigen-specific CTLs could be generated upon direct administration of LVs using HLA-Cw3 as a model antigen (Esslinger *et al.* 2003). Similar results were obtained in HLA-A transgenic mice using a LV encoding a minigene containing the dominant human Melan-A HLA-A*0201 epitope (Chapatte *et al.* 2006) or human telomerase reverse transcriptase (hTERT) (Adotevi *et al.* ; Rusakiewicz *et al.*). Using OVA as an antigen, it was confirmed that direct administration of LVs resulted in a higher number of IFN-γ producing $CD8^+$ T cells, which had a higher lytic capacity as compared to those primed with *ex vivo* transduced DCs (Dullaers *et al.* 2006). Memory CTL responses were also significantly stronger upon LV administration. Other studies with tumour antigens such as NY-ESO (Palmowski *et al.* 2004), TRP-2 (Kim *et al.* 2005), TRP-1 (Liu *et al.* 2009) and CEA (Loisel-Meyer *et al.* 2009), have also shown potent immune responses upon vaccination with LVs. Comparison of *in vivo* administration of LVs to the peptide or DNA vaccination strategy was performed in a HLA-A transgenic mice, by Chapatte *et al* (Chapatte *et al.* 2006) and Rusakiewicz *et al* (Rusakiewicz *et al.*), demonstrating that stronger tumour-specific immune responses were elicited when immunization was performed with LVs.

Reference	Dose	Route	Antigen
Esslinger et al	2 x 10^7 EFU	sc (footpad, tail base)	Cw3, mini Melan-A
Palmowski et al	1 x 10^6 PFU	iv (tail vein)	NY-ESO
Kim et al	1.6 x 10^6 PFU	sc (footpad)	TRP-2
Rowe et al	1 x 10^7 IU	iv (tail vein)	OVA
Dullaers et al	2 x 10^7 TU	sc (footpad)	OVA
He et al	1 x 10^6 TU	sc (footpad)	OVA
Chapatte et al	2 x 10^7 EFU	sc (tail base)	Melan-A
Liu et al	2.5 x 10^7 TU	sc (footpad)	TRP-1
Loisel-Meyer	0.15 × 10^6 TU	sc (footpad)	CEA
Rusakiewicz et al	1 x 10^7 TU	sc (footpad)	hTERT

Table 1. **Summary of the studies evaluating LVs as an off-the-shelf vaccine.**

Although $CD4^+$ T cell responses were shown to be necessary for the priming and maintenance of CTLs when DCs are used for vaccination, not much data is available on the role of $CD4^+$ T cell help in the induction of CTLs upon immunization with LVs. Esslinger *et al* (Esslinger *et al.* 2003) showed that CD4 depletion reduces the primary CTL response upon direct administration of LVs. Similarly, we (Dullaers *et al.* 2006) showed that although there was a larger requirement for $CD4^+$ T cell help during the primary response in case of immunization with *ex vivo* transduced DCs compared to direct administration of LVs; $CD4^+$ T cell depletion strongly reduced the capacity to mount a recall CTL response in both cases. Interestingly, Marzo *et al* (Marzo *et al.* 2004) showed that in the case of a VSV infection, a

functional $CD8^+$ T cell memory response can be generated in the absence of $CD4^+$ 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 *L. monocytogenes* antigens need to be cross-presented. Since, the currently applied LVs are pseudotyped with VSV envelopes, it needs to be further examined to what extent the CTL response is $CD4^+$ T cell-dependent.

The generation of specific T cell responses is a convenient read-out for the success of a vaccination strategy however; there are many examples of discrepancies between immune responses and anti-tumour responses (Rosenberg *et al.* 2005). Therefore, it is critical to evaluate the effect of LV vaccination on tumour growth. Rowe *et al* (Rowe *et al.* 2006) showed significantly improved protection of mice vaccinated with LVs encoding OVA against a subsequent tumour challenge. Similarly, Dullaers *et al* (Dullaers *et al.* 2006) showed that direct administration of LVs 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 *et al.* 2005), TRP-1 (Liu *et al.* 2009) or CEA (Loisel-Meyer *et al.* 2009) as tumour antigen, demonstrated improved survival of tumour bearing mice receiving LVs encoding these tumour antigens. Liu *et al* (Liu *et al.* 2009) showed that this type of immunization could result in complete regression of small subcutaneous tumours, which correlated with enhanced numbers of functional $CD8^+$ T cells in the tumour environment. Therefore, these studies highlight the potential of LVs encoding tumour antigens as an anti-cancer therapeutic.

These studies demonstrate that administration of LVs doesn't provoke immunological tolerance, but rather elicits powerful CTL responses against transgene-encoded proteins. This suggests a certain degree of immunogenicity of LVs or components within LV preparations, leading to activation of innate viral-sensing pathways and as a consequence induction of strong adaptive immunity. Therefore, it is not surprising that several studies addressed the immunogenicity of LVs. LVs are generally derived from HIV-1, for which activation of pDCs through TLR7 triggering has been demonstrated (Fonteneau *et al.* 2004; Beignon *et al.* 2005). It was demonstrated *in vivo* that activation of pDCs by recombinant LVs is mediated by several mechanisms. Brown *et al.* (Brown *et al.* 2006a) reported a TLR7-dependent type I IFN response, whereas a role for TLR9 was demonstrated by Pichlmair *et al* (Pichlmair *et al.* 2007), who demonstrated that VSV.G pseudotyped LV preparations contain tubulovesicular structures of cellular origin, which carry nucleic acids. These structures triggered TLR9 in pDCs, whereas LVs pseudotyped with a γ-retroviral envelope didn't (Lopes *et al.* 2008), suggesting that this particular mechanism is not necessary for potent immune stimulation. More recently, Rossetti *et al* (Rossetti *et al.*) demonstrated that also human blood-derived pDCs are activated in a TLR7/9-dependent way by LVs *in vitro*. These observations were not surprising as the pDC subset is the DCs subset that is best equipped to sense viral infections. However, recombinant LVs also target cDCs. Therefore, this DC subset should not be neglected when the LV immunogenicity is discussed. Gruber *et al* (Gruber *et al.* 2000) reported that transduction of cDCs at low MOI didn't result in DC activation, whereas Tan *et al.* (Tan *et al.* 2005) described that transduction of cDCs at high MOI results in up-regulation of adhesion, stimulatory and antigen presenting molecules. Furthermore, these DCs displayed enhanced allo-stimulatory capacities and an altered cytokine secretion pattern. To clarify these results, we (Breckpot *et al.* 2007b) transduced DCs with LVs at varying MOI, confirming that transduction of DCs with LVs at low MOI results in considerable transgene delivery, without activation, whereas transduction at high

MOI indeed leads to DC maturation. A role for protein kinase R, a cytosolic receptor that interacts with double stranded RNA during LV replication, and several TLRs was suggested (Breckpot *et al.* 2007b). In our recent *in vivo* study, we demonstrated that cDCs are activated upon LV infection. More importantly, we showed that this activation was dependent on retroviral reverse transcription and critically dependent on the signal adaptor molecules MyD88 and TRIF, hence TLR signalling. Experiments with TLR knock out DCs demonstrated that both TLR3 and TLR7 are involved in the DC activation (Breckpot *et al.*).
It is important to stress that induction of therapeutic anti-tumour immunity is critically dependent on an inflammatory environment in order to overcome tolerance, and active inhibitory mechanisms exerted by suppressive immune cells, such as Treg, as well as tumour cells. Such an inflammatory environment can be achieved by strong activation of the innate arm of the immune system, in particular through the engagement of TLRs. This was highlighted by Yang *et al* (Yang *et al.* 2004) and by Lang *et al* (Lang *et al.* 2005), who demonstrated that tolerance of antigen-specific CTLs could be broken by persistent TLR ligation. In that respect, we demonstrated that DCs activated by LVs *via* TLR3 and TLR7, efficiently expanded antigen-specific CTLs, whereas DCs lacking either TLR lacked this CTL inducing capacity (Breckpot *et al.*). Furthermore, it has been described that signalling through certain combinations of TLRs on DCs not only provided a synergy with respect to the production of stimulatory cytokines such as IL-12, which is essential in the differentiation of $CD4^+$ T cells to a T_H1 phenotype (Gautier *et al.* 2005; Napolitani *et al.* 2005), but also offered protection from suppressive Treg that actively quench the anti-tumour immune response (Warger *et al.* 2006). As a consequence, much research efforts have been put in designing approaches that enhance the intrinsic immunogenicity of LV-based vaccines. Some of these will be discussed in the next section.

4.3 Engineering lentiviral vectors to enhance the immune stimulatory capacity of dendritic cells

To enhance the immunogenic potential of LVs, and concomitantly prevent the actions of tolerogenic mechanisms over transduced DCs, LVs can be engineered to not only deliver the tumour antigen but also deliver molecules that enhance DC activation. Based on our growing knowledge on the importance of TLRs for DC activation and which activation pathways are triggered by these TLRs, several TLR-based strategies have been developed to enhance the immune stimulatory capacity of DCs upon LV transduction.
Over the years LPS, which binds to TLR4 has been extensively used to activate DCs *in vitro* (Ardeshna *et al.* 2000; Arrighi *et al.* 2001; da Silva Correia *et al.* 2001). LPS-mediated activation remarkably enhances stimulation of DC-mediated immune responses *in vitro*, and overcomes suppression by Treg, a critical factor in anti-tumour immunology (Pasare & Medzhitov 2003). However, its clinical use is limited by its cytotoxicity. Therefore, the feasibility of using RVs encoding a constitutive active TLR4 (caTLR4) for DC maturation has been evaluated (Xu *et al.* 2007). This was achieved by linking the cytoplasmic domain of TLR4 to the extracellular single-chain immunoglobulin anti-erbB2. However, no activation of an immortalized DC line, JAWSII was observed, although a similar strategy, *i.e.* electroporation with mRNA encoding caTLR4, was recently shown to activate human DCs, resulting in priming of Melan-A CTL responses (Bonehill *et al.* 2008). Using a similar cloning strategy Xu *et al* (Xu *et al.* 2007) generated RVs encoding MyD88 or IRAK-1, two major adaptor molecules in TLR triggered activation pathways. Again they used the JAWSII DC line to evaluate the chimeric proteins, demonstrating that only the IRAK-1 chimera

mediated IL-12 and TNF-α secretion, and enhanced OVA-specific $CD4^+$ T cell responses. Akazawa *et al* (Akazawa *et al.* 2007) expressed MyD88 and TRIF, another major TLR signal transduction molecule in mouse DCs using LVs. MyD88-modified DCs produced IL-6 and IL-12, but didn't up-regulate phenotypic markers, whereas TRIF expression stimulated IFN-β production and increased levels of CD86. Both MyD88 and TRIF increased the allo-stimulatory capacity of the modified DCs, and tumour outgrowth was delayed after immunization with these DCs.

Introduction of MyD88 or IRAK-1 in DCs activates the NF-κB pathway. NF-κB is a well-studied transcription factor that targets genes associated with DC maturation. Sustained NF-κB activation in DCs using LVs has been achieved by expressing Kaposi's sarcoma associated human herpes virus vFLIP (Rowe *et al.* 2009). In this case, DC maturation was enhanced by up-regulation of MHC, adhesion (ICAM-1) and co-stimulatory molecules (CD80, CD86, CD40), and increased secretion of TNF-α and IL-12. vFLIP-modified DCs significantly increased antigen-specific CTL responses resulting in enhanced anti-tumour immunity (Karwacz *et al.* 2009; Rowe *et al.* 2009). Another effective approach leading to sustained NF-κB activation consists of down-regulating the negative feedback molecule, A20 of which the expression is under the immediate control of NF-κB. A20 deactivates several adaptor molecules of the TNFR, IL-1/TLR signalling pathways by ubiquitinating/de-ubiquitinating activity (Vereecke *et al.* 2009). Therefore, A20 down-regulation could result in prolonged NF-κB activation, resulting in DCs with enhanced stimulatory capacity. LV delivered A20-targeted shRNA (Song *et al.* 2008) and direct introduction of siRNA (Breckpot *et al.* 2009) were applied to down-regulate A20 in mouse and human DCs, respectively. These approaches showed that A20 controls DC maturation, cytokine production and immunostimulatory potency. Human DCs with down-regulated A20 expression had an increased NF-κB activity and showed enhanced and sustained IL-10 and IL-12 secretion. These DCs were more potent in stimulating Melan-A CTL responses (Breckpot *et al.* 2009). Mouse DCs with down-regulated A20 expression showed enhanced expression of co-stimulatory molecules and pro-inflammatory cytokines. Moreover, these DCs hyper-activated tumour-specific CTL and T_H cells, which were refractory to Treg suppression (Song *et al.* 2008).

Besides LVs that target the NF-κB pathway, LVs have been engineered to increase the DC's immunogenicity by introducing specific genes that modulate intracellular MAPK pathways. p38 was activated by expressing MKK6 mutants containing glutamate and aspartate residues in their activation loop, mimicking phosphorylated serine or threonine residues (Raingeaud *et al.* 1996). A fusion protein between MKK7 and JNK1 was expressed to achieve constitutive JNK1 phosphorylation (Escors *et al.* 2008). Expression of constitutive activators prevents inactivation by phosphatase-dependent negative feedback mechanisms, which may 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 *et al.* 2008). Particularly, there was specific up-regulation of co-stimulatory molecules and absence of significant secretion of pro-inflammatory cytokines (Escors *et al.* 2008). Interestingly, co-expression of OVA with the p38 activator in DCs significantly increased antigen-specific $CD4^+$ and $CD8^+$ T cell responses leading to increased anti-tumour immunity (Escors *et al.* 2008; Karwacz *et al.* 2009). Additionally, MAPK p38 constitutive activation also increased $CD8^+$ T cell responses to human tumour antigens NY-ESO in a humanized HLA-A2 mouse model and Melan-A in a human DC-T cell culture (Escors *et al.* 2008). Specific activation of JNK1 in DCs showed only

a moderate up-regulation of co-stimulatory molecules and no significant secretion of pro-inflammatory cytokines, confirming previous studies, which suggested that JNK marginally controls DC maturation (Nakahara *et al.* 2004; Escors *et al.* 2008). On the other hand, increased antigen-specific $CD8^+$ 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 DCs *in vivo* (Escors *et al.* 2008).

5. Exploitation of lentivirally transduced dendritic cells for the induction of tolerance

There are many ways in which DCs have been utilised for the treatment of autoimmune disorders. This chapter will focus on genetic modification using LVs, rather than providing an extensive review of all DC-based methods. Because the achievement of immune suppression is more challenging than inducing activation, there are a limited number of reports on the use of LVs as immunosuppressive (tolerogenic) therapeutic agents.

An obvious approach to genetically modify DCs for the treatment of autoimmune disorders is to express potent immunosuppressive cytokines. In fact, there are a few reports of DC modification using mainly RVs expressing immunosuppressive cytokines for the treatment of inflammatory diseases (Lee *et al.* 1998; Takayama *et al.* 1998; Morita *et al.* 2001). The equivalent approach has been undertaken by transduction of DCs using LVs expressing IL-10 in an OVA-dependent model of experimental asthma (Henry *et al.* 2008). *In vivo* intratracheal injection of OVA-pulsed DCs modified with LVs expressing IL-10 effectively inhibited airway inflammation and asthma-associated symptoms. Interestingly, it was demonstrated that host IL-10 expression was absolutely required for the IL-10 DCs to inhibit asthma (Henry *et al.* 2008). Therefore, IL-10 expression from DCs was playing an indirect role in inhibiting disease. Interestingly, a significant increase in $Foxp3^+$ Treg expressing IL-10 were expanded, and their adoptive transfer prevented OVA-sensitized mice from eosinophilia after OVA challenge (Henry *et al.* 2008).

An attractive option for programming tolerogenic DCs is to modulate signalling pathways involved in differentiation of immunosuppressive DCs. This approach regulates expression of gene clusters, which act in a concerted action in physiological functional tolerogenic DCs. There is quite a wide range of experimental evidence linking MAPK ERK activation to immune suppression and tolerance (Agrawal *et al.* 2006; Caparros *et al.* 2006; Dillon *et al.* 2006; Escors *et al.* 2008). Constitutive activation of the ERK pathway can be readily achieved by expression of constitutively active MEK1 mutants, the upstream ERK kinase (Pages *et al.* 1994; Raingeaud *et al.* 1996; Escors *et al.* 2008; Anastasaki *et al.* 2009). Particularly, DCs modified with a LV expressing a MEK1 mutant with a deletion in the coding region of the nuclear export signal, together with two activating mutations resulted in DCs with a marked immature phenotype (Escors *et al.* 2008). ERK-activated mouse and human DCs showed a pronounced CD40 down-regulation and secretion of significant amounts of TGF-β (Escors *et al.* 2008; Arce *et al.* 2010). These DCs were strongly immunosuppressive, leading to differentiation of antigen-specific $Foxp3^+$ Treg *in vivo* and *in vitro* (Arce *et al.* 2010). These differentiated Treg strongly proliferated after a second antigen encounter in inflammatory conditions. A LV vaccine based on an ERK activator was successfully applied for the treatment of inflammatory arthritis in a mouse model (Arce *et al.* 2010). This therapeutic approach could be applied even when the specific arthritogenic antigen was not specifically targeted. Application in human therapy could follow a similar approach in which

simultaneous ERK activation and expression of an endogenous antigen could be used to inhibit arthritis even though the arthritogenic antigens are not well characterized and may vary between patients.

Interestingly, constitutive activation of the type I IFN signalling pathway was also immunosuppressive in DCs. Expression of a constitutively active IRF3 mutant (IRF3 2D) in mouse DCs induced expression of high levels of IL-10 (Escors *et al.* 2008). Interestingly, vaccination with a LV co-expressing IRF3 2D with an OVA-containing transgene resulted in systemic expansion of OVA-specific Foxp3$^+$ Treg (Escors *et al.* 2008). In physiological conditions, phosphorylated IRF3 dimerizes and translocates to the nucleus where it transactivates type I IFN promoters, leading to IFN-β production, a potent antiviral cytokine (Fitzgerald *et al.* 2003). Interestingly, it has been known for some time that components of the type I interferon pathway are in fact immunosuppressive (Chang *et al.* 2007). This is also the basis of the use of type I IFNs for the treatment of multiple sclerosis (Comabella *et al.* 2002; Billiau 2006). Very interestingly, production of both IFN-β and IL-10 share a common pathway when activated by TLR signalling (Hacker *et al.* 2006; Chang *et al.* 2007). It has been proposed that phosphorylated IRF3 may link IFN-β production with IL-10 secretion through a MyD88-dependent pathway (Escors *et al.* 2008). Taking advantage of this, activators of the type I IFN pathway could be expressed in DCs for the treatment of autoimmune disorders such as multiple sclerosis.

An alternative to constitutive activation of immunosuppressive pathways is to specifically inhibit pro-inflammatory signalling pathways. Possibly, one of the major pro-inflammatory pathways in DCs is NF-κB (Breckpot & Escors 2009b). Consequently, silencing of components from the NF-κB pathway could theoretically prevent DC maturation and induce immune suppression and tolerance. For instance, this has been successfully applied by silencing Rel-B in DCs by delivery of a specific shRNA using LVs (Zhang *et al.* 2009). Rel-B silencing was sufficient to confer DCs resistance to TLR-derived maturation signals and to inhibit experimental autoimmune myasthenia gravis in a mouse model (Zhang *et al.* 2009). Interestingly, just by inhibiting NF-κB, DCs acquired tolerogenic activities characterised by inhibition of T cell proliferation and differentiation of Foxp3$^+$ Treg.

Another interesting approach is the exploitation of naturally occurring negative feedback mechanisms of pro-inflammatory pathways. This has been achieved by over-expressing suppressor of cytokine signalling 3 (SOCS-3) in DCs using LVs (Li *et al.* 2006). SOCS comprise a family of cytoplasmic proteins induced by cytokine-mediated signal transduction. They form part of a negative feedback mechanism that limits cytokine-induced signalling. Expression of SOCS3 in mouse DCs results in immature DCs with down-regulated MHC molecules and reduced CD86 (Li *et al.* 2006). These modified DCs exhibit an impaired signalling by IL-12 and IL-23, and reduced expression of these cytokines. More importantly, enhanced secretion of IL-10 was observed, which polarised T cell responses towards a T_H2 type. Interestingly, SOCS3-expressing DCs could efficiently inhibit the development of EAE, an experimental model for human multiple sclerosis (Li *et al.* 2006).

Recently, a LV-based shRNA delivery system was successfully applied for the treatment of experimental collagen-induced arthritis without specific targeting of the arthritogenic antigen (collagen) (Lai Kwan Lam *et al.* 2008). Direct administration of a LV encoding a siRNA specific for B cell activating factor (BAFF) to the inflamed joint was sufficient to inhibit arthritis. BAFF is a member of the TNF family which is mainly involved in regulating B cell maturation and functions (Batten *et al.* 2000; Yang *et al.* 2010). Interestingly, elevated BAFF levels have been found in the serum of patients suffering from several autoimmune

disorders including rheumatoid arthritis. Very interestingly, it was demonstrated that LVs preferentially transduced DCs in the inflamed joint, and that BAFF silencing in these DCs interfered with DC maturation. Local BAFF silencing inhibited pro-inflammatory T cell development and inhibited production of pro-inflammatory cytokines such as IL-17, IL-23 and IL-6 (Lai Kwan Lam *et al.* 2008). Importantly, these authors clearly demonstrated that (i) LVs can be directly administered to the site of inflammation, (ii) they preferentially transduce local DCs and (iii) it is not strictly necessary to target the arthritogenic antigen.
In addition to manipulation of signalling pathways, there are small peptides with broad activities including immune suppression. Direct intraperitoneal immunisation with a LV encoding vasointestinal peptide (VIP) reduced the severity of collagen-induced arthritis in a mouse model (Delgado *et al.* 2008). Interestingly, vaccination with this LV significantly inhibited the secretion of a wide array of pro-inflammatory cytokines both systemically and in the joint. This tolerogenic LV-vaccination expanded Foxp3$^+$ Treg (Delgado *et al.* 2008). However, VIP has a variety of physiological functions apart from its immunosuppressive properties. Therefore, in this case, it would be desirable to modify DCs *ex vivo* with a VIP-expressing LV followed by *in vivo* transfer (Toscano *et al.* 2010). In fact, VIP expression in DCs was sufficient to keep them in an immature stage, leading to secretion of high levels of IL-10 (Toscano *et al.* 2010). *In vivo* administration of VIP-expressing DCs had beneficial therapeutic effects in EAE mice and in the cecal ligation and puncture model, both models relevant for multiple sclerosis and sepsis in humans (Toscano *et al.* 2010).

6. Limitations of lentiviral vectors for direct *in vivo* application

Although the HIV-based vector system is by far the best developed among the various LVs, a variety of quality, safety, efficacy, regulatory and ethical concerns slacken the frequent employment of HIV-based vectors in a clinical setting. In view of DC modulation, the scope of this review will be limited to the biological risks and immunogenic consequences of LV-based vaccination. Safety seems the most pressing issue as LVs are derived from an integrating pathogenic agent, lethal in humans. As mentioned, one of the main adverse events to consider is the potential generation of RCLs. However, to date no RCLs have been reported for LV packaging systems. This can be partially explained by the separation of *cis*- and *trans*-acting sequences during LV production, but also by the fact that SIN LVs are less likely to produce RCLs (Pauwels *et al.* 2009). A major setback for viral gene therapy clinical trials was caused by the development of leukaemia in five patients of two separate γ-RV gene therapy trials for X-linked SCID as a consequence of insertional activation of the LMO2 gene (proto-oncogene) by the LTR enhancer. As genomic integration is common to all retroviruses, the associated risk of insertional mutagenesis and/or transactivation of adjacent sequences must be taken into account for LVs as well (Howe *et al.* 2008). However, as these observations were made with γ–RV it would be to hasty to extrapolate this risk to the multiply attenuated recombinant LV system used today. An *in vitro* mapping study comparing RV and LV integration in transduced human HSCs revealed that RV but not LV hot spots were highly enriched in proto-oncogenes, cancer-associated and growth-controlling genes, suggesting that LVs have a lower propensity for integrating in potentially dangerous regions within the human genome (Cattoglio *et al.* 2007). Furthermore, an *in vivo* genotoxicity assay using a tumour-prone murine model, also showed differences in the oncogenic potential of RVs and LVs. Herein, it was shown that LTRs co-determine the vector's genotoxic potential, supporting the choice of SIN LVs (Montini *et al.* 2009). Recently,

immune-deficient mice received LV transduced HSCs, and were followed for 2-6 months. No LV-associated adverse events were observed, and none of the mice had detectable levels of HIV p24 antigen in their sera (Bauer *et al.* 2008). More recently also the concept of clonal dominance came forward for LVs in a phase I/II clinical trial using HSCs transduced by a SIN LV with chromatin insulators (Bank *et al.* 2005). This clonal dominance seems to be due to growth and/or survival advantage conferred by gene-activating or -suppressing effects of the integrated LV. Importantly, such induced clonal dominance didn't lead to malignant transformation (Fehse & Roeder 2008). Additional potential adverse events are seroconversion of the subject to components of the HIV-1 vector, mobilization and subsequent spread of mobilized vector particles to previously untransduced cells and tissues, and transmission of exogenous DNA to cells of the germ line (Romano *et al.* 2003).

In view of LV based vaccination, the induced innate and adaptive immune reactions against the LV particle, transduced cells and/or its transgene product, act as a double-edged sword. This immunogenicity may aggravate a robust immune reaction, which is possibly advantageous in the context of *e.g.* anti-tumour immunotherapy. However, unwanted immune responses may curtail the induction of tolerance, which is desired for the treatment of *e.g.* transplantation rejection or autoimmune diseases. As broad tropism LVs are capable of transducing DCs but also other cell types, this can have important consequences in view of the immunological outcome. On the one hand transgene expression by non-DCs could lead to uptake of cellular remains from these cells by DCs followed by antigen presentation in the context of MHC class II and *via* cross-presentation in the context of MHC class I. In this way not only a CTL response is triggered, but also T_H cells, which are important to induce a proper CTL response (Matrai *et al.* 2010). On the other hand DCs also play an important role in the induction and maintenance of peripheral tolerance against self-antigens (Steinman 2007). In this context, persistent expression of the transgene by a non-DC, as a consequence of LV integration in a terminally differentiated long-living cell, could also lead to induction of peripheral tolerance (Follenzi *et al.* 2004). Thus, upon broad tropism LV vaccination, the antigen-specific immune response could be initiated by transduced DCs (direct priming), and theoretically by non-lymphoid cells (cross-priming). However, the latter might also counteract the envisaged immune response, which makes it hard to predict the immunological outcome. Therefore, targeting of LVs to DCs, the cell type of choice to induce immunity or tolerance has been explored. Two different approaches have been successfully attempted (i) transcriptional targeting in which DC-specific promoters are exploited and (ii) transductional targeting in which DC-specific LV entry is achieved. Transcriptional targeting has been extensively discussed by Frecha *et al* (Frecha *et al.* 2008).

We believe that transductional targeting of LVs to DCs is a very interesting strategy to tackle safety and efficacy concerns all at once, since: (i) DCs have a relative short half-life, which decreases the chance for malignant transformation after insertional mutagenesis, since this is a multi-step process (Fehse & Roeder 2008), (ii) there is no risk for transmission of exogenous DNA to the germ line, (iii) non-DC transduction is prevented which can overcome unwanted interference with the desired immune response, (iv) DC subset-specific targeting paves the way to fundamental research on the exact role of each DC subset in the development of diseases, as well as their therapeutic importance.

As the LV tropism is determined by the envelope glycoprotein, pseudotyping allows the generation of LVs with a specific transduction pattern. To date, there is no natural DC-

specific envelope glycoprotein identified. In first instance, it was attempted to modify existing envelope proteins by means of genetic engineering to obtain DC-specific binding without disrupting membrane fusion. An example of this application is the construction of a LV where the VSV.G envelope is replaced by a chimeric version of a scFv, coupled to a (i) N-terminal end of the VSV.G protein (Dreja & Piechaczyk 2006), (ii) the amphotrophic MLV protein (Gennari *et al.* 2009), (iii) the sindbis virus envelope protein (Morizono *et al.* 2009; Zhang *et al.* 2010) or (iv) the H-protein of the measle virus envelope, which is than combined with its F-protein (Ageichik *et al.*). However, these manipulations result in viral particles with low stability and limited binding- and fusion capacities. Alternatively, the sindbis glycoprotein can be mutated in its heparin sulphate receptor (HSR) binding part whereby it exclusively binds to DC-SIGN, a DC-specific glycoprotein (Yang *et al.* 2008). Importantly, with this strategy DC-specific transduction could be demonstrated *in vivo.* It was moreover demonstrated that a strong T cell response could be generated that induced tumour regression. An alternative method to target LVs to specific cell types was proposed by Chandrashekran *et al* (Chandrashekran *et al.* 2004). Since RVs and LVs obtain their envelope after budding from the cell membrane of the producer cell line, every glycoprotein that is expressed on this membrane, will be incorporated in the viral envelope. Through this mechanism a membrane bound form of stem cell factor (mbSCF) was incorporated in the membrane of a RV producing cell line, which produced ecotropic RVs. This envelope doesn't allow transduction of human cells. However, binding of the mbSCF onto its receptor, c-kit, resulted in the transduction of human c-kit$^+$ cells by the mbSCF-containing and ecotropic envelope pseudotyped RVs. This strategy was later on expanded by Yang *et al* to LVs (Yang *et al.* 2006). They incorporated a fusogenic protein (derived from influenza A or sindbis virus), as well as an antibody against CD20 in the plasmamembrane of the LV producing cell line. Subsequently LVs were produced, which transduced B cells both *in vitro* and *in vivo.* Importantly, Zhang *et al* (Zhang et al.) engineered a truncated version of VSV.G (VSV.GS), which resulted in a binding-defective, but fusogenic envelope, which still confers vector stability hence allows production of high titer LVs. It was shown that LVs that incorporate the VSV.GS together with mbSCF in their viral surface efficiently transduced c-kit$^+$ cells. This strategy could be translated to DCs, where DC-specific molecules, such as BDCA-2 or DEC-205 are targeted (Dzionek *et al.* 2001; Bonifaz *et al.* 2004; Yang *et al.* 2008).

As molecular cloning of classic antibodies or fragments thereof is challenging, alternatives have been explored. One of these is the use of antibodies from 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-Casterman *et al.* 1993). The antigen-binding part of the molecule is composed of only one single variable region (termed VHH, or Nanobody, Nb). These antigen-specific antibody fragments offer many advantages: (i) they are highly soluble, (ii) they can refold after denaturation whilst retaining their binding capacity, (iii) cloning and selection of antigen-specific Nbs obviate the need for construction and screening of large libraries, (iv) as Nbs can be fused to other proteins, it should be possible to present them on the cell membrane of a producer cell line, thus generating LVs that incorporated a DC-specific Nb in their envelope during budding as described above .

7. Conclusion and future perspectives

To date, numerous studies have demonstrated that LVs are excellent candidates for the treatment of immunological pathologies such as cancer and infectious diseases. This can be

explained by their capacity to transduce DCs *in vivo*, which is followed by efficient expression, processing and presentation of the introduced transgenes *via* MHC class I and class II molecules, thereby (re) activating an effector and T_H1 response, respectively. Although LVs comprise an intrinsic immunogenic potential, crucial for effective activation of transduced DCs, they have been exploited as a means to induce antigen-specific tolerance for the treatment of diseases with an autoimmune aetiology and transplantation rejection as well. Although this seems contradictory, this duality endows the LV with the potential to become the vaccine tool of choice with an infinite number of application possibilities. Although we are convinced of the LVs' potential as an immunotherapeutic, we give a concise overview of the extra improvements we believe are necessary to moderate the translation of the LVs from bench to bedside.

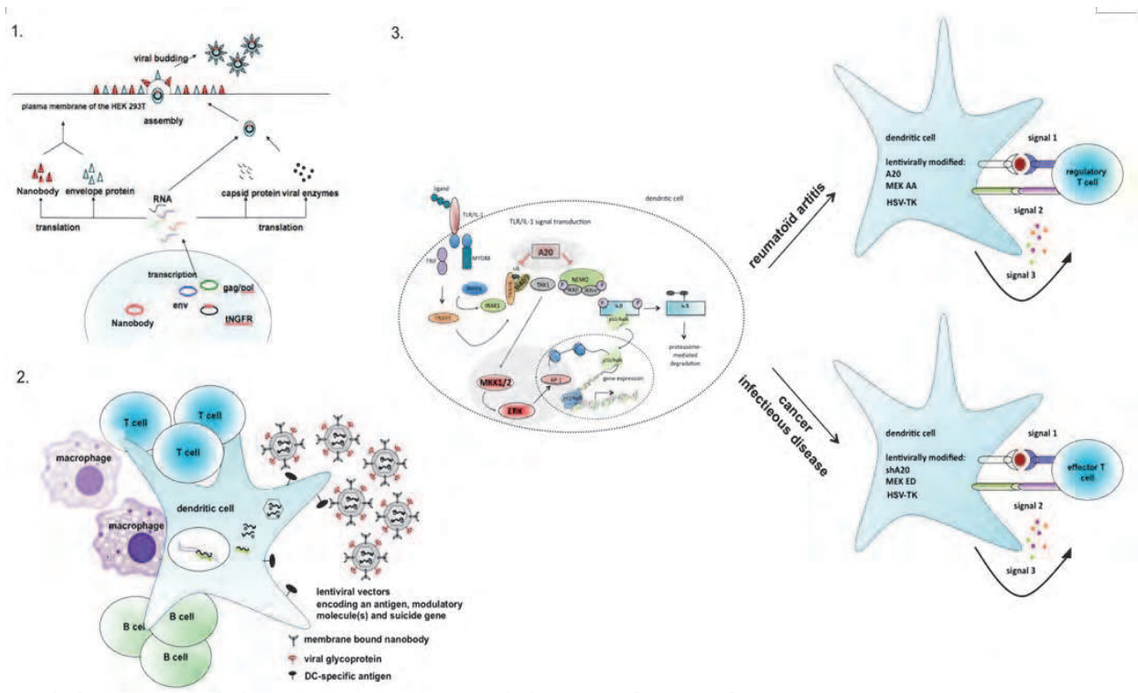

Fig. 4. **Schematic representation of what we believe is the ultimate strategy to successfully exploit the advantageous of LVs and DCs for immunotherapeutic purposes.** Herein, LVs encoding antigen and immune modulating molecules are pseudotyped with a binding-defective but fusogenic envelope glycoprotein, as well as a DC-specific Nb (1). The latter will dictate binding of the LVs to DCs, after which the envelope mediates fusion (2). Subsequently, the DCs are rendered immunogenic or tolerogenic, depending on the envisaged therapy, and the DCs present antigen-derived peptides in the context of MHC class I and class II molecules, hence induce effector T cells or Treg (3).

A first improvement will be the overall use of third generation LVs to curtail the risk of RCLs. Secondly, the use of NILVs is believed to be an inevitable strategy to diminish the LVs' potential to induce insertional mutagenesis. A third improvement to increase both safety and efficacy lies within transductional targeting of the LV to DCs *in vivo*. Therefore we propose to exploit the Nb display technology to target LVs to DCs in combination with

LV-based modulation of two signal transduction pathways within the DCs (Fig. 4). The Nb display technology is based on the natural budding mechanism of LVs as they are released from the LV producing cell. This mechanism allows the incorporation of a binding-defective but fusogenic glycoprotein together with a DC-specific Nb in the plasmamembrane of the LV producer cells and subsequently in the viral envelope. The DC-specific Nb will bind to its antigen on the DC surface whereupon the fusogenic envelope induces transduction. In this way LVs shuttle their cargo into the DC. Subsequently DCs can be conditioned to become either stimulatory or tolerogenic for the introduced transgene(s). For this purpose we can modulate adaptor molecules, such as the MAPK ERK, which switches on a tolerogenic pathway (Arce *et al.*) or A20, which inhibits the stimulatory NF-κB pathway (Song *et al.* 2008). Down-regulation of both will elevate the DC' immunogenic potential, whereas their up-regulation could ameliorate its tolerogenic potential. We strongly believe that this strategy can pave the way toward a safe and multifunctional LV toolbox.

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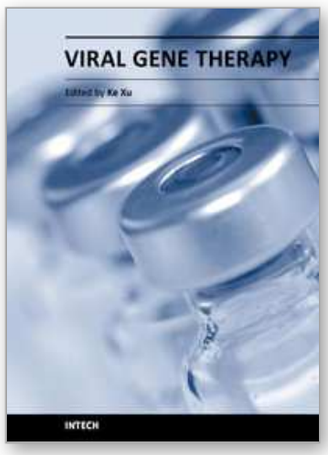

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The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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