Consequences of persistent antigen presentation following administration of HIV-1-derived lentiviral vectors

Frederick Arce Vargas

Thesis submitted to the University College of London
for the degree of Doctor of Philosophy

2010

Division of Infection and Immunity
University College of London

DECLARATION

I, Frederick Arce Vargas, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Lentiviral vectors (LVs) are promising tools for *in vivo* gene delivery, either to correct genetic defects or for vaccination. Intravenous administration of LVs results in stable transduction and expression of the transgene in antigen presenting cells (APCs) from the spleen. Therefore, it was decided to investigate the reasons for and the consequences of sustained antigen expression in these cells after systemic *in vivo* administration of LVs. Intravenous injection of a LV encoding green fluorescent protein (GFP) resulted in transduction of lymphocytes, macrophages and all subsets of dendritic cells (DCs) in the spleen, detected 5 days later. In the case of macrophages and DCs, the percentage of transduced cells increased between 5 and 30 days after injection. The transduction of dividing precursors contributes to the persistence of the transgene-expressing DCs, as shown by BrdU incorporation. Expression of ovalbumin (OVA) resulted in a reduced number of transgene-expressing cells after 30 days. However, the remaining transduced cells stimulated proliferation and activation of OVA-specific $CD8^+$ T cells up to 3 months after LV administration, in spite of a reduction in the activation status of transduced DCs over time. Mice also maintained cytolytic activity against OVA-pulsed targets following a single immunisation. In conclusion, this thesis shows that LVs can transduce DCs and macrophages, leading to persistent antigen expression. These modified APCs are functional and capable of activating T cells. Therefore, LVs can be used as tools for persistent genetic modification of APCs, opening the opportunity for their use in long-term immunomodulation.

ACKNOWLEDGEMENTS

I am deeply grateful with Mary Collins for supervising this PhD and for being supportive since the first time I met her. Her constant optimism motivated me throughout the PhD and her excellent advice made this project possible.

I also acknowledge my gratitude to Benny Chain for his co-supervision, consistent interest in the project, his insights and critique.

It has been a privilege to be part of the Collins lab and I am very thankful with all the past and present members of the group. Thanks to Helen Rowe for the patience of training me at the beginning of the PhD. Special thanks to Luciene Lopes, David Escors and Karine Breckpot for being always keen to share their knowledge, time and friendship. Many thanks also to my fellow colleagues, Kasia, Sean and Doug (the latter also had the patience of proofreading this thesis), for their support and for the many scientific and non-scientific discussions we had along these years; to Ranbir, whose invaluable work made things always run smoothly in the lab, and to the rest of the members of the group: Sam, Sasha, Akira, who contributed to create a stimulating working environment. They will all remain my friends.

I am also grateful with Hans Stauss and Antony Antoniou for their advice in my MPhil/PhD transfer examination. I must also recognise the help provided by Anne Cachat, who contributed importantly to this project during her brief rotation as my student, and by all the staff of the Windeyer Institute, especially the members of the IT department, the administration and the Biological Services Unit.

It has been a honour to form part of UCL during these years, and even more to receive the financial support from the Graduate School throughout the program, which made this PhD possible.

Living in London has given me the opportunity to meet some wonderful people, and I would like to thank Jhen, Marie, Tobias, Anta, Paolo and Bal for their invaluable friendship. My deepest gratitude to Gaz for his constant and unconditional support, for proofreading this thesis and for being so patient all these years. I must of course thank my friends from afar—Gaby, José P., José M., Nayla, Adolfo and Cristina—that have been close to me at different moments in spite of the distance.

I am eternally grateful with my family, who have always being supportive to my projects with unconditional love and encouragement. They have always been my inspiration and to them I dedicate this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AAV	adeno-associated virus
APCs	antigen presenting cells
APOBEC3G	apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G
AVV	adenoviral vector
BM	bone marrow
BMDCs	bone marrow-derived dendritic cells
CCR	chemokine C-C motif receptor
cDNA	copy deoxyribonucleic acid
CLIP	class II-associated invariant chain peptide
CMV	cytomegalovirus
cPPT	central polypurine tract
CTL	cytotoxic T lymphocyte
DCs	dendritic cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
Dox	doxycycline
dsDNA	double-stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eGFP	emerald green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ELISPot	enzyme-linked immunospot assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
Flt3	FMS-like tyrosine kinase 3
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hank's buffered salt solution
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IFN	interferon
Ii	invariant chain
IiOVA	ovalbumin/invariant chain fusion
IL	interleukin
iv	intravenous
IRES	internal ribosomal entry site
KRAB	Krüppel-associated box
KSHV	Kaposi sarcoma virus
LB	Luria Bertani
LCMV	lymphocytic choriomeningitis virus

LN	lymph node
LTR	long terminal repeat
LV	lentiviral vector
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MLV	Moloney murine leukaemia virus
MoDCs	monocyte-derived dendritic cells
moi	multiplicity of infection
MVA	modified vaccinia virus Ankara
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK	natural killer cells
NOD	nucleotide oligomerisation domain
NYVAC	New York vaccinia virus attenuated from Copenhagen
mRNA	messenger ribonucleic acid
OVA	ovalbumin (chicken)
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
pre-DC	dendritic cell precursor
PRR	pattern recognition receptor
RANK	receptor activator of nuclear factor κB
RCL	replication competent lentiviral vector
RIG-I	retinoid acid-like inducible gene I
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
rTetR	reverse tetracycline repressor protein
RTmut	inative mutant of reverse transcriptase
rtTA	reverse tetracycline repressor
SD	standard deviation
SFFV	spleen focus-forming virus promoter
shRNA	short hairpin RNA
SIN	self-inactivating
SIV	simian immunodeficiency virus
TAA	tumour-associated antigen
TAP	transporter associated with antigen processing
TCR	T cell receptor
Tet	tetracycline
TetR	Tet repressor
TGF	transforming growth factor
$T_H1/2$	T helper 1 or 2
TLR	Toll-like receptor
TRE	tetracycline-responsive element
TRIF-1	Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β-1

T_{reg}	regulatory T cell
TNF	tumour necrosis factor
tTA	tetracycline-controlled transcriptional activator
UBI	human ubiquitin promoter
vFLIP	viral (Fas-associated death domain-like IL-1β-converting enzyme)-like inhibitory protein
VSV-G	vesicular stomatitis virus glycoprotein
VV	vaccinia virus
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element

CHAPTER 1

1. INTRODUCTION

Vaccines are valuable tools for the prevention and, potentially, for the treatment of infectious diseases and cancer. Despite its many successes—as highlighted by the eradication of smallpox—vaccination has not yet proved effective against a wide variety of conditions. Alternative immunisation strategies are needed especially for chronic infections or malignant diseases, where an inadequate immune response must be reactivated.

The ideal vaccines should assure optimal antigen delivery to professional antigen presenting cells (APCs) in the right activation context, in order to induce potent primary and memory immune responses with minimal side effects. This has been typically achieved with preparations of protein antigens together with adjuvants. The APCs play a pivotal role in this process by bridging the adaptive and innate immunity.

In recent years, genetic modification of APCs has transformed vaccine design and development. This approach is based on the delivery of antigen genes into APCs, which process and present them on major histocompatibility complexes (MHC) after their transcription and translation. Increasing knowledge in the biology of APCs and the improvement of gene therapy techniques have made of this a promising strategy. In the past few decades, most of the interest has concentrated on dendritic cells (DCs), one of the most potent APCs (Steinman & Banchereau 2007). Hence, many of the studies concerning genetic modification of APCs have focused on DCs, although other professional APCs also play an important role.

Gene delivery to DCs, both *in vitro* and *in vivo*, has been achieved with viral and non-viral vectors. Among the latter, recombinant lentiviruses (LVs) have been shown to generate a stable and high expression of the antigen gene. At the same time, they convey a series of advantages that make them good candidates for clinical use in

immunisation, as will be further discussed. This thesis will explore the immune consequences resulting from antigen-gene delivery using LVs. Due to the fact that LVs can integrate into the host's genome, this project will focus on the effects of stable antigen expression in APCs after systemic *in vivo* administration using a murine model.

1.1. Genetic modification of dendritic cells as a strategy for immunisation

Genetic modification of DCs is an attractive approach for immunisation since it results in superior antigen processing as compared with other vaccination strategies. One of its advantages is that the antigens are produced in their native conformation and then processed by the DC. This favours a multispecific immune response. In addition, the identification of specific immunogenic peptides is not completely necessary (Germain & Margulies 1993; Esslinger *et al.* 2002; Collins & Cerundolo 2004).

Genetic modification of DCs can be performed *in vitro* and *in vivo*. In the first method, DCs are generated *ex vivo* from blood or bone marrow precursors using specific cytokine combinations. Autologous DCs are then genetically modified and re-injected in the donor. This approach has been especially useful in understanding DC biology and function, and effective enough to justify its testing in the clinic. However, this process is costly and laborious since it requires specific vaccine preparation in each individual case. Direct *in vivo* administration of gene vectors bypasses this problem because the same vector preparation can be applied to different recipients. Nevertheless, this approach also implies a risk of 'vector spillage' and transduction of other cells besides the desired targets. To overcome this limitation, several strategies have been developed to engineer specific targeting to particular cell types (Frecha *et al.* 2008).

A variety of gene vectors have been used to transduce DCs (Breckpot *et al.* 2004). These can be grouped in two broad categories: viral and non-viral vectors.

1.1.1. Non-viral vectors

Nucleic acids encoding antigen genes can be delivered directly into DCs. Both DNA and mRNA have been used with this purpose, exhibiting a good safety profile and minimal risk of insertion in the host's genome. Besides, nucleic acids production is straightforward and can be scaled-up at low cost (Breckpot *et al.* 2004; Kutzler & Weiner 2008). However, difficulties in vector delivery, low transgene expression levels and limited success in clinical trials have inhibited their use.

1.1.1.1. DNA vaccines

DNA vaccines that encode specific antigen genes have been used with relative success in pre-clinical studies in generating immunity against multiple types of cancer (Rice *et al.* 2008) as well as infectious diseases, such as human immudeficiency virus (HIV) (Center *et al.* 2009), influenza (Szécsi *et al.* 2009), and malaria (Daubersies *et al.* 2008). The gene products are presented to T cells by APCs that have been transfected directly or have picked up exogenous antigen shed from other cell types, or phagocytosed it from dead cells. This generates both humoral and cellular immune responses (Kutzler & Weiner 2008).

An important advantage of DNA vaccines is that they also contain pathogen-associated molecular patterns that stimulate innate immunity through Toll-like receptors (TLRs). Particularly, engagement of TLR-9 by hypomethylated CpG motifs present in DNA has been involved in DC activation (Klinman 2006). TLR-9 is expressed both in conventional and plasmacytoid DCs in mice, but only in the latter in humans. However, *Tlr9*$^{-/-}$ mice can be effectively immunised with DNA vaccines, indicating that this is not the only immunogenic mechanism involved (Spies *et al.* 2003). DNA also activates DCs via its recognition by retinoic acid-like inducible gene-I (RIG-I) receptors and nucleotide-binding oligomerisation domain (NOD)-like receptors (Koyama *et al.* 2009).

A caveat of DNA vaccination is its difficult delivery to APCs. Transfection of *ex vivo*-generated DCs is inefficient, reaching not more than 12% of the cells even when using electroporation (van Tendeloo *et al.* 1998). To overcome this problem, particle-mediated delivery, gene gun and intramuscular injection with or without

electroporation have been applied *in vivo* with variable results (Fuller *et al.* 2006; Rice *et al.* 2008).

Multiple clinical trials with DNA vaccines against a variety of infectious agents and cancers have demonstrated their safety, but failed to show high levels of vaccine-specific immunity (Kutzler & Weiner 2008). Consequently, several strategies to improve their effectiveness are being developed. Examples include the using 'prime-boost' protocols in combination with other vectors (Peters *et al.* 2007), or co-expression of adjuvants (*e.g.*, fragment C of tetanus toxin) to activate DCs, or direct expression of co-activators (*e,g*, CD86) (Rice *et al.* 2008).

1.1.1.2. mRNA vaccines

An alternative vaccination strategy is the use of antigen gene-encoding mRNA. The principle of mRNA immunisation is similar to that of DNA, although expression is transient as these molecules are degraded by cellular ribonucleases. mRNA activates APCs through recognition by TLR-7 in mice and TLR-8 in humans (Heil *et al.* 2004; Diebold *et al.* 2004). It can be delivered directly *in vivo* either as 'naked' molecules, in liposomes, in particles with the gene gun and in the form of virus-derived replicative RNA (Pascolo 2004).

Another option is *ex vivo* transfection of DCs. In contrast to DNA, incorporation of mRNA into *ex vivo*-generated DCs is more efficient; up to 81% and 62% of human and murine DCs, respectively, can be transfected (Dullaers *et al.* 2004). Passive diffusion, lipofection, electroporation and transferrin receptor (CD71)-mediated endocytosis have been effective in delivering total mRNA from tumoral cells or molecules encoding specific antigens (Breckpot *et al.* 2004).

Using this technique, both humoral and cellular responses have been generated against tumour-associated antigens (TAAs), as well as antigens from infectious agents, including HIV, human papilloma virus and influenza, among others. Results have been promising so far and several trials have shown partial clinical response (Pascolo 2004; Grünebach *et al.* 2005)).

Co-transfection with mRNA encoding co-stimulatory molecules is being tested to enhance these vaccines. For example, Bonehill *et al.* (2009) have reported a

better immune response by DC co-transfection with mRNA cassettes encoding CD40, CD70 and a constitutively active form of Toll like receptor 4 (TLR4), together with melanoma-associated antigens. However, one of the major concerns of mRNA-based vaccines is that the antigen will only be expressed transiently. Although this characteristic favours its safety, it can also impair its efficacy.

1.1.2. Viral vectors for vaccination

More than half of the vectors used in clinical gene therapy trials today are recombinant viruses (Clinical Trials Worldwide, 2009, in www.wiley.co.uk/genetherapy/clinical/). Different viral vectors have been used to transduce DCs *in vitro* and *in vivo*, resulting in effective immunisation against infectious diseases and cancer. It has also been shown that viral transduction is more effective for the induction of tumour-reactive $CD8^+$ and $CD4^+$ T cells than DC transfection with DNA or mRNA (Lotem *et al.* 2006).

The most commonly viral vectors employed for gene delivery to DCs are adenoviruses, adeno-associated viruses, poxviruses, herpes viruses, oncoretroviruses and lentiviruses. Their main characteristics are summarised in Table 1.1

Table 1.1. Key features of viral vectors used for transduction of DCs.

Parental virus	Genome	Envelope	Packaging capacity	Main advantages	Main disadvantages	Immunisation clinical trials
Adenovirus	dsDNA	No	30 kb	-High titers -Episomal -High transduction efficiency of APCs	-Anti-vector immunity	Yes
Poxvirus	dsDNA	Yes	30 kb	-High titers -Cytoplasmic translation -Moderate transduction efficiency of APCs	-Anti-vector immunity -Safety concerns in immune-compromised hosts	Yes
Adeno-associated virus	ssDNA	No	~5 kb	-Non-pathogenic parental virus -Episomal	-Limited packaging capacity -Anti-vector immunity	No[a]
Herpes virus	dsDNA	Yes	40 kb 150 kb in amplicons	-High titers -High packaging capacity	-Anti-vector immunity (?)	No
Oncoretrovirus	ssRNA+	Yes	8-10 kb	-Sustained gene expression	-Risk of insertional mutagenesis -Cell-cycle dependent	No[a]
Lentivirus	ssRNA+	Yes	8-10 kb	-Sustained gene expression -No anti-vector immunity	-Risk of insertional mutagenesis	No[a]

[a]Used in clinical trials for gene replacement therapy.

1.1.2.1. Adenoviral vectors

Adenoviridae is a family of non-enveloped viruses that have a ~30-35 kb double-stranded linear DNA genome contained within an icosahedral capsid. They infect various species of vertebrates including humans, where 54 serotypes, grouped in 7 species, have been identified (Lasaro & Ertl 2009).

Their genome contains early (E) and late (L) genes. First generation adenoviral vectors (AVVs) are produced by substituting regions E1 and/or E3 by an expression cassette. E1 gene products have regulatory functions and are indispensable for viral replication. Therefore, they have to be provided in *trans* for vector production. E3 gene products subvert the host's immune responses and are dispensable for viral replication (Danthinne & Imperiale 2000). Vectors with additional deletions in early genes have been engineered to reduce the immune response to the vector, prolong antigen expression and improve safety. This is the case of second generation AVVs, which have further deletions in E2 and/or E4, and 'fully gutted' (or 'gutless') vectors, where all the viral coding sequences have been removed (Ehrhardt & Kay 2005).

AVVs efficiently transduce DCs and stimulate the innate immune system through several mechanisms. Most of the immunisation studies have been carried out with E1- or E1/E3-deleted vectors derived from serotypes 2 or 5. In pre-clinical models, these vectors have been shown to induce potent transgene-specific T ($C8^+$ and $CD4^+$) and B cell responses (Lasaro & Ertl 2009). However, their main limitation is the high immunogenicity of the viral particle. This hampers the magnitude of the desired immune response to the transgene by reducing its expression and precludes the use of repeated immunisations with the same vector (Sakurai *et al.* 2008). Life-threatening systemic inflammatory reactions have also been reported in some patients (Thacker *et al.* 2009).

In spite of this, AVV vaccines have been developed for the treatment of different diseases and tested in several clinical trials, although with disappointing results. For example, in the phase IIb STEP clinical trial, immunisation with an AVV-5 encoding several antigens of HIV-1 did not decrease the rates of infection or

post-infection viral load. Furthermore, a trend towards increased HIV-1 acquisition was observed in vaccine recipients, particularly in individuals with pre-existing anti-AVV-5 neutralising antibodies. The underlying mechanisms to explain this apparent increased susceptibility remain unknown (Buchbinder *et al.* 2008).

Several modifications in vector design are being developed to improve AVV safety and efficiency as vaccine vectors, especially to overcome the anti-vector immunity. These include the use of rare human or chimpanzee serotypes and co-expression of antigens and immunomodulators. Structural modifications of the viral particle have also been employed to target the vector to specific cells and to eliminate the capsid epitopes recognised by neutralising antibodies (Lasaro & Ertl 2009; Thacker *et al.* 2009).

1.1.2.2. Poxvirus-derived vectors

Members of the *Poxviridae* family have large double-stranded DNA genomes that encode several hundred proteins, many of which are not essential for viral replication and can be deleted or replaced by large inserts of foreign DNA (up to ~30 kb). Their replication occurs in the cytoplasm using the translation machinery of the infected cell, without integration in the host's genome. Poxvirus-derived vectors are stable, easy to manufacture and administer and have a broad tropism (Gómez *et al.* 2008).

Two attenuated strains derived from vaccinia virus have been safely used as gene vectors. Modified vaccinia virus Ankara (MVA) was derived from a smallpox vaccine strain after serial passage in primary chicken embryo fibroblasts. New York vaccinia virus attenuated from Copenhagen (NYVAC) was also derived from a smallpox vaccine strain in which 18 open reading frames were deleted from the virus genome. In both cases, the viruses have lost their replication capacity and part of their broad tropism in mammalian cells, while keeping their ability to drive high-level gene expression. An expression cassette can be inserted by recombination in sites of naturally occurring deletions or in other loci, such as those encoding thymidine kinase or haemagglutinin (Drexler *et al.* 2004).

Poxvirus vectors can efficiently transduce DCs and elicit humoral and cellular immune responses. They mimic viral infections and therefore stimulate the innate immune system. The loss of immunomodulatory genes from the parental virus accounts for their strong immunogenicity (Gómez *et al.* 2008). Both MVA and NYVAC have been shown to induce protective immunity. However, while the first tends to induce both $CD8^+$ and $CD4^+$ T cell responses, the latter predominantly induces a $CD4^+$ T cell response (Mooij *et al.* 2008).

An important drawback in their use is that the expressed viral proteins compete with the transgene-derived epitopes. This hampers transgene-specific cytotoxic T lymphocyte (CTL) responses, especially after repeated immunisations, and can explain the failure to generate comparable T cell responses to those observed in natural viral infections (Smith *et al.* 2005). To avoid this problem, combined prime/boost protocols using MVA and DNA vaccines or other recombinant viruses have been employed.

MVA and NYVAC have been studied in multiple pre-clinical assays as vaccines against infectious agents and cancer, showing different degrees of protection in most models. Phase I and II clinical trials with MVA-based vectors delivering antigens against infectious agents (*e.g.*, HIV-1, malaria, tuberculosis) or TAAs have demonstrated the safety of these vectors, but the clinical outcomes have been variable (Gómez *et al.* 2008). Phase III trials are currently open (Gene Therapy Clinical Trials Worldwide, 2009, in www.wiley.co.uk/genetherapy/clinical/).

Other poxviruses, such as recombinant avipox (fowlpox and canarypox), have also been tested for immunisation. Fowlpox-derived vectors have been used in clinical trials as a booster following immunisation with vaccinia virus in the treatment of advanced cancer, showing a good safety profile and generation of specific CTLs (Gulley *et al.* 2008; Lechleider *et al.* 2008). Canarypox vectors encoding tumoral antigens (Spaner *et al.* 2006) or HIV epitopes (Russell *et al.* 2007) have shown poor immunologic responses. Expression of co-stimulatory molecules together with the antigen is being assayed to overcome this limitation (Liu *et al.* 2008).

1.1.2.3. Adeno-associated viral vectors

Recombinant adeno-associated virus (AAV), a nonenveloped parvovirus, has also been extensively used as a gene vector. This virus requires the functions of a helper virus (usually adenovirus or herpesvirus) for replicative infection. In its absence, AAV replication is limited and it can establish latency by specific integration into the chromosomal locus 19q13.4 (Schultz & Chamberlain 2008). Its main advantage is that the parental virus is non-pathogenic in humans.

AAV has a single-stranded DNA genome with two coding regions flanked by inverted terminal repeats (ITRs), which have a series of regulatory functions. In AAV-derived vectors, the coding regions are replaced by foreign genes of up to ~5 kb. Despite its limited packaging capacity, strategies such as *trans*-splicing and self-complementary AAVs have been developed to express larger proteins (McCarty 2008). In the actual constructs, the regions necessary for genome integration have been removed, so the vector persists in the nucleus as extrachromosomal elements (Daya & Berns 2008).

Several AAV serotypes have been used as vectors, although most of the studies have been based on AAV-1 and AAV-2. These vectors can transduce DCs with variable efficiencies and induce specific CTL responses (Mahadevan *et al.* 2007; Veron *et al.* 2007; Yu *et al.* 2008). AAV-based vectors have been used in gene therapy clinical trials. One of the observed limitations is the prevalence of neutralising antibodies against the vector in some human populations (Daya & Berns 2008).

1.1.2.4. Herpes virus

Herpes simplex virus (HSV) is an enveloped virus with a linear double-stranded DNA genome that encodes more than 80 viral proteins. The wild-type virus can produce a lytic infection or persist in a latent stage, whereby the viral genome persists as an epichromosomal form with minimal transcriptional activity in the nucleus of the host cell. In replication-defective vectors, mutation or deletion of essential genes renders the virus unable to grow except in transformed cell lines, where these elements are provided in *trans*. These deleted genes can be replaced by

foreign DNA of up to ~40 kb. In amplicon vectors, the HSV-1 particles carry a concatemeric form of a plasmid DNA instead of the viral genome, with no viral coding sequences and multiple copies of the same or different transgenes (Marconi *et al.* 2008).

HSV vectors efficiently transduce DCs (>90%), induce their partial maturation without affecting their viability and elicit strong CTL responses (Chiu *et al.* 2009). For example, replication-defective HSV-1 encoding simian immunodeficiency virus (SIV) antigens resulted in partial protection against a challenge with SIV in macaques (Murphy *et al.* 2000). However, their clinical application for antigen gene delivery has not yet been evaluated.

A potential drawback of HSV-based vectors is the presence of pre-existing immunity against the parental virus, although this issue is controversial. While a study has shown that the vectors can induce immune responses even in the presence of pre-existing host immunity (Brockman & Knipe 2002), another has demonstrated that they could be hampered depending on the route of inoculation (Lauterbach *et al.* 2005).

1.1.2.5. Retrovirus

Retroviruses belong to the family *Retroviridae,* a group of RNA viruses that replicate through a dsDNA intermediate that integrates into the host genome. Once integrated, the viral RNA can be transcribed during the life of the cell resulting in long-term gene expression. An advantage of retroviral vectors is the absence of expression of most viral proteins, which reduces anti-vector immune responses.

Because of historical and practical reasons, the most commonly used retroviral vectors derive from Moloney murine leukaemia virus (MLV). These vectors are difficult to produce in titers comparable to other vectors and only transduce dividing cells because they require disruption of the nuclear membrane to integrate into the host's DNA (Roe *et al.* 1993). This would potentially limit the transduction of DCs, which are generally regarded as terminally-differentiated, non-dividing cells. However, transduction of monocyte-derived DCs using MLV vectors has been reported, with an efficiency of 30-60% (Aicher *et al.* 1997). Another

approach relies on the transduction of dividing $CD34^+$ haemopoietic precursors, which can then be differentiated into DCs. This strategy has resulted in transduction of 40-70% of $CD34^+$-derived DCs (Heemskerk *et al.* 1999; Movassagh *et al.* 1999).

The cell-cycle dependence of retroviral vectors has been circumvented with the use of lentiviral vectors (LVs), derived from other members of the *Retroviridae* family. LVs have the key ability to transduce non-dividing cells, resulting in efficient gene delivery to DCs. This has led to promising results in the fields of gene therapy and genetic immunisation.

1.2. Lentivirus

Lentiviruses are complex retroviruses of the genus *Lentiviridae* that cause several slow-onset diseases. They have been extensively studied because of the impact that HIV, a member of this genus, has had in public health in the past decades.

LVs have been engineered to become efficient and safe gene transfer vehicles. As compared with other gene delivery technologies, they convey several advantages that have overcome some of the limitations of other vectors: (1) they can transduce non-dividing cells, (2) the elimination of all dispensable viral proteins from the vector genome minimises their cellular toxicity and immunogenicity, (3) the exposure of the host to the parental viruses is low, reducing the possibility of pre-existing anti-vector immunity and (4) LVs can be pseudotyped with different viral envelopes, providing more stability to the viral particle, the possibility of targeting specific cells and the potential to repeat immunisations using different envelopes (Kim *et al.* 2005; Loisel-Meyer *et al.* 2009).

LVs have been derived from equine infectious anaemia virus (Olsen 1998), SIV (Stitz *et al.* 2001) and feline immunodeficiency virus (Sakurai *et al.* 2008). However, most studies regarding the use of LVs for immunisation have been performed with vectors derived from HIV-1, which normally infects DCs.

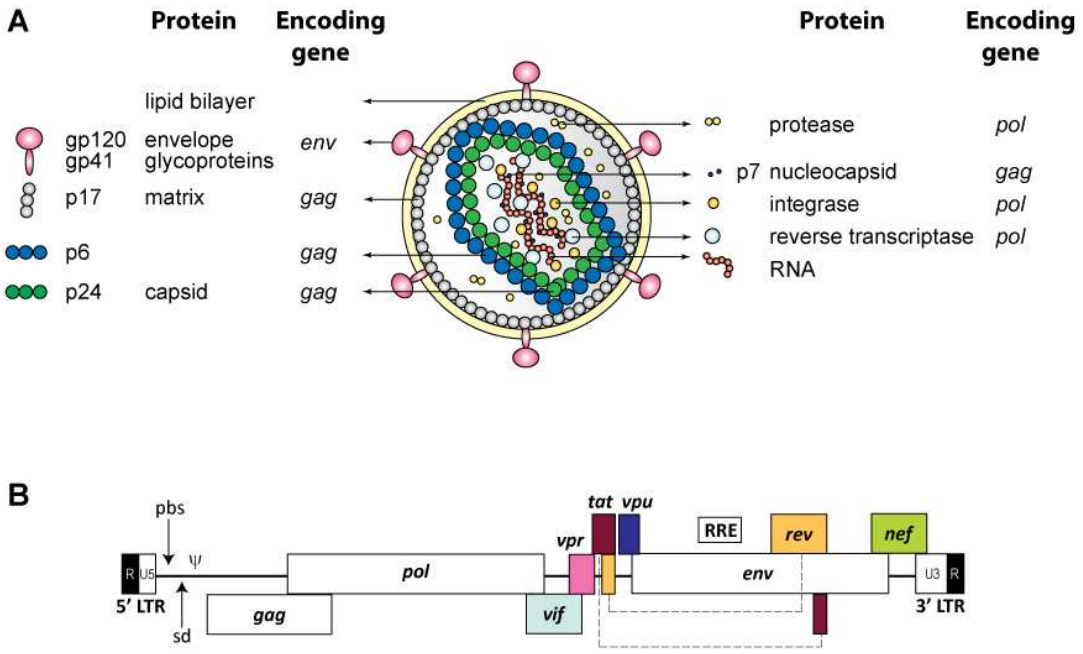


Figure 1.1. Representation of HIV-1 and its genome organisation. HIV-1 particles have an envelope that surrounds a truncated cone-shaped capsid, where the viral RNA is contained. These components are associated with other structural proteins, viral enzymes and cellular factors, some of which are shown (A). Each RNA strand (B) is flanked by long terminal repeats (LTR) that have important regulatory functions. *Gag-pol* encodes structural proteins and viral enzymes. Envelope glycoproteins are encoded by *env*. There are six additional genes have with regulatory functions: *tat*, *rev*, *nef*, *vpu*, *vpr* and *vif*. *Cis*- elements are also represented: primer binding site (pbs), major splice donor site (sd), packaging signal (ψ) and Rev-responsive element (RRE).

1.2.1. HIV-1 structure and life cycle

HIV-1 virions are enveloped spherical particles of 100-120 nm in diameter. The viral envelope is composed of a lipid bilayer, viral envelope glycoproteins and some cellular proteins (Fig. 1.1A). The envelope surrounds a truncated cone-shaped capsid (termed core) made of capsid protein that encloses two genomic RNA molecules complexed with nucleocapsid proteins, viral proteins (protease, reverse transcriptase, integrase) and some cellular factors. The core is further enclosed by a layer of matrix protein present under the viral envelope (Hughes 1997).

1.2.1.1. Genomic organisation

The HIV-1 genome consists of two identical 9.2 kb single-stranded positive RNA molecules. Its organisation is shown in Figure 1.1B. Four main regions can be distinguished:

(1) The long terminal repeats (LTRs) are important regulatory regions that flank the 5' and 3' ends of the genomic RNA. After reverse transcription, each LTR consists of 3 segments: unique 3 (U3), repeat (R) and U5. The U3 region contains binding sites for cellular transcription factors. The R segment includes the transactivation response element (TAR), implicated in Tat-mediated transactivation (Berkhout & Jeang 1992; Richter *et al.* 2002).

(2) The *Gag-pol* gene encodes two polyprotein precursors. Gag polyprotein (p55) is proteolytically processed into 3 viral structural proteins: matrix (p17), capsid (p24) and nucleocapsid (p7), and the polypeptide p6. A -1 ribosomal frameshift leads to translation of Gag-pol that additionally encodes the viral enzymes necessary for replication: protease, reverse transcriptase (RT)/RNase H and integrase (Sierra *et al.* 2005).

(3) The *env* gene encodes the gp160 polypeptide precursor, which consists of transmembrane gp41 and surface gp120 subunits. Together with a lipid bilayer derived from the cell membrane, these glycoproteins form the viral envelope that

mediates virus entry to the cell by interaction with receptors and co-receptors (Sierra *et al.* 2005).

(4) Six additional genes are present in the HIV-1 genome: the regulatory genes *Tat* and *Rev*, and the accessory genes *Nef*, *Vpu*, *Vpr* and *Vif*, which have different functions that will be mentioned later.

1.2.1.2. Life cycle

The HIV life cycle can be divided into two phases. The early phase begins with the attachment of the virus to the cell and finishes with the integration of the viral genome into the DNA of the host cell. The late phase starts with the transcription of viral genes and continues until new virions are released. The main steps of each phase are shown in figure 1.2A.

1.2.1.2.1. Cell entry

HIV-1 cell entry is a complex multi-step process that starts with proteoglycan-dependent adsorption of the virus to the cell. In DCs, adsorption is also mediated by cell surface lectins, such as DC-SIGN. Following this initial binding step, HIV-1 envelope glycoproteins (gp120 and gp41) recognise their primary receptor, CD4. This restricts the tropism of the virus to $CD4^+$ T cells, monocytes, macrophages and DCs. Binding of gp120 and CD4 triggers conformational changes in these molecules and recruitment of co-receptors, CXCR4 and CCR5 (Feng *et al.* 1996). This results in further conformational changes leading to gp41-mediated fusion of the viral envelope with the cell membrane.

1.2.1.2.2. Uncoating and reverse transcription

After membrane fusion, the viral core is released into the cytoplasm and disassembled. The viral RNA, associated with several viral and cellular proteins, is released in the form a reverse transcription complex, in which the viral RNA is reverse transcribed to double-stranded DNA by the virion-packaged reverse transcriptase. The trigger for reverse transcription is not completely understood, but

the high concentration of cytoplasmic deoxyribonucleotides seems to play a significant role (Goff 2001). The reverse transcriptase presents additional RNAse H activity, which degrades the viral template RNA as it is reverse transcribed.

It is worth noting that the host cells present several anti-retroviral mechanisms, such as the antiviral APOBEC3G protein. This interferes with reverse transcription by inducing deamination of cytidine bases in the minus DNA strand. In the case of HIV-1, the accessory protein Vif, which is incorporated within the viral core, counteracts this effect by reducing APOBEC3G incorporation into the virions and inducing its ubiquitination and subsequent degradation (Sheehy *et al.* 2003).

1.2.1.2.3. Nuclear import and integration

The completion of reverse transcription results in the formation of a pre-integration complex, which is actively transported from the cytoplasm to the nucleus through the nuclear pore complexes. Consequently, in contrast with other retroviruses, HIV-1 does not require disruption of the nuclear membrane during mitosis to reach the host cell DNA. Although the mechanism of nuclear import is still not clear, it has been shown that four different components of the pre-integration complex participate in the transport through the nuclear pore: the integrase, the matrix protein, Vpr and the viral DNA. The first three contain nuclear localisation signals and interact with components of the nuclear pore complex. Vpr in addition has been proposed to mediate an unconventional mode of nuclear entry by disruption of the nuclear envelope. However, studies so far have been controversial (Suzuki & Craigie 2007; Nisole & Saïb 2004).

A triple-stranded DNA flap also seems to participate as a nuclear import signal by an undefined mechanism. This structure originates during reverse transcription, when DNA synthesis is initiated concomitantly from a central polypurine tract (cPPT) and a 3' PPT, resulting in a 99 nucleotide-long strand overlap. In fact, the presence of cPPT in HIV-based vectors significantly enhances nuclear entry in quiescent cells (Firat *et al.* 2002).

Once in the nucleus, integration into the host DNA is catalysed by the integrase in a two-step process. The first stage is known as 3' processing and takes

place in the cytoplasm. Here the integrase cleaves a dinucleotide adjacent to the attachment (att) sites at the ends of the viral DNA, which exposes recessed 3'OH groups. In the second step, called strand transfer, the integrase catalyses a nucleophilic attack to phosphodiester bonds in the target DNA and ligates the viral DNA. Finally, host DNA repair enzymes remove the two nucleotide overhangs and repair the gaps in the DNA (Engelman *et al.* 1991). The HIV-1 genome preferentially integrates in genes highly transcribed by RNA polymerase II, as studied by Schröder *et al.* (2002).

1.2.1.2.4. Retroviral gene expression

HIV-1 early gene expression starts at the 5'LTR using the cellular transcriptional machinery. The LTR U3 region contains binding sites for several transcription factors (*e.g.*, NFκB) which transactivate the viral promoter, resulting in basal transcription of the viral genome. Viral transcription begins from nuclear pre-integration viral forms (linear, 1-LTR and 2-LTR circles) and continues after incorporation of the provirus to the host's genome (Wu 2004). However, elongation of the nascent mRNA is inefficient at this stage. During early gene expression, Tat is accumulated and then binds to the transactivation response element (TAR) in the R region of the LTR and to other cellular transcriptional co-activators. Thus, Tat recruits transcription elongation factors to the HIV-1 promoter and also interacts with mRNA capping proteins, all of which stabilises nascent RNA transcripts (Harrich *et al.* 1997).

1.2.1.2.5. Splicing and nuclear export

Transcripts from HIV-1 undergo multiple alternative splicing events. In the early phases, only multiply-spliced mRNAs are produced, resulting in expression of Tat, Nef and Rev. Nuclear export of full-length viral genomic RNA is, however, required for the expression of Gag-pol proteins and for its packaging into new virions. This is achieved when there is enough expression of Rev, which is critically important for the nuclear export of full-length and incompletely-spliced HIV-1 transcripts. Rev recognises the Rev-responsive element (RRE), an RNA element

present in the *env* coding region that folds into a complex secondary structure similar to TAR. Rev interacts with cellular transport proteins and mediates nuclear export of RRE-containing singly-spliced and unspliced transcripts (Pollard & Malim 1998).

1.2.1.2.6. Late events of the life cycle

Viral structural components are translated from the viral mRNA in the form of three polyproteins: Env, Gag-pol and Gag. These proteins translocate to the cell membrane after post-traslational modifications and start to assemble, directed by Gag polyprotein. The nucleocapsid portion of Gag contains a RNA-binding domain that recognises the packaging signal and ensures specific incorporation of genomic RNA into the virions. In addition, all required viral enzymes, cellular tRNAlys3 primer, and certain cellular proteins are also incorporated to the immature core (Göttlinger 2001).

Depletion of CD4 from the cell membrane is necessary for viral assembly in order to avoid its interaction with newly-synthesised gp120. This is mediated in part by Nef, which accelerates endocytosis and degradation of CD4 and MHC (Malim & Emerman 2008). Env also contributes in this process by trapping CD4 bound to gp160 in the endoplasmic reticulum, where Vpu induces its degradation (Schubert *et al.* 1996). Nef has also been involved in depletion of MHC-I from the cell surface. It also interacts with several transduction pathways, all of which contributes to HIV-1 virulence (Quaranta *et al.* 2009).

Budding and release of newly formed virions is also mediated by Gag. For this process, the virus uses a cellular pathway involved in the sorting of ubiquitinated receptors in transit to lysosomal degradation called endosomal sorting complex required for transport (ESCRT). The p6 segment of Gag recruits proteins from the ESCRT-III with intrinsic membrane remodelling properties necessary for budding from the cell (Martin-Serrano 2007). Release of the viral particle can also be inhibited by cellular proteins called tetherins; it has been recently demonstrated that Vpu counteracts the effects of tetherins, favouring release of the virions (Neil *et al.* 2008).

Budding virions from the cell are immature and non-infectious. The HIV viral protease cleaves Gag and Gag-pol polyproteins, inducing morphologic changes that result in mature, infectious virions (Sierra *et al.* 2005).

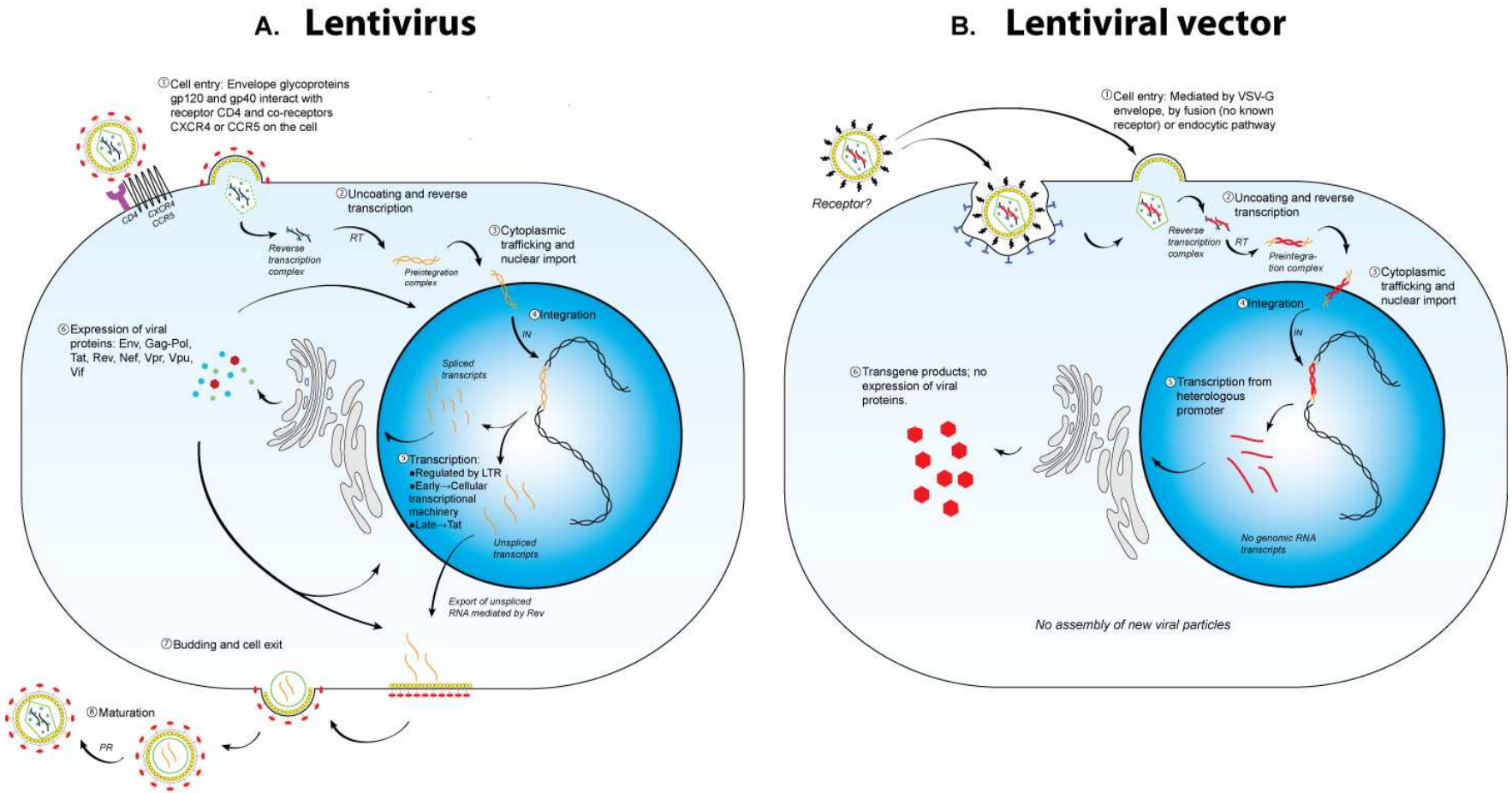


Figure 1.2. Comparison of the cellular cycle of wild-type HIV-1 and a lentiviral vector.

1.3. Lentivirus attenuation and vector design

For the development of HIV-1-based LVs, the HIV genome has been modified to abrogate its pathogenic and replication capacity while retaining its ability to transfer and integrate in the host genome.

Firstly, the HIV-1 *trans*-acting elements (the sequences that encode for enzymatic, structural, accessory and envelope proteins) are segregated from *cis* elements (the non-coding sequences required for vector RNA synthesis, reverse transcription, integration and packaging) (Naldini *et al.* 1996). These are provided in separate expression plasmids that are transiently co-transfected in a packaging cell line where the viral particle is assembled. In this way, the possibility of generating replication competent lentivectors (RCLs) is minimised, since at least two recombination events between the separated viral elements would be required.

Secondly, dispensable viral genes are removed, especially those contributing to HIV-1 pathogenesis. Elimination of Vpr, Vif, Vpu, Nef and Tat leads to fully-efficient transducing particles, while reinforcing the safety of LVs (Dull *et al.* 1998; Zufferey *et al.* 1997).

Thirdly, many of the HIV transcriptional elements in the LTR are eliminated to generate self-inactivating (SIN) vectors. This is attained by a deletion of part of the U3 region in the 3' LTR, which serves as a template and is transferred to the 5' LTR of the proviral DNA during reverse transcription. The use of SIN vectors reduces even more the possibility of RCL generation, hampers the likelihood of recombination with wild type HIV-1 in the host and avoids transcriptional interference with promoters controlling transgene expression in the transfer vector (Zufferey *et al.* 1998).

Based on these approaches, the current production of LVs is carried out by co-transfection of packaging cells with three (or four, in the latest generation of LVs) different expression systems: envelope, packaging and transfer vectors (Fig. 1.3).

1.3.1. Packaging vector

This vector encodes the enzymatic, structural and accessory proteins necessary to assemble the viral particle. Its components have been modified in the past decade to improve its biosafety (Fig. 1.3A-C).

'First generation' packaging constructs consist of the human cytomegalovirus (CMV) immediate early promoter, driving the expression of all the viral proteins required in *trans*, except the viral envelope and Vpu (Fig. 1.3A). The packaging signal (ψ) and adjacent sequences have been eliminated in the 5' end and the 3' LTR was substituted by a polyadenilation signal (Naldini *et al.* 1996). Deletion of all the accessory genes and preservation of *tat* and *rev* led to a safer 'second generation' packaging vector (Fig. 1.3B) (Zufferey *et al.* 1997). Further modifications included deletion of *tat* and separation of *gag/pol* and *rev* in two different cassettes (Fig. 1.3C) (Dull *et al.* 1998). For the production of this 'third generation' vectors, a fourth plasmid (encoding *rev*) is required. This curtails even more the risk of RCL generation, which would then require three recombination events. Furthermore, using a vector containing a synthetic *gag-pol* with a codon-optimised sequence, gene expression can be achieved independently of Rev (Wagner *et al.* 2000).

1.3.2. Envelope vector

Lentiviruses are enveloped viruses that can be pseudotyped with a variety of glycoproteins derived from other enveloped viruses encoded in *trans* in a separate vector. Pseudotyping allows the explotation of the natural tropism of the envelope and restricts transduction to specific cells or tissues. It also prevents the effects of neutralising antibodies against viral envelopes present in the host and facilitates vector production and purification (Cockrell & Kafri 2007; Cronin *et al.* 2005).

Vesicular stomatitis virus glycoprotein (VSV-G) is the most commonly employed envelope protein to pseudotype LVs (Fig. 1.3D). VSV-G enables transduction of a wide range of cell types. The mechanism of cell entry is not completely clear. It involves fusion to the cell membrane, although no receptor has been identified. Clathrin-mediated endocytosis has also been recently described

(Cureton *et al.* 2009). In addition, VSV-G stabilises the viral particle and resists concentration by ultracentrifugation without shedding. However, complement and antibody-mediated immune responses against VSV-G can counteract transduction by the pseudotyped particles (DePolo *et al.* 2000).

There is an ever-growing list of glycoproteins that have been successfully used for pseudotyping of lentivectors. Examples are those from *Retroviridae, Rhabdoviridae, Arenaviridae, Flaviviridae, Paramyxoviridae, Baculoviridae, and Filoviridae* families (Bouard *et al.* 2009). Although each of these glycoproteins preferentially interacts with specific cell types, finding a natural envelope for DC-specific targeting has been unsuccessful. Therefore, envelopes have been engineered in several ways to re-direct their tropism towards specific cell types.

One way of doing this is by genetically modifying the envelope glycoproteins with ligands that bind to receptors present in the target cells. However, ligand-fused glycoproteins often result in poor infectivity due to the inability of the retargeted envelope to induce membrane fusion and to the sequestration of the viral particles by cell surface molecules (Frecha *et al.* 2008).

Another alternative is the modification of existing envelopes to re-direct receptor attachment without affecting membrane fusion. Yang *et al.* (2008) showed that by introducing a mutation to the envelope of Sindbis virus, its affinity to an ubiquitous receptor (heparan sulphate) was ablated while preserving its capacity of binding to DC-SIGN, a lectin-type receptor present on some DC subsets. LVs encoding ovalbumin (OVA) pseudotyped with this modified envelope specifically transduced DCs *in vivo* and induced immune responses against OVA-expressing tumors.

Conjugation of the viral envelope with single-chain antibodies (scFv) specific for surface proteins present on the target cells has also been used for this purpose. For example, specific targeting of DCs has been achieved with antibodies directed against C-type lectins, such as DEC-205 (Bonifaz *et al.* 2002) and DC-SIGN (Dzionek *et al.* 2001). Display of scFv in the context of measles virus envelope glycoproteins seems to be a promising approach since measles virus enters cells through direct fusion with cell membranes. In this system, the natural tropism of measles virus is modified by introducing a mutation in the contact residues of

haemagluttinin and fusing a scFv to its ectodomain (Nakamura *et al.* 2005). The resulting virus infects cells that express the cognate cell-surface antigen. Funke *et al.* (2008) demonstrated target versus non-target cell discrimination *in vitro* by using LVs pseudotyped with re-targeted measles virus envelope and different scFv.

1.3.3. Expression vector

This component consists of a full-length vector RNA molecule that contains the *cis* acting elements required for efficient packaging, reverse transcription, nuclear import and integration, as well as the expression cassette of interest (internal promoter and transgene sequence). Improvement of transgene expression and transduction efficiency has been attained by the incorporation of two *cis* elements: the cPPT and the woodchuck hepatitis virus post-transcriptional element (WPRE) (Fig. 1.3E).

The function of cPPT was described before. When included in the LVs, it enhances transduction efficiency by 2-10 fold in dividing and non dividing cells (Zennou *et al.* 2001; Firat *et al.* 2002). WPRE also increases expression of the transgene (Zufferey *et al.* 1999). It has been proposed that WPRE could incite oncogenic activity because it contains enhancer/promoter elements and encodes part of the woodchuck hepatitis virus X protein, associated with hepatic oncogenesis (Kingsman *et al.* 2005). However, recent data on insertional mutagenesis do not support this and the risk has been further reduced by abrogating the X protein open reading frame of WPRE (Modlich *et al.* 2009; Zanta-Boussif *et al.* 2009).

The gene of interest driven by a heterologous promoter is also included in the expression vector. Tissue-specific promoters have been used to target gene expression to specific cell types. For example, LVs with B7-DC and CCL17 promoters result in transgene expression in murine bone marrow-derived DCs (BMDCs) but not in macrophages (Gorski *et al.* 2003). Gene expression has also been specifically attained in some DC subpopulations using the dectin-2 promoter. A LV encoding an antigen gene driven by the dectin-2 promoter resulted in an immune response comparable to that obtained with LVs containing a strong constitutive promoter (Lopes *et al.* 2008).

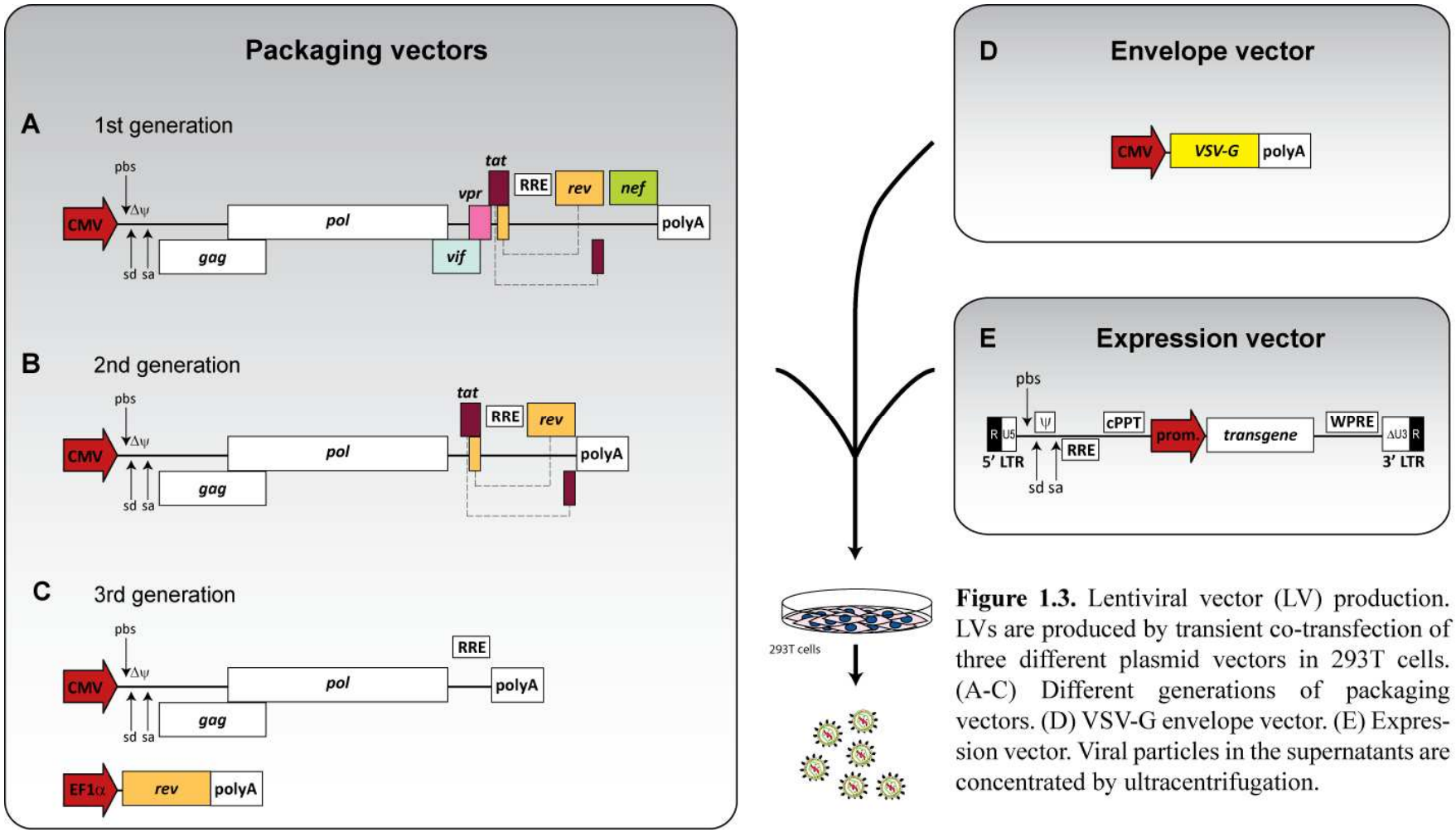


Figure 1.3. Lentiviral vector (LV) production. LVs are produced by transient co-transfection of three different plasmid vectors in 293T cells. (A-C) Different generations of packaging vectors. (D) VSV-G envelope vector. (E) Expression vector. Viral particles in the supernatants are concentrated by ultracentrifugation.

1.4. Lentiviral vectors for immunotherapy

The first evidence for the potential use of LVs in immunotherapy came from the observation that they transduce DCs. Murine BMDCs as well as human DCs derived from monocytes or $CD34^+$ haemopoietic precursors can be transduced *in vitro* with VSV-G-pseudotyped LVs with efficiencies ranging from 35 to 90% (Chinnasamy *et al.* 2000; Cui *et al.* 2002; Esslinger *et al.* 2002; Oki *et al.* 2001; Rouas *et al.* 2002; Schroers *et al.* 2000; Unutmaz *et al.* 1999; VandenDriessche *et al.* 2002). LV-transduced DCs show no obvious signs of cellular toxicity and keep their ability to present allogeneic antigens, respond to maturation stimuli such as lipopolysaccharide (LPS) or CpG, and secrete stimulatory cytokines (*e.g.*, interleukin-12) (He *et al.* 2005).

The second relevant aspect is that the transgene product is also processed and presented in the context of the major histocompatibility complex (MHC). This has been demonstrated with model antigens such ovalbumin (OVA), TAAs (Melan-A, tyrosinase related protein, NY-ESO) and antigens from various infectious agents, including flu or lymphocytic choriomeningitis virus (LCMV) (Dyall *et al.* 2001; Lopes *et al.* 2006; Metharom *et al.* 2001; Palmowski *et al.* 2004; Zarei *et al.* 2004). DCs transduced with LVs encoding these antigens induced proliferation and activation of both class I or class II-restricted T cell lines or transgenic lymphocytes bearing the cognate T cell receptor.

The results of *in vitro* experiments are reflected *in vivo* following injection of LV-transduced DCs in mice. Esslinger *et al.* (2002) observed proliferation and activation of antigen-specific $CD8^+$ T cells in mice after injecting DCs transduced with a LV encoding HLA-Cw3, a model antigen. The response was better than that observed using an adenoviral vector. In a similar model, He *et al.* (He *et al.* 2005), showed that the *in vivo* lytic T cell response against OVA was stronger and lasted longer with LV-transduced DCs as compared with peptide-pulsed cells. Indeed, immunisation with LV-transduced DCs protected mice from a challenge with OVA-expressing tumoral cells and inhibited growth of established tumours.

DC transduction after direct administration of LVs has been demonstrated *in vivo* both in the spleen and in lymph nodes after systemic or subcutaneous injections, respectively (Esslinger *et al.* 2003; Palmowski *et al*. 2004; VandenDriessche *et al.* 2002). Following these studies, the effectiveness of direct administration of LVs has been shown by several research groups (Table 1.2). Although there has been great interest on tumoral models, some have also investigated the potential use of LVs against infectious diseases.

Table 1.2. Overview of studies involving direct *in vivo* administration of LVs for immunisation.

Antigen	Dose & route of administration	Boosting	Characterisation of immune response	Endpoint analysis	Reference
Cw3 Melan-A	2 x 10^7 EFU sc footpad or base of tail	No	$CD8^+$ T cell response, $CD4^+$ T cell-dependent in the case of Melan-A but not Cw3	Elimination of targets in *in vivo* cytotoxicity assay Poor secondary response to challenge with same LV (anti-vector immunity?)	Esslinger *et al.* 2003
NY-ESO-1	5 x 10^7 IU iv tail vein	NY-ESO-1-VV, day 8	$CD8^+$ T cell response	Elimination of targets in *in vivo* cytotoxicity assay; boosting improved response	Palmowski *et al.* 2004
Trp2/hsp70 Neu/hsp70	1.6 x 10^7 PFU sc footpad	No	$CD8^+$ T cell response	Decreased growth of small established B16 or G-26 tumours Decreased growth of established spontaneous mammary gland tumours in BALB/c-Neu transgenic mice	Kim *et al.* 2005
Full-length HIV-1 Rev/Env Codon-optimized HIV-1 gp120	1 x 10^7 RT units im	No	$CD8^+$ T cell response Humoral response	*In vitro* cytotoxicity assay	Buffa *et al.* 2006
Melan-A	4 x 10^6 EFU sc base of tail	No	$CD8^+$ T cell response	Elimination of targets in *ex vivo* and *in vivo* cytotoxicity assays Effective secondary response to challenge with peptide	Chapatte *et al.* 2006
OVA	1 x 10^6- 1 x 10^7 iu sc	OVA-LV, day 150	$CD8^+$ and $CD4^+$ (T_H1) T cell response; CTL response partially dependent on $CD4^+$ T cells	Elimination of targets in *in vivo* cytotoxicity assay Decreased growth of established EG.7 tumours	Dullaers *et al.* 2006
E-glycoprotein West Nile virus	500 ng p24 ip	No	Humoral response (primary and memory)	Protection against challenge with West Nile virus	Iglesias *et al.* 2006
OVA	1 x 10^6 sc	No	$CD8^+$ T cell response	Elimination of targets in *in vivo* cytotoxicity assay Protection from B16-OVA tumour challenge	He *et al.* 2006
OVA	1 x 10^7 iu iv tail vein	OVA –VV, week 3	$CD8^+$ and $CD4^+$ T cell response Humoral response	Complete protection against EG.7 tumour challenge	Rowe *et al.* 2006

Antigen	Dose & route of administration	Boosting	Characterisation of immune response	Endpoint analysis	Reference
HIV-1-derived restricted polyepitopes	0.2-1 x 10^8 TU ip	No	$CD8^+$ and $CD4^+$ T cell response; CTL response partially dependent on $CD4^+$ T cell priming	Elimination of targets in *in vivo* cytotoxicity assay	Iglesias *et al.* 2007
Trp2	2 µg p24 iv	Trp2-LV, day 7	$CD8^+$ T cell response	Increase in survival following tumour challenge with B16 cells using CMV promoter was used, but not with MHC-II specific promoter	Kimura *et al.* 2007
Codon-optimized HIV-1 gp120[a]	1.3 x 10^7 RT units im	No	$CD8^+$ T cell reponse Humoral response	*In vitro* cytotoxicity assay	Negri *et al.* 2007
NY-ESO-1	4 x 10^6 TU sc base of tail	No	$CD8^+$ and $CD4^+$ T cell response Humoral response	No functional assays	Garcia Casado *et al.* 2008
NY-ESO-1	0.001-1 x 10^8 iu sc base of tail or 1 x 10^8 iu iv	NY-ESO-1-VV, week 3	$CD8^+$ and $CD4^+$ T cell response	No functional assays	Lopes *et al.* 2008
Mutated Trp-1	2.5 x 10^7 TU sc footpad	No	$CD8^+$ T cell response	Elimination of targets in *in vivo* cytotoxicity assay Protection against challenge with B16 cells Elimination of early-stage and inhibition of growth in established B16 tumours	Liu *et al.* 2009
CEA	0.15 x 10^6 TU sc in footpad	CEA-LV, weekly x 3 doses	$CD8^+$ and $CD4^+$ (T_H1 and T_H2) T cell response Humoral reponse	Regression of CEA-expressing tumours; poor long-term protection (loss of cellular response and tumour regrowth after day 36)	Loisel-Meyer *et al.* 2009
SIVmac239 gag non-secreted protein [b]	0.25-1 x 10^8 TU sc	Gag-LV with a different envelope, day 79	$CD8^+$ and $CD4^+$ T cell response Humoral response	Protection against intrarrectal challenge with SIVmac251	Beignon *et al.* 2009
OVA[a]	150 ng RT sc	No	$CD8^+$ T cell response	Partial regression and increased survival of mice bearing EG.7 tumours.	Karwacz *et al.* 2009
Secreted hepatitis B virus surface antigen	1 x 10^7 iu im	No	$CD8^+$ T cell and humoral responses		

[a] Immunisations with non-integrating LVs. [b] Study performed with macaques; the rest were done in mice.

1.4.1. Interaction of LVs with DCs

The regulation of the cellular immune response by DCs depends on the delivery of different 'signals' to receptors present in lymphocytes (Janeway & Bottomly 1994). 'Signal 1' is antigen-specific and depends on the recognition of the MHC/peptide complex by cognate T cell receptors. Activation and expansion of T cells also requires a co-stimulatory 'signal 2', which results from the balance of several positive and negative signals delivered to particular receptors in the T cells. Classical co-stimulatory molecules in DCs are CD80 (B7.1) and CD86 (B7.2), which interact with CD28 in T cells and induce their clonal expansion, cytokine secretion and effector functions (Seliger *et al.* 2008). DCs also provide a third signal ('signal 3') through cytokines that exert multiple functions, such as regulation of the CTL activity of $CD8^+$ T cells, polarisation of the $CD4^+$ T cell response and generation of regulatory T cells, among others (Curtsinger *et al.* 1999; Curtsinger *et al.* 2003; Kaliński *et al.* 1999). Thus, a vaccine should provide both the antigen to be presented and th components necessary to induce DC maturation in order to deliver adequate signals 2 and 3. There is evidence that immunisation with LVs provides these signals.

1.4.1.1. Processing of LV-encoded antigens: Delivery of 'signal 1'

Antigen processing and presentation has classically been divided in two different pathways. Endogenous antigens are processed through an intracellular pathway that results in presentation to $CD8^+$ T cells in the context of MHC-I. Exogenous antigens follow an endocytic pathway and are presented to $CD4^+$ T cells in the context of MHC-II. However, cross-talk between the two pathways can take place. Exogenous peptides can be presented by MHC-I (cross-presentation) and endogenous antigens by MHC-II ('biosynthetic' pathway). This cross-presentation is particularly important in the context of immunity against infectious diseases and cancer (Basta & Alatery 2007).

Processing of the antigen genes delivered by LVs reach the MHC-I following the same pathway as 'endogenous' products, such as cellular or viral proteins produced in infected cells. A proportion of these newly synthesised proteins are degraded by the proteasome and immunoproteasome. The resulting peptides can then be translocated to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) and by other TAP-independent mechanisms (Yewdell *et al.* 1998). As expected, TAP plays an important role in the processing of LV-encoded antigens, since no class I antigen presentation is observed in TAP-deficient cells transduced with LVs (Zarei *et al.* 2002).

In the ER, the peptides are loaded onto MHC-I heterodimers through peptide-loading complexes, a mechanism that is highly regulated by a variety of chaperones and other proteins. Once class I molecules are loaded with stably-bound peptides, they are released from the peptide-loading complex and shuttled to the cell surface via the Golgi complex for antigen presentation (Buckwalter & Albert 2009; Van Kaer 2002; Vyas *et al.* 2008).

On the other hand, 'exogenous' antigens are internalised in APCs by phagocytosis, endocytosis or both, and then enzymatically degraded in endocytic compartments. Here they encounter the MHC-II molecules. After its synthesis in the ER, the MHC-II is associated with a pre-assembled trimer called invariant chain (Ii) that blocks the peptide-binding cleft and prevents binding of endogenous peptides in the ER, among other functions. The Ii is degraded by the endosomal hydrolases, except for a short fragment—the class II-associated invariant chain peptide (CLIP)—which remains bound to the peptide-binding cleft. CLIP is then exchanged for processed peptides in the endosomal compartments, a mechanism that is regulated by other 'non classical' MHC-II molecules. The stable MHC-II/peptide complexes are finally transported to the plasma membrane for antigen presentation (Vyas *et al.* 2008; Jensen 2007). Class II antigen presentation is important in vaccination, as $CD4^+$ T cells play a critical role in the immune response.

Proteins encoded by LVs can reach MHC-II through several pathways. Secreted proteins can be taken up directly by DCs or neighbouring cells and enter the endocytic pathway, although this mechanism is probably inefficient. Membrane-bound proteins enter the recycling pathway, which leads to endosomal localisation

and processing. Proteins can also be traslocated to the ER and then passed to the Golgi, where they can go directly into endosome and be processed. If the protein does not enter the ER and remains in the cytoplasm, there is very little class II processing. However, these proteins can occasionally reach this pathway during autophagy by fusion of autophagosomes with the MHC-II-loading compartments (Paludan *et al.* 2005).

Several molecular approaches have also been employed to improve class II processing of intracellular peptides encoded by LVs or other vectors. One strategy is engineering chimeric genes that encode the antigen plus specific proteins of the endocytic route, such as lysosomal-associated membrane protein 1 (LAMP-1) or the invariant chain (Gregers *et al.* 2003; Sanderson *et al.* 1995; Wu *et al.* 1995). This method enhances MHC-II presentation and increases the efficacy of the immune response to certain antigens (Rowe *et al.* 2006).

Exogenous antigens can also be cross-presented by MHC-I by some APCs, in particular by DCs. Although the exact cellular mechanism is not completely understood, it involves direct transport of the antigen from the phagosome or early endosomes to the cytoplasm for proteasome processing, or direct fusion of phagosomes with the ER (Jensen 2007). This mechanism seems to be important for the presentation of antigens derived from apoptotic cells or from transduced non-professional APCs following vaccination with DNA or viral vectors (Pang *et al.* 2009; Schulz *et al.* 2005).

1.4.1.2. DC maturation and delivery of signals '2' and '3' by LVs

Antigen presentation in the context of appropriate co-stimulation ('signal 2') and cytokine stimulus ('signal 3') are critical for the generation of an adequate immune responses. This is directly related to the maturation state of the DCs. It is widely accepted that immature DCs, which present antigen but do not deliver co-stimulatory signals and stimulatory cytokines, induce T cell tolerance. Instead, the presence of co-stimulation and activating cytokines results in potent induction of immunity (Jonuleit *et al.* 2001; Probst *et al.* 2003; Reis e Sousa 2006). This has been confirmed in multiple experiments in which antigen presentation by adoptively

transferred immature DCs or antigen delivery to DCs in the steady state result in T cell anergy and/or induction of regulatory T cells (T_{reg}) (Bonifaz *et al.* 2002; Dhodapkar *et al.* 2001; Dhodapkar & Steinman 2002; Hawiger *et al.* 2001). In contrast, *ex vivo* activated DCs or concomitant *in vivo* administration of DC activators and antigen induce potent immune responses (Banchereau & Steinman 1998; Dhodapkar *et al.* 1999; Probst *et al.* 2003).

1.4.1.2.1. Overview on dendritic cell activation

Maturation of DCs is regulated by a variety of signals, such as microbial patterns, 'danger' signals and inflammatory cytokines. They are sensed by pattern recognition receptors (PRRs) that include Toll-like (TLRs), nucleotide-binding oligomerisation domain (Nod)-like, retinoic acid induced gene (RIG)-I-like and C-type lectin receptors (Fritz *et al.* 2006; H. Kato *et al.* 2005; Geijtenbeek & Gringhuis 2009). These sensors play an important role in linking the innate and adaptive immune responses. Sensing the 'danger' signals results a series of phenotypic changes in DCs, which switch from an antigen-capturing to an antigen-presenting and T cell-stimulating cell. During the process of DC maturation, the cells migrate to secondary lymphoid organs, assemble peptide-MHC complexes and produce cytokines (Reis e Sousa 2006).

Among the PRRs, the TLRs have been extensively studied in DCs. These are a family of receptors present on the cellular and endosomal membranes that recognise several pathogen-associated molecular patterns (PAMPs). They are differentially expressed among DC subsets. For example, $CD8^+$ DCs express TLR3, but not TLR5 or TLR7, while pDCs express mainly TLR7 (in mice and humans) and TLR9 (in mice), but very low level of TLR3 (Reis e Sousa 2004). Thus, different types of ligands can activate different DC subtypes.

The role of TLRs in the activation of adaptive immunity is well established (Iwasaki & Medzhitov 2004). TLR signalling controls antigen uptake, processing and loading onto MHC molecules (Blander & Medzhitov 2006; Cella *et al.* 1997). It also downregulates chemokine receptor (CCR)-5 and upregulates CCR-7, facilitating DC migration to lymph nodes (Means *et al.* 2003). In many cases, TLR stimulation

results in a T helper 1 (T_H1)-polarised $CD4^+$ T cell response characterised by secretion of inflammatory cytokines like interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α and IL-12 (van Duin *et al.* 2006). Importantly, it has been shown that stimulation of TLRs is necessary to break tolerance to self- and TAAs by counteracting T_{reg} (Lang *et al.* 2005; Yang *et al.* 2004).

One of the consequences of TLR stimulation is the upregulation of the co-stimulatory molecules CD80 (B7.1), CD86 (B7.2), CD40 and CD70 on DCs (Iwasaki & Medzhitov 2004). CD80 and CD86 belong to the 'B7 family', which also includes the co-inhibitory molecule programmed death ligand (PDL)-1. These molecules bind receptors from the CD28 family on T lymphocytes. Engagement of CD28 by CD80 and CD86 results in cytokine secretion and inhibition of apoptosis, consequently enhancing the immune response. Interestingly, these molecules also have inhibitory functions. Either engagement of PDL-1 to its receptor, PD-1, or of CD80 and CD86 to another receptor of the CD28 family, cytotoxic T lymphocyte antigen (CTLA)-4, attenuate the T cell response and prevent T cell hyperactivation (Seliger *et al.* 2008).

CD40 is a receptor of the TNF receptor superfamily. Its ligand, CD40L (CD154), can exist in a soluble form or on the cell membrane of activated T cells, B cells, platelets, monocytic cells, natural killer (NK) cells, mast cells and basophils. Upon engagement, CD40 induces cytokine production, expression of co-stimulatory molecules and antigen cross-presentation by DCs (Elgueta *et al.* 2009). CD40 signalling also promotes DC survival and is involved in the protection of APCs from CTL-mediated elimination (Mueller *et al.* 2006). The cellular response against specific antigens can be enhanced by induction of CD40L expression in DCs using mRNA electroporation or viral vectors (Bonehill *et al.* 2009; Koya *et al.* 2003; Liu *et al.* 2008; Thacker *et al.* 2009).

CD70 binds to its receptor, CD27, expressed on the surface of $CD8^+$ and $CD4^+$ T cells, NK cells and activated B cells. This receptor is also member of the TNF receptor superfamily and delivers important signals for cell survival and differentiation of T cells (Borst *et al.* 2005). CD70 expression in DCs contributes to T cell priming (Schildknecht *et al.* 2007) and can break $CD8^+$ T cell tolerance (Keller *et al.* 2008). For this reason, it has been used in some vaccination strategies (Keller *et al.* 2009; Bonehill *et al.* 2008).

1.4.1.2.2. Dendritic cell activation by LVs

Based on the above evidence, it is clear that to elicit an immune response LVs should not only deliver the antigen but also activate the DCs. The latter may be achieved by (1) components of the viral particle itself, (2) components of the viral preparation other than the viral particle and (3) delivery of transgenes involved in activation pathways.

1.4.1.2.2.1. DC activation by LV particles

Although LVs do not express viral proteins, some of the vector components could potentially stimulate the innate immune system. Single stranded RNA contained in the viral core is a ligand of TLR7. Double stranded DNA, generated after reverse transcription of the viral genome, can engage TLR9.

Some evidence supports LV-mediated activation of DCs. HIV-1, the parental virus of most LVs, induces pDC maturation and type I IFN secretion *in vitro* through TLR7 stimulation (Beignon *et al.* 2005; Fonteneau *et al.* 2004). Type I IFN induces autocrine maturation of pDCs and also of 'bystander' myeloid DCs. This has also been shown *in vivo*, where administration of LVs induced a rapid and transient secretion of type I IFN initiated by pDCs; when the ability to respond to IFN-α/β was absent, the clearance of transduced cells was abolished (Brown *et al.* 2007). These studies contrast with others in which transduction of pDCs by LVs did not affect their activation phenotype (Veron *et al.* 2009).

The effects of LVs on conventional DCs (cDCs) are less clear. In several studies, no changes in the immunophenotype of cDCs were observed upon transduction with LVs or infection with HIV-1 (Beignon *et al.* 2005; Schroers *et al.* 2000; Gruber *et al.* 2000). In contrast, Harman *et al.* (Harman *et al.* 2006) reported that infection of monocyte-derived DCs (MoDCs) with live or inactivated HIV-1 resulted in a partially mature phenotype. Breckpot et al (Breckpot *et al.* 2007) also reported upregulation of co-stimulatory (CD25, CD80, CD83) and HLA molecules, as well as enhanced secretion of pro-inflammatory cytokines (IL-6, TNF-α) and allostimulatory capacity on MoDCs transduced with LVs. Engagement of TLR2,

TLR3 and TLR8 seem to mediate this process. However, in this study, DC activation was only observed at high multiplicity of infection.

More recently, it has been shown that LVs induce activation of murine myeloid DCs through a TLR-3/TLR-7-dependent mechanism (Breckpot *et al*., submitted). These discrepancies among studies could be explained by differences in experimental designs, including the origin of the DCs, the way the virus is produced and concentrated and the amount of virus used.

1.4.1.2.2.2. DC activation by other components of the viral preparation

By-products present in the vector preparation can affect the immunostimulatory properties of LVs. Viral preparations of VSV-G-pseudotyped LVs contain tubulo-vesicular structures that contain nucleic acids, that stimulate TLR9, induce type I IFN production by pDCs and elicit T and B cell responses to co-administered proteins (Pichlmair *et al.* 2007). The presence of residual foetal calf serum (FCS) in concentrated viral pellets can also be immunogenic. Bao *et al.* (2009) showed generation of $CD4^+$ T cells specific for epitopes in FCS components which could potentially affect the $CD4^+$ T cell help in the immune response.

1.4.1.2.2.3. Delivery of transgenes involved in activation pathways

DC function can be modulated by delivering genes involved in DC differentiation and maturation pathways. This can be achieved by expressing downstream effectors or intermediate molecules that stimulate or inhibit different signalling cascades. The most common targets are TLR signalling, mitogen-activated protein kinase and (MAPK) and nuclear factor κB (NF-κB) pathways.

TLR signalling can be emulated in the absence of ligands by expression of adaptor molecules associated with this pathway. For example, transduction of BMDCs with LVs encoding Myd88 or TRIF-1 results in secretion of IL-6 and IL-12 or IFN-α production, respectively, and enhanced cytotoxicity (Akazawa *et al.* 2007).

Activation of the NF-κB pathway has been achieved using LVs encoding the Kaposi sarcoma-associated herpes virus (KSHV) FLICE-like inhibitory protein (vFLIP), which activates the classical and alternative NF-κB pathways. Co-

expression of this molecule with OVA resulted in DC activation, enhanced $CD8^+$ T cell responses, improved tumour-free survival in a tumour therapy model and reduction of parasite load after a challenge with OVA-leishmania (Rowe *et al.* 2009). NF-κB activation has also been attained by inhibiting A20, which inhibits NF-κB by deactivation of some of the adaptor molecules that take part in the signalling of TLRs, TNF and IL-1 receptors. Transduction with a LV encoding a short hairpin RNA (shRNA) against A20 resulted in upregulation of co-stimulatory molecules and pro-inflammatory cytokines in DCs, enhanced $CD8^+$ T cell response and inhibition of T_{reg} (Breckpot *et al.* 2009; Song *et al.* 2008).

The MAPK pathway has also been manipulated by using constitutively activated or dominant negative mutants. Escors *et al.* (2008) showed that activation of p38 and JNK1 with constitutively active mutants of MKK6 and the fusion protein MKK7-JNK1, respectively, resulted in upregulation of co-stimulatory molecules (CD40 and CD80) in DCs, although no increase in secretion of pro-inflammatory cytokines. Co-expression of these molecules with OVA or other human relevant antigens resulted in a better $CD8^+$ T cell response and improved survival in murine tumoral models. In contrast, activation of ERK1/2 pathway with an active mutant of MEK1, and activation of IRF3 resulted in inhibition of the immune response and increase in the number of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells. These results have been confirmed in the context of immunisation with non-integrating lentivectors (Karwacz *et al.* 2009).

1.4.2. Clinical application of LVs

Until now, LVs have not been employed clinically as vaccine vectors. However, they have been used in gene therapy clinical trials, showing encouraging results regarding their safety and efficacy.

In the first reported clinical trial, LVs were used to express an antisense gene against the HIV envelope in autologous $CD4^+$ T cells that were then transferred to infected patients. Sustained gene transfer and no evidence of insertional mutagenesis were shown in a follow up of 21-36 months. The data also showed self-limiting mobilisation of the vector and improvement of the immune function in four out of

five treated subjects (Levine *et al.* 2006). LVs are also being used in other gene therapy clinical trials for the treatment of X-linked adrenoleukodystrophy, β-thalassemia, sickle-cell anaemia and Parkinson's disease (D'Costa *et al.* 2009).

1.5. Justification and aims of the PhD project

LVs have several advantages that make of them promising tools for immunotherapy. These include (1) efficient transduction and stable gene expression in professional APCs, particularly in DCs, (2) low toxicity, (3) relatively high packaging capacity, (4) low antivector immunity and (5) the possibility of regulating the immune response by the viral particle itself or by co-expression of immunomodulators. Their effectiveness has been demonstrated in multiple pre-clinical models in mice and, more recently, in macaques (Table 1.2). Furthermore, reports of early gene therapy clinical trials have documented their safety. Although they have not yet been employed for immunotherapy, all this evidence supports the possibility of their application, possibly for the treatment of serious conditions such as malignant diseases.

Integration into the host genome allows LVs to sustain expression of the transgene during the lifespan of the target cell and its progeny. This has been shown in different organs and tissues, *e.g.*, liver (Kang *et al.* 2005a), neurons (Naldini *et al.* 1996), retina (Ikeda *et al.* 2009) and cells of the haemopoietic system (Marangoni *et al.* 2009). Sustained expression is a favourable feature that has been exploited for the correction of genetic diseases. However, for genetic immunisation, this property is questionable since sustained expression of antigen is not absolutely necessary to elicit an efficient immune response and the consequences of prolonged antigen presentation are not always favourable.

Two key unanswered questions in the use of LVs are for how long do DCs produce antigen and what are the consequences of sustained transgene expression in professional APCs. This thesis will attempt to address these questions. Therefore, the main objectives of this PhD are:

- Determine the duration of antigen presentation by professional APCs following antigen gene delivery by lentiviral vectors.

- Study the immune consequences of persistent antigen in the long term.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Molecular biology techniques

2.1.1. Molecular buffers and bacterial media

The buffers and bacterial media are summarised in table 2.1. For general purposes, plasmid DNA was stocked in solution in EB or TE buffer at 1 µg/µL.

2.1.2. Restriction digestions and ligations

All restriction enzymes used were purchased from Promega (Madison, WI) or from New England Biolabs (Ipswich, MA). Digestions of plasmid DNA were done for at least one hour in the conditions recommended by the manufacturers for each combination of enzymes in a final volume of 10 µL (1 µL DNA, 16 µL water, 0.5 µL each enzyme, 2 µL buffer 10X) or 30 µL (10 µL DNA, 15 µL water, 1 µL each enzyme, 3 µL buffer 10X).

DNA fragments were ligated overnight at ~17º C in presence of T4 DNA ligase and its respective buffer (New England Biolabs). Ligations were done in a final volume of 10 µL (4 µL of each DNA fragment, 1 µL T4 DNA ligase, 1 µL buffer 10X).

Table 2.1. Buffers and media for molecular biology

Buffers/media	Composition
1X phosphate-buffered saline (PBS)	137 mM NaCl, 2mM KCl, 10 mM sodium hydrogen phosphate (dibasic), 2 mM potassium hydrogen phosphate (dibasic), pH 7.4
Tris-EDTA buffer (TE)	10 mM Tris.Cl, 1mM EDTA, pH 8.0
EB buffer	10 mM Tris.Cl, pH 8.5
1X tris-acetate EDTA buffer (TAE)	40 mM Tris (pH 7.8), 20 mM sodium acetate, 1 mM EDTA
Luria Bertani agar	LB broth plus bacto-agar 15 g/L
Luria Bertani broth	1% bacto-tryptone, 0.5% bacto-yeast extract, 10% NaCl, pH 7.0
6X gel loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water
Transformation buffer (TFB)-I	30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM magnesium chloride, 15% glycerol, pH 5.5 with acetic acid
TFB-II	10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol, pH 6.5 with KOH

2.1.3. Agarose gel electrophoresis

Electrophoreses were done in 1% agarose (Invitrogen, Carlsbad, CA) gels with 5 µg/mL ethidium bromide (Dutscher Scientific, Essex, UK). A 1-Kb Plus DNA ladder (Invitrogen, Carlsbad, CA) was run in parallel to indentify the band sizes.

For DNA purification after electrophoresis, the section of the gel containing the desired DNA fragment was cut out and the DNA purified using QIAquick Gel

Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.1.4. Preparation and transformation of competent bacteria

Competent XL1-Blue *E. coli* were prepared by selection in tetracycline (10 µg/mL) using the rubidium chloride method (buffers TBF-I and TBF-II) as described elsewhere (Bokhoven 2008). Aliquots of 100 µL were kept at -80º C until used.

For transformation, competent bacteria were thawed on ice and inoculated with 1-3 µg of plasmid DNA or 3-5 µL of ligation reaction. After incubation on ice for 20 minutes, the bacteria were heat shocked for 2 minutes at 37º C and put back on ice for further 2 minutes. Transformed cells were then grown overnight in LB agar plates at 37º C. All clones were selected based on ampicillin resistance (50 µg/mL).

2.1.5. DNA purification and quantification

Single colonies were picked from LB agar plates and grown overnight at 37º C in 3 mL (minipreps), 150 mL (midipreps) or 400 mL (maxipreps) of LB broth with ampicillin (50 µg/mL). Plasmid DNA was purified using QiaPrep Spin Miniprep, Plasmid Midi and Plasmid Maxi kits (Qiagen) according to manufacturer's instructions.

The concentration of DNA was determined using a Hitachi U-1500 spectrophotometer and the concentration calculated using the following formula:

$$\text{Concentration (ng/µL)} = \text{Absorbance}_{260\ \text{nm}} \times \text{Dilution factor} \times 50$$

2.1.6. Polymerase chain reaction (PCR)

PCR reactions were done using Hotstar Taq DNA polymerase (Qiagen). Table 2.2 summarises the reagents used for one reaction in a volume of 50 µL. Reactions were run in a Hybraid thermal cycler, performing 25-30 cycles with the parameters shown in Table 2.3.

Table 2.2. Polymerase chain reactions (for one reaction of 50 µL).

Reactive	Stock concentration	Volume added	Final concentration
10X PCR buffer	10X	5.25 µL	1X
dNTPs	2.5 mM	1.5 µL	200 µM of each dNTP
Forward primer	1 µg/ µL	1 µL	0.5 µM
Reverse primer	1 µg/ µL	1 µL	0.5 µM
Taq polymerase	5 U/µL	0.3 µL	2.5 U/100 µL reaction
MgCl2	25 mM	4 µL	1.5 mM
DTT	1 M	0.05 µL	1µM
DNA	1 µg		
Distilled water		Complete volume to 50 µL	

Table 2.3. Thermocycler parameters for PCR (25-30 cycles).

Phase	Time	Temperature
Initial activation	10 minutes	94º C
Denaturation	45 seconds	94º C
Primer annealing	30 seconds	~5º C below Tm of primers
Extension	1 min/kb of product	72º C
Final extension	10 min	72º C

2.1.7. Sequencing

DNA sequences were verified using the Imperial College and University College Sequencing Services, either using standard or customised primers.

2.2. Tissue culture

2.2.1. Cell lines

293T cells were used for viral packaging and testing expression of LVs. They are an easily transfectable and highly transgene-expressing cell line derived from human embryonic kidney cells, which express the large T antigen of simian virus 40 (DuBridge *et al.* 1987; Pear *et al.* 1993). 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK), supplemented with 10% Foetal Calf Serum (FCS) (Serotec, Oxford, UK), 2 mM L-glutamine (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cells were passaged 1:3-1:4 using trypsin/EDTA (Gibco) every 2-3 days.

For the *in vitro* experiments in which the tetracycline conditional expression system was used, media were supplemented with 10% Tet System Approved Foetal Bovine Serum (Clontech, Mountain View, CA), free of tetracycline or doxycycline traces.

RF33 cells, a mouse T cell hybridoma that expresses the T cell receptor specific for OVA class I epitope SIINFEKL, was cultured in Roswell Park Memorial Institute (RPMI, Gibco) medium supplemented with 10 % FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

2.2.2. Generation of bone marrow-derived dendritic cells

Bone marrow-derived DCs were produced as described by Talmor *et al.* (1998). Briefly, the bone marrow of C57BL/6 mice was flushed from leg bones with Hank's buffered salt solution (HBSS, Gibco) using a syringe. After lysing the red blood cells and washing twice, the cells were resuspended at 5-7.5 x 10^5 cells/mL in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol (Gibco) and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ). Medium was replaced every 3 days.

Transductions were performed on day 5-6 with a multiplicity of infection (moi) of 20 (Palmowski *et al.* 2004).

2.3. Lentiviral vectors

2.3.1. Vector production

Vectors were produced by transient co-transfection of 293T cells with a transfer vector, a second generation HIV-1 derived packaging plasmid (p8.91) and a plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) (pMD.G), as shown in figure 1.3 and described before (Demaison *et al.* 2002; Rowe *et al.* 2006). p8.91, pMD.G (Fig. 1.3B-C, respectively) as well as pHRSIN-CSGW and pDUAL-IiOVA-GFP were produced by Plasmid Factory (Bielefeld, Germany); the rest of the plasmids were generated in the laboratory.

The day before transfection, 10^7 293T cells were plated in 15 cm^2 plates to reach a confluency of 80-90% the following day. Transfection was done with Fugene 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) using a mix with the following components (amounts per 15 cm^2 plate):

p8.91	2.5 µg
pMD.G	2.5 µg
Transfer plasmid	37.5 µg
Optimem (Gibco)	500 µL
Fugene 6	45 µL

The mix was incubated 15 minutes at room temperature and added dropwise to the cells in fresh medium. The medium was changed again 24 hours later. The supernatants were collected after 72 hours and passed through a 45 µm filter.

The viral particles were concentrated 100-200-fold by two rounds of ultracentrifugation (115 000 x *g* for 2 hours at 4º C) in a Sorvall ultracentrifuge, then resuspended in HBSS and kept at -80º C until usage.

2.3.2. Transfer constructs

A schematic representation of the lentiviral transfer plasmids employed is shown in Fig. 2.1. The pHRSIN-CSGW construct (Fig. 2.1A) was provided by A. Thrasher and is described in Demaison *et al.* (2002). This vector is a self-inactivating derivative of HIV-1 subtype B, isolate HXB2, with a 400 bp deletion in the 3'LTR U3 region. It contains an insert encoding emerald green fluorescent protein (eGFP, referred from now on as GFP) driven by a spleen focus-forming virus (SFFV) promoter, and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).

pDUAL-IiOVA-GFP (Fig. 2.1B) was described by Rowe *et al.* (Rowe *et al.* 2006[a]). It contains a SFFV promoter that drives expression of a fusion of the C-terminal portion of the invariant chain and amino acids 242-353 of chicken ovalbumin (OVA). The class I H2-K^b-restricted SIINFEKL ($OVA_{257-264}$) and class II H2-IA/IE^b-restricted ISQAVHAAHAEINEAGR ($OVA_{323-339}$) epitopes are encoded within the IiOVA insert. A second promoter, the human ubiquitin promoter (UBI), drives the expression of GFP.

For the Thy1.1 (CD90.1)-encoding vectors (Fig. 2.1C), a Thy1.1 insert was amplified by PCR from pMiT (Mitchell *et al.* 2001), kindly provided by P. Marrack, using the primers BamHI-Thy1.1-FW and Thy1.1-NotI-RV (Table 2.4). The PCR product was subcloned into a pGEM-T Easy Vector (Promega) and then into the BamHI-NotI restriction sites under control of the SFFV promoter to generate pHRSIN-Thy1.1, pDUAL-Thy1.1-GFP and pDUAL-Thy1.1-IiOVA.

The regulatable gene expression LV was kindly provided by O. Danos and M. A. Zanta-Boussif. It contains a conditional promoter consisting of a tetracycline responsive element (TRE) and a cytomegalovirus minimal promoter (CMV_{min}) driving the expression of the insert of interest. A human phosphoglycerate kinase promoter (hPGK) drives constitutive expression of a tetracycline-responsive transactivator (rtTA2^S-M2) (Barde *et al.* 2006). Upon the presence of tetracycline or its analogue, doxycycline (DOX), the transactivator protein undergoes conformational changes, binds to TRE and induces expression of the gene of interest (TetOn system). GFP or IiOVA cassettes were generated by PCR as MluI-MluI

fragments using MluI-GFP-FW and GFP-MluI-RV, or MluI-IiOVA-FW and IiOva-MluI-RV primer sets (Table 2.4). The PCR product was subcloned into pGEM-T Easy vector and then into the MluI-MluI restriction sites of the LV to generate pSIN-TRE-GFP-hPGK-rtTA2S-M2 and pSIN-TRE-IiOVA-hPGK-rtTA2S-M2 (Fig. 2.1D). Correct orientation of the insert in the final constructs was verified by DNA sequencing.

2.3.3. Site directed mutagenesis of p8.91

Two mutations (D185A and D186A) in the highly conserved YMDD motif of the catalytic core of the reverse transcriptase were introduced in the packaging plasmid p8.91 in order to ablate the activity of this enzyme (Kaushik *et al.* 1996; Lowe *et al.* 1991). This mutant was generated using the the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the primers 8.91-RTmut-FW and 8.91-RTmut-RV (Table 2.4) according to the manufacturer's instructions.

Table 2.4. Primers (5' → 3').

Name	Sequence
BamHI-Thy1.1-FW	**GGATCC**GCCACCATGAACCCAGCCATCAGCG
Thy1.1-NotI-RV	**GCGGCCGC**TCACAGAGAAATGAAGTCCAGGG
MluI-GFP-FW	**ACGCGT**ACCGGTCGCCACCATGGTGAGC
GFP-MluI-RV	**ACGCGT**TTTACTTGTACAGCTCGTCC
MluI-IiOVA-FW	**ACGCGT**CACTAGTAACGGCCGCCA
IiOva-MluI-RV	**ACGCGT**TCACAGGGTGGCAGCATC
8.91-RTmut-FW	TCCAGACATAGTCATCTATCAATACATG**GCAGCA** TTGTATGTAGGATCTGACTTAGAAATAGG
8.91-RTmut-RV	CCTATTTCTAAGTCAGATCCTACATACAA**TGCTGC** CATGTATTGATAGATGACTATGTCT GGA

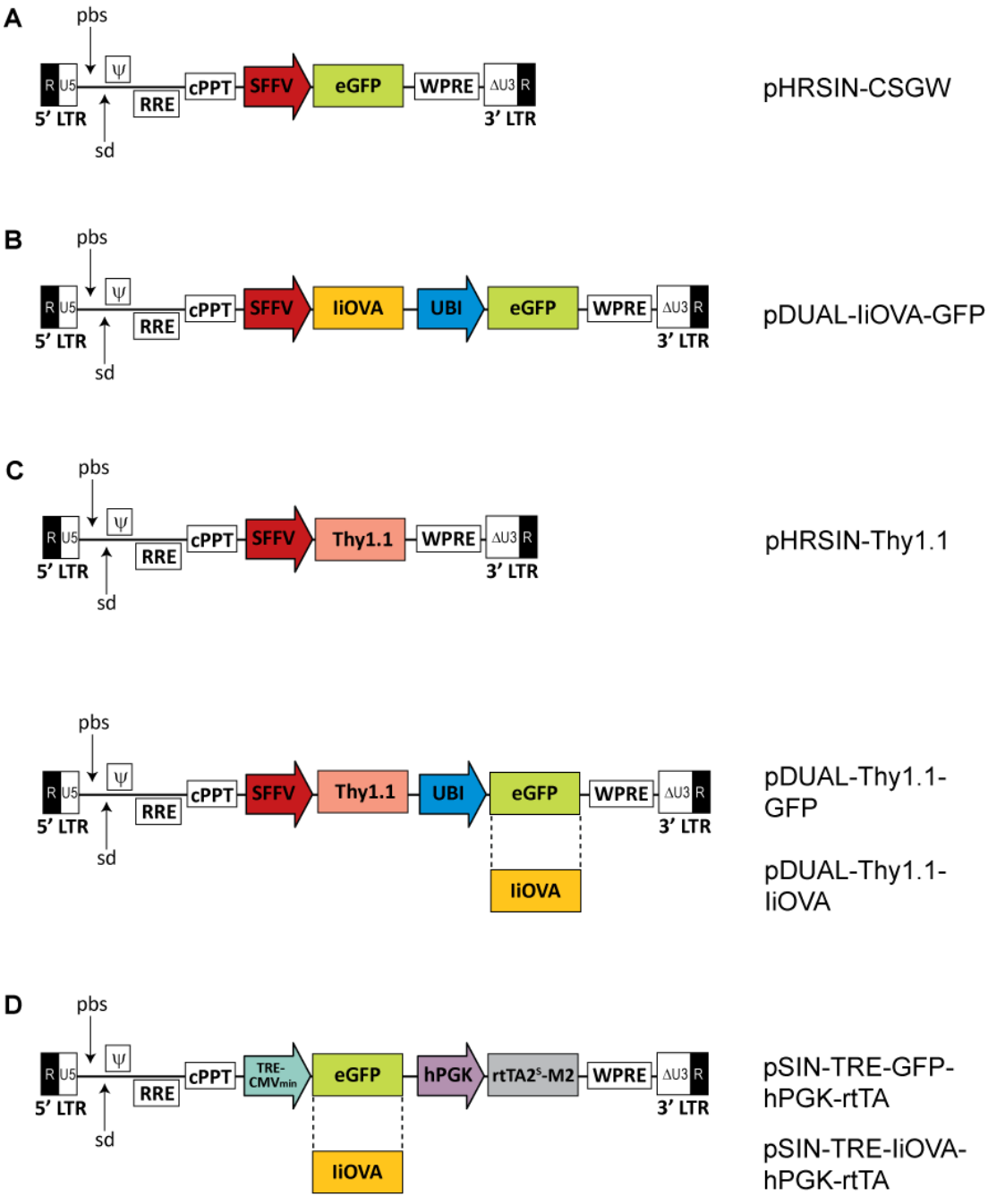


Figure 2.1. Lentiviral constructs.

2.3.4. Viral titration

Viral titers were measured with the following methods:

2.3.4.1. Expression of GFP in 293T cells

For GFP-encoding LVs, 2 x 10^5 293T cells were transduced with 25 µL of serial 1:5 dilutions of lentivectors in 24-well plates. 72 hours later, the percentage of GFP-expressing cells was determined by FACS. Using a sample with 10-30% of transduced cells, the titer was calculated as follows:

$$\text{Viral titer (iu/mL)} = 2 \times 10^5 \text{ cells} \times \% \text{ transduced cells} \times (\text{dilution factor} / 25\ \mu\text{L}) \times 1000$$

Titers of concentrated virus typically ranged between 10^8-10^9 iu/mL.

2.3.4.2. Quantification of reverse transcriptase

The concentration of reverse transcriptase (RT) was measured using a Reverse Transcriptase Assay colorimetric kit (Roche), according to the manufacturer's instructions. For this assay, concentrated virus was diluted 1:20-1:100 and incubated for 3 h for the RT reaction. In general, a correlation of ~10^5 iu/ng RT was observed between these to titration methods.

2.3.4.3. Quantification of p24

Concentration of p24 was measured by ELISA using a QuickTiter Lentivirus Quantitation Kit HIV p24 (Cell Biolabs, San Diego, CA). Concentrated viruses were diluted 1:500. This titration method was only used for experiments involving viruses containing the inactive mutant of RT.

2.4. *In vivo* and *ex vivo* experiments

2.4.1. Mice

C57BL/6 mice were bred in UCL Biological Services facilities and used at 6-8 weeks of age. OT-1 mice were a kind gift of A. Noble (King's College of London) and then bred in UCL facilities. Animal experiments were performed with local ethical approval, following institutional guidelines and under UK Home Office License.

2.4.2. Immunisations

Mice were injected with 10^8 iu or 500 ng RT LVs (unless otherwise specified), or its equivalent in ng p24, either intravenously (iv) or subcutaneously (sc). Negative controls were injected with PBS. Some mice were also injected with 50 µg of OVA class I peptide (SIINFEKL, Proimmune, Oxford, UK) plus 30 µg of monophosphoryl lipid A (MPL, Sigma-Aldrich, St. Louis, MO) as adjuvant, both iv.

2.4.3. Harvesting spleens

Spleens were collected in HBSS and mashed through a 70 µm nylon mesh (BD Falcon). Cells were washed in HBSS containing 2% FCS, resuspended in red blood cell lysis buffer (Sigma-Aldrich) for 5 minutes at room temperature and washed twice before using.

2.4.4. Splenectomies

Mice were anaesthetised with 5% isoflurane and maintained with 1-3% of the same anesthetic gas. In aseptic conditions, spleens were removed through a subcostal incision. Bleeding was controlled by electrocauterisation. The incision was sutured in two layers. The animals were recovered at 37°C and closely monitored immediately and the days after the procedure. For pain control, 0.1 mg

buprenorphine was injected sc 30 minutes before anaesthesia and every 12 hours thereafter, or upon distress signs.

2.4.5. Purification of dendritic cells from spleens

Spleens were collected, cut into small pieces and then incubated in a solution of 2 mg/mL collagenase IV (Worthington, Lakewood, NJ) and 10 mM Hepes buffer (Gibco) in HBSS for 30 minutes at 37°C. Splenocytes were obtained as described before. Fc receptors (FcR) were blocked with an anti-mouse FcR (CD16/32) antibody produced in house from a rat hybridoma cell line. DCs were purified by positive selection using magnetic CD11c (N418) microbeads (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions.

2.4.6. Surface staining for flow cytometry

Cell surface staining was performed on ice and with ice-cold buffers. Incubations were done in the dark. The antibodies employed and their respective dilutions are shown in table 2.5. An additional blocking step with anti-FcR and HBSS with 10% mouse serum (Serotec) was done for 20 minutes prior to staining DCs or before using anti-Thy1.1 to reduce unspecific antibody binding.

Each sample (1-5 x 10^6 cells) was stained in a volume of 50 µL (96-well plate) or 100 µL (FACS tube). Antibodies were diluted in staining buffer (HBSS, 2% FCS, 0.1% sodium azide), incubated for 30 minutes and washed twice. When needed, samples were labelled with stretavidin-conjugated fluorochromes for 20 minutes. Cells were fixed with Cytoperm/Cytofix solution (BD Biosciences) and resuspended in HBSS with sodium azide 0.1%. Fluorescence activated flow cytometry (FACS) was done using either FACScalibur or LSR flow cytometers (BD, San José, CA). The results were analysed using CellQuest (BD) or FlowJo 7.5 (Tree Star Inc., Ashland, OR) softwares.

Table 2.5. Antibodies for FACS.

Antibody	Clone	Company	Dilution
CD3-APC	145-2C11	eBioscience	1:100
CD4-PE	GK1.5	eBioscience	1:200
CD4-PECy7	GK1.5	eBioscience	1:200
CD8a-APC	53-6.7	eBioscience	1:200
CD8a-PE	53-6.7	eBioscience	1:200
CD11c-APC	HL3	BD Pharmingen	1:400
CD11c-biotin	HL3	BD Pharmingen	1:400
CD19-PE	6D5	eBioscience	1:200
CD40-biotin	1C10	eBioscience	1:100
CD44-APC	IM7	eBioscience	1:100
CD80-biotin	16-10A1	eBioscience	1:100
CD86-biotin	B7-2	BD Pharmingen	1:100
F4/80-PE	BM8	eBioscience	1:500
Gr-1 (Ly-6G)-PECy7	RB6.8C5	eBioscience	1:200
IFNγ-APC	XMG1.2	BD Pharmingen	1:100
MHC-II (IA/IE^{b})-PE	M5/114.15.2	eBioscience	1:1000
MHC-II (IA^{b})-FITC	AF6-120.1	BD Pharmingen	1:1000
PDCA-1-PE	JF05-1C2.4.1	Miltenyi	10 µL/sample
Thy1.1 (CD90.1)-PE	HIS51	eBioscience	1:500
Thy1.1 (CD90.1)-biotin	HIS51	eBioscience	1:500
Vα2-PE	Cat# RM5004	Caltag	1:200
Vβ5.1, 5.2-biotin	MR9-4	BD Biosciences	1:200
Isotype controls			
Hamster IgG-biotin	G235-2356	BD Pharmingen	1:200
Mouse-IgG2a-biotin	M2a	eBioscience	1:200
Rat IgG2a-biotin	R35-95	BD Pharmingen	1:200
Streptavidin conjugates			
sva-FITC		DakoCytomation	1:500
sva-PE		eBioscience	1:500
sva-APC		eBioscience	1:500
sva-PECy7		eBioscience	1:500

2.4.7. BrdU labelling

To label *in vivo* dividing cells, mice were injected intraperitoneally (ip) with 200 µg of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) in HBSS and then continuously given BrdU (0.8 mg/mL) in drinking water, that was changed daily. Experiments were terminated after 10 days of BrdU administration. Then spleens were harvested and $CD11c^+$ cells were purified using magnetic microbeads as described before. $CD11c^+$ and $CD11c^-$ cells were stained for surface markers and nuclear BrdU using a BrdU Flow Kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. In brief, after surface antigen staining, cells were fixed and permeabilised with Cytofix/Cytoperm (BD Biosciences). Then they were treated with 300 µg/mL of DNase I (Worthington) in DPBS (Gibco) for one hour at 37°C, washed, re-blocked with 10% mouse serum and stained with an APC-labelled anti-BrdU antibody for FACS analysis.

2.4.8. CFSE labelling and adoptive transfer of OT-1 cells

Spleen cells from OT-1 mice were resuspended at 5-10 x 10^6 cells/mL in HBSS plus 0.1% bovine serum albumin and then labelled with 5 µM carboxyfluorescein succinimydil ester (CFSE, Invitrogen, Carlsbad, CA) for 10 minutes at 37°C. 5-10 x 10^6 CFSE-labelled cells were transferred iv into immunised and control C57BL/6 mice. Five days later they were tracked by FACS in peripheral blood mononuclear cells (PBMCs) or in spleens after staining with antibodies against the Vα2 (Caltag) and Vβ5.1, 5.2 (BD Pharmingen) chains of the T cell receptor. Cell proliferation was quantifed by FACS by measuring dilution of CFSE in labelled cells.

2.4.9. Enzyme-linked immunospot assay

Enzyme-linked immunospot assay (ELISpot) plates (Millipore, Billerica, MA) were covered overnight with 10 µg/mL of purified anti-IFNγ (BD Pharmingen, San Diego, CA) at 4ºC overnight. The day after, the plate was washed and blocked for at least 2 hours with RPMI medium with 2% FCS, 2 mM L-glutamine, 100 U.mL

penicillin and 100 µg/mL streptomycin. Splenocytes were resuspended in RPMI and added in different amounts per well (usually 5 x 10^5 or 1 x 10^6, in duplicates). The plate was incubated for 20-24 hours at 37ºC, either in the presence of 50 ng/mL OVA class I peptide (SIINFEKL, Peprotech, Rocky Hill, NJ) or medium alone. The day after, the cells were washed and IFNγ production was determined with 0.5 mg/mL biotinilated anti-IFNγ antibody (BD Pharmingen), 1:10000 streptavidin-conjugated alkaline phosphatase (Caltag, Burlington CA) and an Alkaline Phosphatase Conjugate Substrate Kit (BioRad, Hercules, CA). Spots were counted using AID ELISPOT counter and software.

2.4.10. Pentamer staining

Blood samples were collected in tubes with heparin and diluted 1:5 with HBSS. PBMCs were obtained by density separation using M-Lympholyte (Cedarlane, Canada) following the manufacturer's instructions. Cells were stained with APC-conjugated anti-CD8 and washed twice. Then 10 µL of PE-conjugated MHC Pentamer H2-K^b SIINFEKL (Proimmune, Oxford, UK) were added to each sample and incubated for 10 minutes at room temperature. Cells were washed twice and resuspended in running buffer for FACS.

2.4.11. *In vivo* cytotoxicity assays

Spleen cells from naïve mice were resuspended in HBSS (5 x 10^6 cells/mL) and pulsed with $OVA_{257-264}$ peptide (5 µg/mL) during 2 hours at room temperature. Then they were labelled with 10 µM CFSE as described before. They were mixed in a 1:1 ratio with non-pulsed cells, labelled with 3 µM CFSE. The mixture was injected in control and immunised mice at different time points. Specific lysis of the pulsed cells was analysed 18 hours later by FACS in cells isolated from peripheral blood or from the spleen. The percentage of killing was calculated with the following formula:

$$1-[(\%\ CFSE^{high}/\%CFSE^{low})_{immunised}/(\%CFSE^{high}/\%CFSE^{low})_{non\ immunised}]$$

2.4.12. Isolation of transduced DCs with magnetic microbeads

Isolation of tranduced DCs in the spleen was done based on expression of Thy1.1 and using an Anti-biotin Multisort magnetic separation kit (Miltenyi) according to the manufacturer's instructions. Briefly, splenocytes of mice injected with Thy1.1-GFP or Thy1.1-IiOVA LV at different time points (5 or 30 days before the experiment) were prepared as described before and labelled with anti-CD11c-biotin and anti-Thy1.1-PE antibodies. In a first positive selection step, $CD11c^+$ cells were purified using anti-biotin detachable microbeads. After releasing these magnetic particles with a "release buffer", cells were labelled with anti-PE microbeads to separate $CD11c^+Thy1.1^+$ cells. The purity of the final samples ranged from 60 to 70% of $CD11c^+Thy1.1^+$ cells. However, a population of $CD11c^{hi}Thy1.1^-$ cells was observed in two separate experiments, from which the anti-biotin microbeads probably did not detach.

The obtained cells were used for*ex vivo* antigen presentation assays or DC transfer experiments.

2.4.13. *Ex vivo* antigen presentation assays

DCs isolated from spleens of mice injected with IiOVA-GFP or GFP LVs were co-cultured with splenocytes from OT-1 mice or T cell hybridoma RF33.

In the case of the DC/OT-1 co-culture, 5×10^5 isolated DC were plated in each well of an ELISpot plate. The experiments were done both using frozen expanded $CD8^+$ T cells (Rowe, 2006[a]) and with total splenocytes from OT-1 mice. The DC:OT-1 ratios ranged from 10:1 to 1:1. Antigen presentation was measured by IFN-γ spot count after 20-hour incubation in the presence of peptide (SIINFEKL, 50 ng/mL) or medium.

In the case of the DC/RF33 co-cultures, 5×10^4 RF33 cells were cultured overnight with different numbers of DCs ($5 \times 10^4 - 1 \times 10^3$) in 96-well plates, in the presence of SIINFEKL (10 ng/mL) or medium. To measure antigen presentation, IL-2 was quantified in the supernatants by ELISA (eBioscience, San Diego, CA).

2.4.14. DC transfer experiments

Spleen DCs from mice injected with LVs or with PBS at different time points (5 or 30 days before the experiment) were transferred to naïve mice and tested for their ability to generate a progeny of transgene-expressing DCs or to present antigen in the recicipents. For the first, 3-5 mice were injected with Thy1.1-LV iv and either $CD11c^+$, $Thy1.1^+$ or $CD11c^+Thy1.1^+$ cells were purified from their spleens using magnetic microbeads as described above. Up to 5 x 10^6 DCs, 2 x 10^6 $Thy1.1^+$ cells, or 1 x 10^6 $CD11c^+Thy1.1^+$ cells were trasferred iv to naïve mice in separate experiments. Three days later, DCs were purified from the spleens of the recipient mice and stained with anti-Thy1.1 to identify transduced cells.

For the second, DCs of mice injected iv with IiOVA-GFP LV, GFP LV or PBS at different time points (5 or 30 days) were purified from the spleen and injected (5 x 10^6) sc or iv in naïve mice. The immune response was evaluated by IFN-γ ELISpot ten days later.

2.4.15. Western blots

Buffers and solutions for Western blots are shown in table 2.6. Total cell lysates were prepared with 1% Igepal (Sigma-Aldrich) solution, resuspended in Laemmli buffer and heated at 96º C for 5 minutes. Volumes were standardised according to protein concentrations. Proteins were separated by SDS-PAGE electrophoresis using 4% polyacrylamide stacking gels and 11% polyacrylamide separation gels, in SDS running buffer at 150 volts. Proteins were blotted onto Hybond ECL nitrocellulose membrane (Amersham GE Healthcare, Buckinhamshire, UK) using semi-dry transfer (Pharmacia Biotech) in the presence of transfer buffer, at 300 mA for 45 minutes. Membranes were blocked 1 h with blocking buffer at room temperature and probed overnight with a polyclonal rabbit anti-OVA antibody (1:500 in blocking buffer) at 4ºC. The membrane was washed three times with PBS plus 0.1% Tween 20 before adding anti-rabbit horseradish-peroxidase (HRP)-

conjugated antibody (DakoCytomation, Glostrup, Denmark) diluted 1:3000 in blocking buffer. Membranes were washed twice with PBS plus 0.1% Tween 20, once with PBS, and developed with ECL substrate reagents and Hyperfilm ECL (Amersham GE Healthcare).

2.4.16. Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0. Means were compared using T-student or Mann-Whitney tests, as indicated in each experiment.

Table 2.6. Buffers and gels for Western blots.

Buffer/gel	Composition
Laemmli buffer	2% sodium duodecylsulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.2 mg/mL bromophenol blue, 0.1 M DTT 50 mM Tris (pH 6.8)
11% polyacrylamide gel	11% acrylamide/bis, 125 mM Tris.HCl (pH 8.8), 10% SDS, 0.1% TEMED, 1% ammonium persulphate (APS)
4% stacking gel	4% acrylamide/bis, 125 mM Tris.HCl (pH 6.8), 10% SDS, 0.1% TEMED, 1% APS
Running buffer	25 mM Tris (pH 8.5), 200 mM glycine, 0.1% SDS
Transfer buffer	100 mM Tris, 200 mM glycine, 20% methanol
Blocking buffer	5% semi-skimmed milk, 0.1% Tween 20 in PBS

CHAPTER 3

3. LONG-TERM TRANSGENE EXPRESSION IN LENTIVECTOR –TRANSDUCED ANTIGEN PRESENTING CELLS

3.1. Introduction

It has been shown that after systemic administration, LV integrants persist in the bone marrow, liver and spleen (Pan *et al.* 2002). In the liver and spleen, these vectors transduce APCs, which explains the immune responses against the delivered transgene that are observed after intravenous injection (VandenDriessche *et al.* 2002; Palmowski *et al*. 2004).

There is evidence that expression of LV-delivered transgenes persist in these cells for months (VandenDriessche *et al.* 2002; Kimura *et al.* 2007). In the steady state, the longevity of different APC subpopulations differs. Macrophages in the mouse spleen are mostly long-lived cells (Wijffels *et al.* 1994). In contrast, DCs have a rapid turnover and an average half-life of 2-3 days (Kamath *et al.* 2002). Even the relatively long-lived plasmacytoid DC subset has a lifespan of 14 days (O'Keeffe *et al.* 2002). This raises the question of how is the population of LV-modified APCs maintained in spleen for several months.

Much of the research on LVs as vaccine vectors has focused on genetic modification of DCs because of their functional plasticity and efficient antigen presentation ability of (Banchereau & Steinman 1998). Considering the interest on these cells, the following sections will analyse some aspects of the DC biology in more detail. Since this thesis is based on a murine model, most of the information will refer to the mouse system, unless otherwise specified.

3.1.1. DC classification

DCs comprise a widely distributed network of cells that vary in haematological origin, life cycle and functional properties. They share common specialised features that allow them to be grouped in a single family: (1) they possess all the necessary machinery to pick up, process and present antigens to T cells, (2) they have migratory competence and (3) they are capable of sensing and translating environmental signals into information that ultimately dictates the fate of T cells (Reis e Sousa 2006; Steinman & Banchereau 2007; Shortman & Liu 2002).

DCs can be classified according to different criteria, including developmental stage, life history, anatomical location, presence in a steady-state or inflammatory environment, activation status and into different subtypes based on several markers (Naik 2008). Figure 3.1 shows a general classification of DCs according to these criteria.

3.1.1.1. Conventional DCs

Conventional DCs (cDCs) are fully differentiated cells that present the typical DC morphology and function in the steady state. Based on their life history, cDCs can be divided into migratory or resident DCs. The subclassification of cDCs and their frequency in different lymphoid organs is shown in Table 3.1.

3.1.1.1.1. Migratory DCs

This population of DCs are found in the periphery (*e.g.*, skin, mucosae), where they function as antigen-sampling sentinels. They migrate to the regional draining lymph nodes via afferent lymphatic vessels. During the steady state, migration occurs at a basal rate and is important for the maintenance of peripheral tolerance (Hemmi *et al.* 2001). In the presence of 'danger' signals, DCs migrate to secondary lymphoid organs and progressively mature. Maturation is accompanied by an increase in the antigen presentation ability and loose of migratory capacity (Reis e Sousa 2006).

This category of DCs include the epidermal Langerhans cells ($CD11c^+$ MHC-II^+ $langerin^+$) and interstitial DCs ($CD11c^{int}$ MHC-II^{hi} $DEC\text{-}205^{int}$), present in the mucosae and in the dermis, some of which also express langerin. These cells serve two functions: transportation of the antigen to the lymph node and antigen presentation to cognate T cells. Some studies have suggested that Langerhans cells are not directly involved in T cell priming but only in the transport of antigen to the lymph node, where it is captured and presented by resident DCs (Allan *et al.* 2003; Zhao *et al.* 2003). It has been proposed that these cells are potentially dispensable for the priming of the immune response (Brewig *et al.* 2009; Henri *et al.* 2007). Therefore, more research is necessary to clarify their role as APCs. Instead, interstitial DCs have been involved in T cell priming during hypersensitivity responses (Bennett *et al.* 2007) and certain infections, including HSV-2 (Zhao *et al.* 2003) and cutaneous leishmaniasis (Brewig *et al.* 2009; von Stebut *et al.* 2000).

3.1.1.1.2. Resident DCs

This group refers to DCs that reside in lymphoid organs, where they collect and present foreign and self antigens. They can be divided in subsets that differ in anatomical location, function and expression of CD4, CD8α, CD11b and DEC-205, among other markers (Table 3.1) (Shortman & Liu 2002).

$CD8\alpha^+$ cDCs are concentrated in the T cell areas of the spleen and lymph nodes (Naik 2008). This anatomical location reflects the importance of their interaction with T cells, on which they exert two opposite effects. In the steady state, $CD8\alpha^+$ DCs play an important role in generating peripheral tolerance (Belz *et al.* 2002). Yamazaki *et al.* (2008) have shown that these cells induce IFN-β-dependent generation of regulatory T cells (T_{reg}).

On the other hand, activated $CD8\alpha^+$ DCs are potent initiators of immune responses and are the main T cell-priming APC in some infections (Smith *et al.* 2003). They have a high expression of TLR3 but not of TLR7, which suggests they preferentially recognise double-stranded RNA (Rizzitelli *et al.* 2005). Upon activation, they produce large amounts of IL-12p70, which is crucial for the generation of $CD4^+$ T_H1 responses, cytotoxic $CD8^+$ T cells and memory T cells

(Maldonado-López *et al.* 1999). CD8α$^+$ DCs also have a superior ability for cross-presentation (den Haan *et al.* 2000; Pooley *et al.* 2001), which explains their important role in the immune response against viral infections (Heath *et al.* 2004).

CD8α$^-$ cDCs are present in the marginal zones of lymph nodes and spleen, but migrate to the T cell areas upon activation. These cells are also potent T cell primers, but tend to polarise CD4$^+$ T cells towards a T_H2 response (Maldonado-López *et al.* 1999). CD8α$^-$ DCs can be subdivided into CD4$^+$ and CD4$^-$. The difference between these two subpopulations is not completely understood. CD8$^-$ CD4$^-$ DCs are thought to be an important source of IFN-γ during immune responses (Hochrein *et al.* 2001) and can also produce enough IL-12p70 to elicit a T_H1 response (Shortman & Liu 2002).

In spite of the differences between DC subsets, these cells display a great plasticity in directing T_H development. The same DC subset can trigger a T_H1 or T_H2-polarised response depending on other factors besides their phenotype, such as the type of stimulus they receive (Manickasingham *et al.* 2003).

3.1.1.2. DC 'immediate' precursors

The DC population also includes its 'immediate' precursors, which are the last stage *en route* to DC differentiation. These cells do not have dendritic morphology or full DC function, but they develop into DCs with little or no cell division. The final differentiation steps can take place spontaneously in the steady state, or may require some additional stimulus, such as inflammation or infection (Shortman & Naik 2007). This category includes monocytes, plasmacytoid DCs (pDCs) and DC precursors that differentiate into resident DCs (pre-DCs).

3.1.1.2.1. Monocytes as precursors of DCs

Monocytes are cells from the mononuclear phagocytic system that are present in circulation and in several organs. They derive from a common macrophage-DC precursor (MDP) of myeloid origin, from which a monocyte/macrophage precursor (MMP) originates and differentiates into monocytes. In the mouse, there are two monocyte subpopulations: non-inflammatory Ly6C^{low} and inflammatory Ly6C^{high}.

These correspond to human $CD14^+$ and $CD16^+CD14^+$ monocytes, respectively (Naik 2008).

For a long time monocytes were thought to be the principal DC precursors mainly because *in vitro* they can differentiate into $CD11c^{high}MHC\text{-}II^+CD8^-$ DCs in the presence of GM-CSF (± IL-4). However, *in vivo*, the role of monocytes and GM-CSF in the generation of DCs in the steady state is controversial. The evidence so far indicates that monocytes differentiate into DCs in some tissues only under certain circumstances (Randolph *et al.* 1999; Varol *et al.* 2007) and that $Ly6C^{high}$ monocytes can differentiate into inflammatory DCs (Serbina *et al.* 2003).

3.1.1.2.2. Plasmacytoid DCs (pDCs)

pDCs are a widely distributed type of DCs, found in the blood, thymus, BM, liver and T cell areas of lymphoid organs. (Asselin-Paturel *et al.* 2003). They have a round morphology and low expression of MHC and T cell co-stimulatory molecules, which renders them with a poor antigen presentation ability. Upon stimulation, they acquire dendritic morphology and upregulate expression of MHC and co-stimulatory molecules. Thus, circulating pDCs are considered DC immediate precursors, *i.e.*, they must undergo a few divisions and differentiation steps to become mature DCs (Shortman & Naik 2007).

pDCs can be identified by expression of several markers: $CD11c^{low}$ Gr-1$(Ly6G)^+$ B220$(CD45R)^+$ $CD4^+$ $CD8\alpha^-$ $CD11b^-$ PDCA-1^+ (O'Keeffe *et al.* 2002; Villadangos & Young 2008). However, the most characteristic feature of these cells is their ability to produce large amounts of type I IFN upon viral infection or stimulation with viral nucleic acid motifs, such as unmethylated CpG motifs and viral RNA (Asselin-Paturel *et al.* 2001; Grouard *et al.* 1997; Björck 2001). The response to these pathogens seems to depend on a discrete number of TLRs—particularly TLR-7 and TLR-9—as well as probably other PRRs (Kadowaki *et al.* 2001). Type I IFNs play an important role in the resistance to viral infections. They act as survival factors for pDCs, enhance the maturation and antigen cross-priming ability of cDCs, induce secretion of chemokines and cytokines, activate natural killer

cells and macrophages, and are involved in the generation of memory $CD8^+$ T cells (Fitzgerald-Bocarsly & Feng 2007).

In spite of the controversy in the past years regarding the capacity of pDCs to present antigen to T cells, it is now consensus that they can prime T cells with the same immunogenic or tolerogenic outcomes observed with cDCs. However, the MHC-II/peptide complexes in pDCs are not as long-lived as in cDCs and they are less efficient in cross-presenting antigen. These two traits make of pDCs a weaker APC as compared with cDCs (Villadangos & Young 2008).

3.1.1.3. Inflammatory DCs

A different subpopulation of DCs, called inflammatory DCs (iDCs), emerges during inflammatory processes. These cells characteristically produce TNF-α and inducible nitric oxide synthase (iNOS) and display $CD11c^{int}CD11b^{high}MAC\text{-}3^+$, as opposed to the $CD11c^{high}$ $CD11b^{low}MAC\text{-}3^-$ steady-state spleen DCs. (Serbina *et al.* 2003). iDCs have a different ontogeny from that of cDCs since they derive from circulating $Ly6C^{hi}$ monocytes through GM-CSF stimulation. Indeed, their development can be modelled *in vitro* by culture of BMDCs with this cytokine (Shortman & Naik 2007). Regarding their function, iDCs mediate innate immune responses against bacteria (Serbina *et al.* 2003) and have recently been shown to play an important role in generating T_H1-polarised responses to certain infections (Nakano *et al.* 2009).

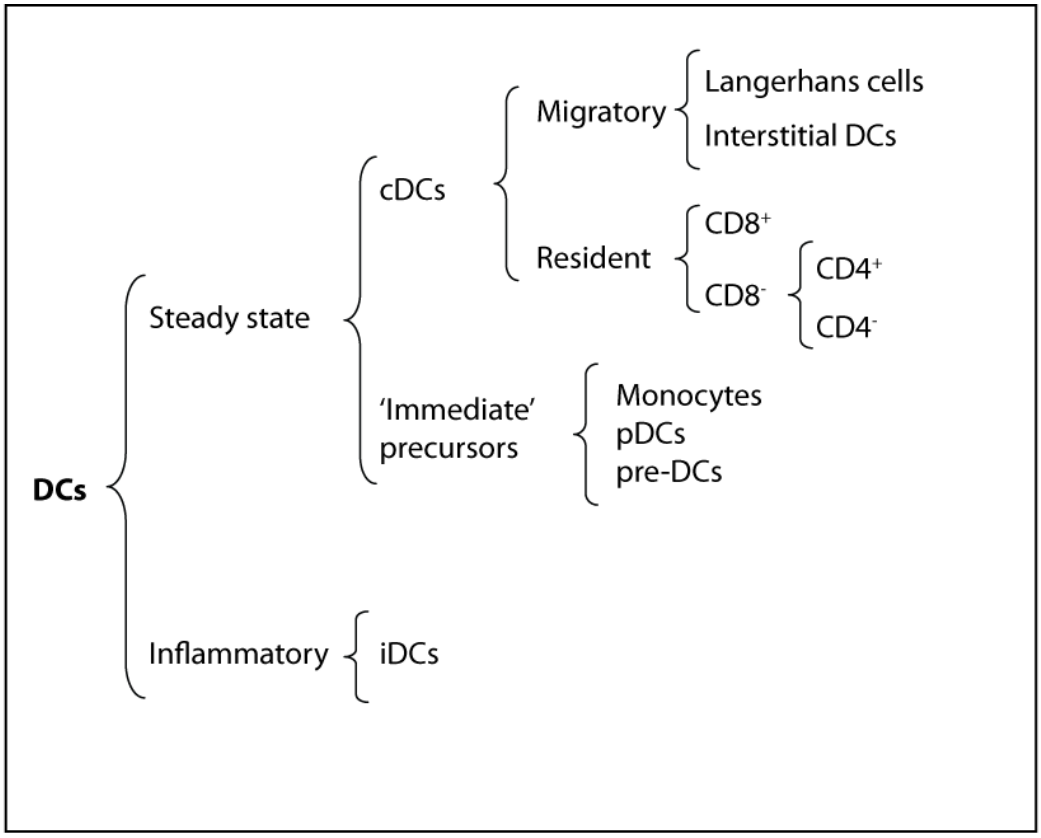


Figure 3.1. Classification of DCs. The mouse DC network comprises several cell subpopulations with phenotypical and functional differences. They are classified according to their presence in the steady state or in inflammatory conditions, their developmental stage, life history, anatomical location and different markers. cDCs = conventional DCs; pDCs = plasmacytoid DCs; pre-DCs = precursors of cDCs; iDCs = inflammatory DCs (Naik 2008; Shortman & Naik 2007).

3.1.2. DC turnover and homeostasis

DCs are short lived cells. Therefore, their homeostasis depends on constant replacement by new cells that derive from dividing precursors. This has been demonstrated by using *in vivo* BrdU incorporation as an indicator of cell division and turnover. These studies have shown that DC turnover rates differ among lymphoid organs and DC subsets (Table 3.2) (Kamath *et al.* 2000; Kamath *et al.* 2002; Ruedl *et al.* 2000). In the steady state, spleen DCs have a half life of 2-3 days. The $CD8\alpha^+$ subpopulation has a higher turnover than other cDC subsets. Furthermore, the life span of DCs is shortened by approximately one third upon antigen capture and maturation (Kamath *et al.* 2002).

The identification of the stages of differentiation and commitment to specific DC lineages has been a matter of debate. Although DC ontogeny is not completely understood, some precursors have been identified in the past decade.

3.1.2.1. Early DC precursors

All DC subpopulations derive from bone marrow haematopoietic progenitors. Both common lymphoid precursors (CLPs) and common myeloid precursors (CMPs) can give rise to all DC subpopulations. Commitment to the DC lineage seems to depend on the expression of the transcription factor fms-related tyrosine kinase 3 (Flt3) and its binding by Flt3 ligand (Flt3L) (Karsunky *et al.* 2003; Onai *et al.* 2007; Waskow *et al.* 2008). $CD8\alpha^+$ and $CD8\alpha^-$ cDCs, as well as pDCs, can be generated *in vitro* by culture of bone marrow cells in the presence of Flt3L. This cytokine plays an important role in maintaining DC homeostasis in the steady state and during inflammation, since it increases the number and mobilization of DCs to the periphery. Nevertheless, the presence of some DC subpopulations in *Flt3*$^{-/-}$ mice and absence of Flt3 expression in some precursors suggests that other factors must be involved in DC development (Tan & O'Neill 2007).

The majority of DCs seem to derive from myeloid precursors, probably because their larger number in the bone marrow (Shortman & Naik 2007). Liu *et al.*

(2009) have recently characterized the progress of DC development from early myeloid precursors, which give rise to monocytes, pDCs and pre-cDCs (Figure 3.2). This and other studies arrive to the following conclusions: (1) cDCs derive from precursors (pre-cDCs) that originate in the bone marrow and migrate to peripheral lymphoid organs, where they undergo their final differentiation steps; (2) monocytes diverge from the cDC lineage in the transition from a common macrophage-DC precursor (MDP) to a common DC precursor (CDP) in the bone marrow; they leave the bone marrow and can differentiate into macrophages or, in presence of inflammation, into DCs; (3) there is so far no identification of a committed pDC precursor in the periphery; instead, pDCs leave the bone marrow and progressively acquire maturation markers in the bloodstream and peripheral lymphoid organs (Diao *et al.* 2006; Liu *et al.* 2007; Liu *et al.* 2009; O'Keeffe *et al.* 2003; Shortman & Naik 2007).

In contrast to other haematopoietic cells, differentiation of DCs does not seem to be a linear process where specific precursors are restricted to give origin to a particular cell type. Instead, they can differentiate into one or other lineage at every stage of differentiation depending on factors that are still not completely understood (Naik 2008).

3.1.2.2. Immediate DC precursors

In the past few years, a broadly distributed population of committed DC precursors (pre-DC) has been identified in lymphoid organs and in circulation. In the steady state, these pre-DCs differentiate *in situ* into cDCs after 0-3 cell divisions. They are also detected in inflamed tissues and may be important to increase the local number of DCs in situations where more antigen processing and presentation is required (Diao *et al.* 2006; Naik *et al.* 2006; del Hoyo *et al.* 2002).

In the spleen, pre-DCs constitute a small population (0.05%) that can generate all the resident cDC subsets from this organ, but not pDCs. They are characterised by an intermediate expression of CD11c, no expression of MHC-II, absence of lineage markers (CD4, CD8, B220, CD3, CD19, Gr-1, F4/80, DX5), and intermediate expression of CD43 and signal-regulatory protein (SIRP)-α (Naik *et al.*

2006). Equivalent cells have been identified in bone marrow, thymus, Peyer's patches and lymph nodes (Diao *et al.* 2006).

Pre-DCs can be divided in two subpopulations. $CD24^+$ pre-DCs give rise to $CD8\alpha^+$ cDCs, while $CD24^-$ pre-DCs give origin to $CD8\alpha^-$ cDCs (Fig. 3.1) (Naik *et al.* 2006). However, the ontogeny of $CD8^-$ $CD4^-$ and $CD8^-$ $CD4^+$ is not clear yet.

Table 3.1. Frequency of DC subtypes in different lymphoid organs[a].

Lymphoid organ	Resident cDCs			Intestitial DCs	Langerhans cells
	$CD8^+$ $CD4^-$ $CD205^+$ $CD11b^-$	**$CD8^-$ $CD4^+$ $CD205^-$ $CD11b^+$**	**$CD4^-$ $CD8^-$ $CD205^-$ $CD11b^+$**	**$CD4^-$ $CD8^-$ $CD205^+$ $CD11b^+$**	**$CD4^-$ $CD8^{lo}$ $CD205^{hi}$ $CD11b^+$**
Spleen	23	56	19	<4	<1
Thymus	70				
Mesenteric LN	19	4	37	26	<4
$Skin^-$ draining LN	17	4	17	20	33

[a] Percentages of each subpopulation in the indicated organ.
Reproduced from Shortman & Liu. *Nature Reviews Immunology*. 2002. 2(3): 151-161, with permission of the publisher.

Table 3.2. Half life[a] of DC subpopulations in the spleen and lymph nodes (LN) based on BrdU incorporation.

DC subset	Spleen	Mesenteric LN	Skin-draining LN
Total DCs	3	3.5	10
$CD8^+$ $CD4^-$	1.5	3	7
$CD8^-$ $CD4^+$	2.9		
$CD8^-$ $CD4^-$	3	3.1	9
Interstitial		4[b]	11[b]
Langerhans cells			21[b]

[a] Half life defined as the number of days required for incorporation of BrdU in 50% of the population.
[b] Since these cells are migratory, these values reflect the total half life of the cell population and not the half life in the lymph node, which is shorter (2-3 days) (Kamath *et al. Blood*, 2002. 100(5) : 1734-1741).

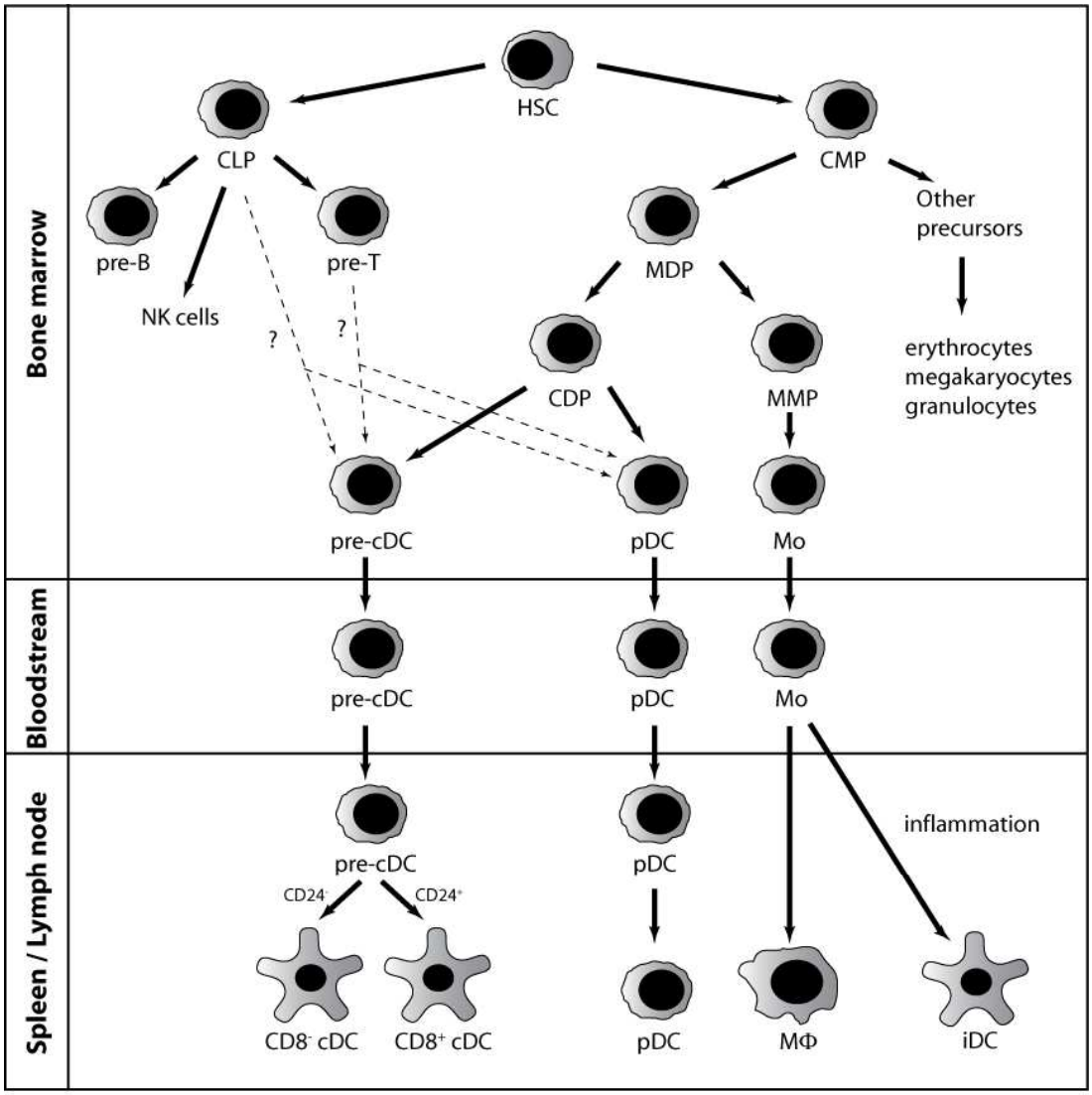


Figure 3.2. Development of murine DC subtypes. DCs derive from both lymphoid and myeloid precursors in the bone marrow, although the myeloid lineage has been better characterised. Committed conventional DC precursors (pre-cDC) originate from a common macrophage/DC progenitor (MDP) and migrate to peripheral lymphoid organs where they differentiate into all cDC subtypes. Plasmacytoid DCs (pDCs) leave the bone marrow and can be found in the bloodstream and peripheral tissues. Under inflammatory conditions, some monocyte subsets differentiate into inflammatory DCs (iDCs). No cells isolated from the mouse bloodstream have been shown to present antigen and activate T cells without prior differentiation. HSC = haematopoietic stem cell; CLP = common lymphoid precursor; CMP = common myeloid precursor; NK = natural killer cells; MDP = macrophage/DC precursor; CDP = common DC precursor; MMP = monocyte/macrophage precursor; pre-cDC = cDC precursor; pDC = plasmacytoid DC; Mo = monocyte; MΦ = macrophage; iDC = inflammatory DC; cDC = conventional dendritic cell.

Modified from Liu et al. *Science*, 2009. 324(5925): 392-397, with permission of the publisher.

DC transduction by LVs is thought to be instrumental for the generation of an immune response to the transgene. The characterisation of different cell subtypes transduced *in vivo* has not been studied in detail, and the persistence of expression in APCs remains unclear. This is important to understand the immune responses or possible risks associated with LVs.

3.1.3. Aims

- Characterise the different subpopulations of cells transduced in the mouse spleen after systemic administration of LVs.

- Determine whether APCs maintain expression of the transgene over time and the possible reasons for this.

3.2. Results

3.2.1. Long term GFP expression in DC subsets in the spleen

Previous studies have shown that expression of reporter genes can be detected in the lymph node after subcutaneous injection of LVs (Lopes *et al.* 2008) and in the spleen after intravenous administration (Rowe *et al.* 2006; Palmowski *et al.* 2004). However, no transgene expression can be detected in the lymph node after 10 days (Dullaers *et al.* 2006), probably because of the short half life or migration patterns of targeted cells. Instead, transduced cells persist for a longer time in the liver, spleen and bone marrow after intravenous administration (Pan *et al.* 2002; VandenDriessche *et al.* 2002).

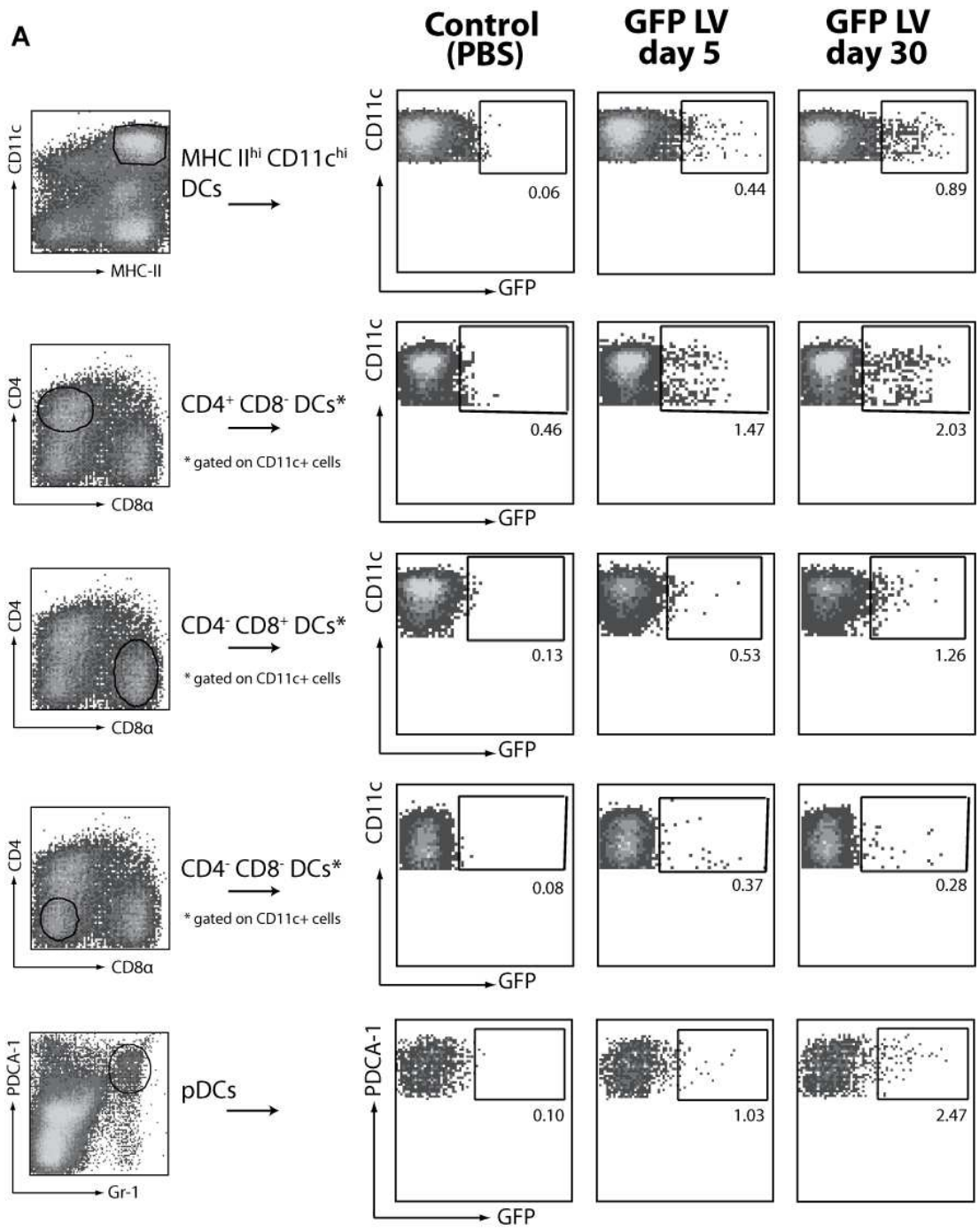
Firstly, the transduction of different APC subpopulations in the spleen following intravenous LV injection was analysed. In these experiments, a LV expressing GFP and a second IiOVA-GFP LV expressing GFP and an ovalbumin fragment fused with the invariant chain were used (Fig. 2.1A-B). GFP expression was then examined both in the short term (5 days) and in the long term (30 days) in

different cells populations in the spleen. Figure 3.3 shows results with the GFP LV; although all the DC subsets present in the spleen were transduced, the percentage was highest in the $CD4^+$ $CD8^-$ subpopulation (Fig. 3.3A), which is also the most abundant in the spleen (Shortman & Liu 2002). Transduction of plasmacytoid DC, macrophages and B cells was also detected (Fig. 3.3B).

Table 3.3 shows that, while there was a considerable variation between experiments, the mean percentage of transduction of DCs and macrophages increased with time using the GFP LV, while the percentage of transduced B cells decreased. T cells were relatively poorly transduced by the LV and almost no transduced T cells were present after 30 days.

The percentage of GFP-expressing cells that could be detected was lower when the IiOVA-GFP LV was used (Table. 3.4), although the pattern of transduction was similar. In this case, the percentage of transduced cells in most DC subsets and macrophages decreased with time, similarly to T and B cells.

Figure 3.3


A
Control (PBS)
GFP LV day 5
GFP LV day 30
CD11c
MHC-II
MHC II^hi CD11c^hi DCs
GFP
0.06
0.44
0.89
CD4
CD8α
CD4+ CD8- DCs*
* gated on CD11c+ cells
0.46
1.47
2.03
CD4- CD8+ DCs*
0.13
0.53
1.26
CD4- CD8- DCs*
0.08
0.37
0.28
PDCA-1
Gr-1
pDCs
0.10
1.03
2.47

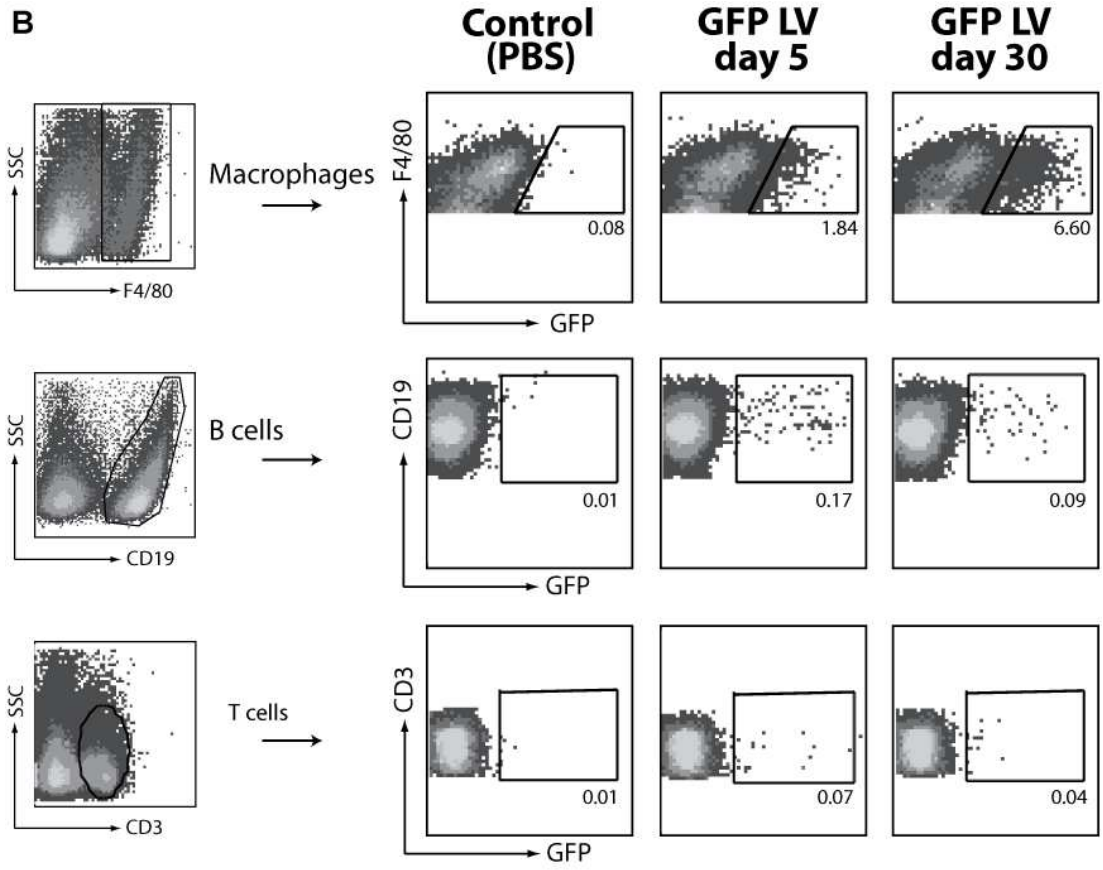


Figure 3.3. Representative experiment of *in vivo* tracking of transduced cell subsets in the spleen. Mice (3 per group) were injected iv with PBS or 10^8 iu of GFP LV. Five or 30 days later, spleens were harvested, pooled and sorted with CD11c magnetic microbeads. Cells were stained with antibodies to analyse transduction of different DC subsets (A), macrophages and lymphocytes (B) by flow cytometry. A summary of 4 similar experiments is shown in table 3.3. The same experiment was performed using IiOVA-GFP LV; the results are shown in table 3.4.

Table 3.3. Percentage of transduced cells[a] in the spleen 5 or 30 days after iv injection of GFP LV.

Cell subset	GFP LV % GFP^+ cells			
	Day 5[b]	***p***	**Day 30**[b]	***p***
MHC II^{hi} $CD11c^{hi}$ DCs	1.15 ± 0.56	0.0018	4.01 ± 3.33	0.0042
$CD4^+$ $CD8^-$ DCs	2.42 ± 1.60	0.0031	3.49 ± 1.85	0.0041
$CD4^-$ $CD8^-$ DCs	0.93 ± 0.16	0.0222	2.25 ± 1.44	0.0106
$CD4^-$ $CD8^+$ DCs	0.71 ± 0.53	0.0314	1.83 ± 1.49	0.042
pDCs	2.07 ± 0.55	0.0358	4.27 ± 0.93	0.0358
Macrophages	7.47 ± 8.12	0.0238	12.69 ± 7.13	0.0238
B cells	1.03 ± 1.11	0.0046	0.24 ± 0.15	0.0123
T cells	0.81 ± 0.75	0.0159	0.19 ± 0.08	0.0655

[a] Percentage of GFP^+ cells after sorting of splenocytes with anti-CD11c microbeads. DCs and macrophages were analysed in the $CD11c^+$ fraction and lymphocytes in the $CD11c^-$ fraction.
[b] Means ± SD. Control (PBS) *vs.* treated groups were compared with Mann-Whitney test.

Table 3.4. Percentage of transduced cells[a] in the spleen 5 or 30 days after iv injection of IiOVA-GFP LV.

Cell subset	IiOVA-GFP LV % GFP^+ cells			
	Day 5[b]	***p***	**Day 30**[b]	***p***
MHC II^{hi} $CD11c^{hi}$ DCs	0.25 ± 0.25	0.0336	0.23 ± 0.21	0.0069
$CD4^+$ $CD8^-$ DCs	0.64 ± 0.75	0.0606	0.25 ± 0.19	0.5120
$CD4^-$ $CD8^-$ DCs	0.25 ± 0.20	0.3605	0.40 ± 0.06	0.0294
$CD4^-$ $CD8^+$ DCs	0.39 ± 0.16	0.0195	0.16 ± 0.10	0.6012
pDCs	0.71 ± 0.19	0.0178	0.25 ± 0.23	0.3539
Macrophages	1.01 ± 1.50	nd	0.63 + 0.01	nd
B cells	0.24 ± 0.11	0.0172	0.07 ± 0.05	0.8114
T cells	0.15 ± 0.09	0.1745	0.02 ± 0.08	0.2016

[a] Percentage of GFP^+ cells after sorting of splenocytes with anti-CD11c microbeads. DCs and macrophages were analysed in the $CD11c^+$ fraction and lymphocytes in the $CD11c^-$ fraction.
[b] Means ± SD. Control (PBS) *vs.* treated groups were compared with Mann-Whitney test; nd = not determined.

3.2.2. LVs transduce dividing DC precursors.

One of the possible explanations for the persistence of antigen presentation after administration of LVs is the transduction DC precursors. To test this hypothesis, the incorporation of BrdU in transduced DCs was evaluated. BrdU is incorporated into dividing DC precursors whose progeny renew the DC population in different lymphoid organs (Kamath *et al.* 2002). To avoid the loss of fluorescence of GFP after intranuclear staining of BrdU, the transduced cells were identified by the expression of the surface marker Thy1.1 (CD90.1) (Fig. 2.1B). Thy1 is a small glycophosphatidylinositol-anchored membrane protein that is expressed in T cells, thymocytes and nervous system in the mouse. It is involved in cell-to-cell interactions both in neural and immunological synapses. Thy1 plays a role in T cell activation, although its function in the immune response is not well characterised since no ligands or receptors have been identified so far. Different mouse strains selectively express one of two allelic forms of Thy1 (Thy1.1 and Thy1.2) that differ in one amino acid in position 89 (arginine in Thy1.1 and glutamine in Thy1.2) (Rege & Hagood 2006; Haeryfar & Hoskin 2004). C57BL/6 mice express Thy1.2. Hence, in these mice, Thy1.1-expressing cells can be identified with an antibody specific for this variant.

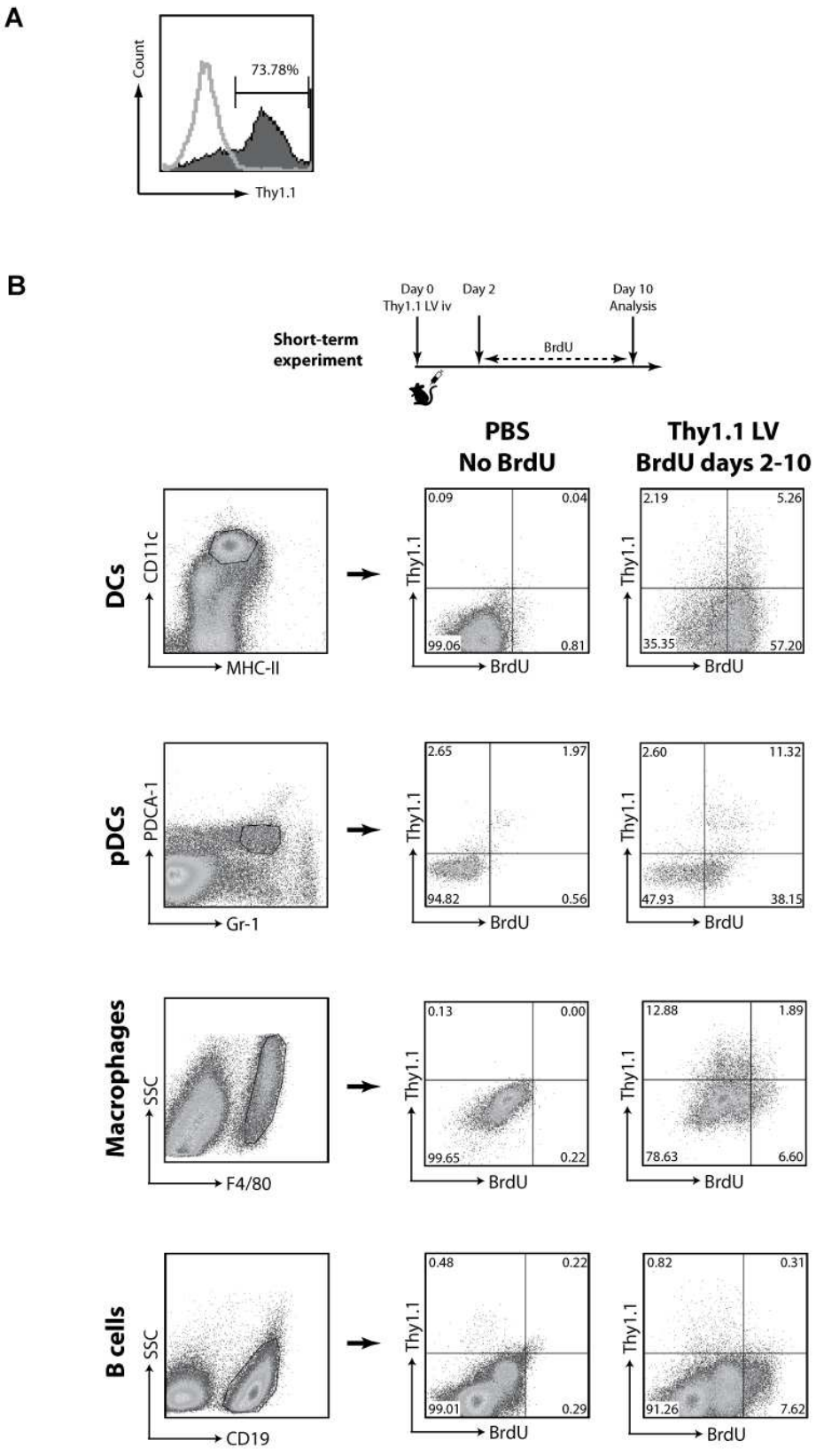
In the first experiment, mice were injected intravenously with a Thy1.1 LV (Fig. 2.1B). Then after 48 hours, BrdU was administered intraperitoneally to ensure immediate access of the label to dividing precursors. BrdU was continually administered in drinking water for ten days (to avoid toxic effects of long-term administration), and then splenocytes were analysed for BrdU incorporation and LV transduction.

Figure 3.4 shows that the majority of splenic DCs and pDCs had proliferated after 10 days, as previously reported (Kamath *et al.* 2002). In contrast only a small fraction of splenic macrophages and B cells had divided at this time point. Strikingly, a large proportion of transduced DCs and pDCs had divided. LV transduction was similar in the BrdU-labelled and BrdU-unlabelled subsets of DCs and higher in the BrdU-labelled subset of pDCs (Table 3.5). BrdU was incorporated at a similar rate

in transduced and non-transduced macrophages. The vast majority of B cells had not divided. However, LV transduction was more efficient in the population that had incorporated BrdU.

In a second experiment, BrdU was administered between day 20 and day 30 after intravenous LV injection. Splenocytes were then analysed on day 30. Consistent with the data in table 3.3, there was an increase in the percentage of transduced DCs over time. A similar difference between the transduced cells was also observed, with the DCs and pDCs having recently proliferated and the macrophages and B cells largely remaining non-dividing during this period. These data demonstrate that LVs can transduce dividing progenitor cells that give rise to DCs after intravenous injection. Such transduced progenitors supply DCs in the spleen for at least 30 days after LV injection.

Figure 3.4


A
Count
73.78%
Thy1.1
B
Day 0
Thy1.1 LV iv
Day 2
Day 10
Analysis
Short-term
experiment
BrdU
PBS
No BrdU
Thy1.1 LV
BrdU days 2-10
DCs
CD11c
MHC-II
0.09
0.04
99.06
0.81
2.19
5.26
35.35
57.20
pDCs
PDCA-1
Gr-1
2.65
1.97
94.82
0.56
2.60
11.32
47.93
38.15
Macrophages
SSC
F4/80
0.13
0.00
99.65
0.22
12.88
1.89
78.63
6.60
B cells
SSC
CD19
0.48
0.22
99.01
0.29
0.82
0.31
91.26
7.62
Thy1.1
BrdU

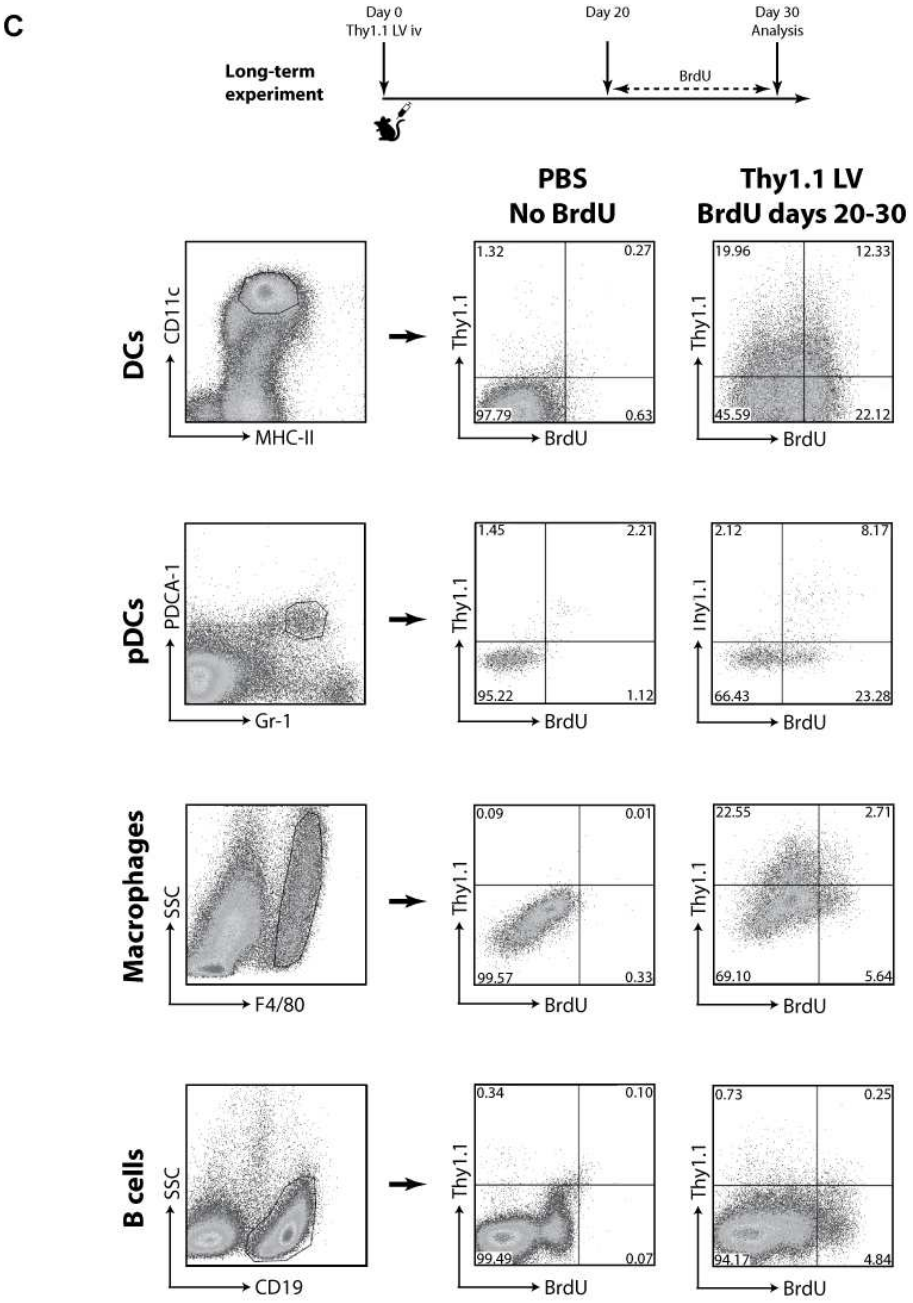


Figure 3.4. BrdU incorporation in transduced cells. A LV encoding Thy1.1 (Fig. 2.1) was used to track transduced cells by antibody surface staining and flow cytometry. Expression of Thy1.1 was first tested *in vitro* (A) by transduction of bone marrow-derived DCs (filled histogram = moi 10; unfilled histogram = non-transduced). Mice (3 per group) were injected iv with Thy1.1 LV. Two days later, they received 200 μg of BrdU ip followed by continuous provision of BrdU (0.8 mg/mL) in the drinking water from days 2 to 12 (B). In a separate experiment, BrdU was provided from days 20 to 30 post LV injection (C). Incorporation of BrdU was analysed by antibody staining and flow cytometry in different subpopulations of the $CD11c^+$ and $CD11c^-$ fractions of spleen cells. The control group was injected with PBS and received no BrdU. Mice were analysed individually and one respresentative case is shown. Results are summarised in Table 3.5.

Table 3.5. BrdU incorporation in transduced cells after iv injection of Thy1.1-LV[a].

	BrdU days 2-12			BrdU days 20-30		
	% transduced[b]	**% BrdU$^+$ among transduced**	**% BrdU$^+$ among non transduced**	**% transduced[b]**	**% BrdU$^+$ among transduced**	**% BrdU$^+$ among non transduced**
DCs	14.86 ± 5.68	64.62 ± 14.39	55.30 ± 15.75	32.55 ± 20.56	41.07 ± 6.80	39.85 ± 8.36
pDCs	6.30 ± 5.39	79.15 ± 6.08	39.57 ± 5.01	4.29 ± 3.31	75.44 ± 5.60	24.75 ± 1.70
Macrophages	26.52 ± 11.66	7.30 ± 4.88	4.78 ± 2.67	22.62 ± 3.60	10.20 ± 0.75	6.96 ± 0.84
B cells	0.79 ± 0.43	31.50 ± 3.53	6.89 ± 1.35	0.30 ± 0.34	24.76 ± 1.07	4.62 ± 0.38

[a] Means ± SD.
b Percentage of Thy1.1$^+$ among different populations of cells pre-sorted with anti-CD11c microbeads. Staining for DCs, pDCs and macrophages was done in the CD11c$^+$ fraction; staining for B cells was done in the CD11c$^-$ fraction.

3.3. Discussion

In vivo transduction of APCs is crucial for the function of LVs as vaccine vectors. Transduction of spleen DCs had been reported following intravenous administration of LVs (Palmowski *et al.* 2004). Here it is shown that in fact, systemic LV administration results in sustained transgene expression in all DC subsets and in macrophages for at least 30 days. This agrees with previous studies that show persistence of LV integrants and transgene expression in liver and spleen in the long term (Esslinger *et al.* 2003; Pan *et al.* 2002; VandenDriessche *et al.* 2002).

A smaller proportion of transduced cells was observed when employing a LV encoding OVA. One explanation for this could be a lower expression of GFP because in the IiOVA-GFP construct its expression is driven by a weaker (UBI). On the other hand, OVA is a stronger immunogen than GFP in C57BL/6 mice. Expression of this molecule in APCs can lead to their elimination by the CTL response in a feedback mechanism like the one described for infectious agents (Wong & Pamer 2003). This has also been suggested by the fact that the percentage of transduced APCs is higher in immunodeficient than in immunocompetent mice (VandenDriessche *et al.* 2002). It also agrees with the fact that the percentage of transduced cells was higher and increased over time when another weak immunogen, Thy1.1, was expressed.

The functional differences among DC subsets are not completely understood. It has been suggested that generation of the immune response probably involves the simultaneous participation of several DC subtypes (Villadangos & Schnorrer 2007). In this sense, genetic modification of the different subpopulations might be important. The majority of transduced DCs are $CD4^+$ $CD8^-$, which can be explained because this subpopulation of DCs is the most abundant in the steady state, accounting for over half of all spleen DCs (Shortman & Liu 2002). Furthermore they have a slightly longer half life than $CD4^-$ DCs (Kamath *et al.* 2000) and are localised in the marginal zone of the spleen, which makes them more accessible for the virus in the bloodstream (McLellan *et al.* 2002). It has been proposed that $CD8^+$ DCs tend

to polarise a T_H1 cell response while $CD8^-$ DCs polarise T_H2 responses (Maldonado-López *et al.* 1999). However, although the effect of targeting specific DC subtypes with LVs remains to be explored, transduction of different DC subtypes with VSV-G-pseudotyped vectors has been reported to generate both $CD4^+$ and $CD8^+$ T cell responses and efficient immunisation with LVs (Dullaers *et al.* 2006; Loisel-Meyer *et al.* 2009; Rowe *et al.* 2006).

LVs also transduce pDCs, whose antigen-presenting function is more controversial. However, it has been shown that these cells are important in the initiation of innate anti-viral responses (Brown *et al.* 2007) and have become a potential target for vaccination.

Transduction of macrophages and B cells probably participate in T cell priming following immunisation with LVs. It is known that macrophages are effective stimulators of memory T cells, although not so effective as primers of naïve T cells (Macatonia *et al.* 1992). B cells can induce both effector or regulatory T cell differentiation and expansion (Chen & Jensen 2008). However, LVs targeted to specifically transduce DCs still generate an effective immune response despite a poor transduction of other APCs (Lopes *et al.* 2008; Yang *et al.* 2008).

Interestingly, transduced DCs were still present 30 days later even when their lifespan is known to be limited to ~2-3 days in the case of cDCs, ~14 days in pDCs or even shorter after they are activated (Kamath *et al.* 2002; O'Keeffe *et al.* 2002). Indeed, the percentage of GFP^+ cells increased over time in all DC subsets and macrophages, but not in T cells and B cells. This contrasts with other studies that have shown a decrease in transgene expression (Kimura *et al.* 2007) or in the number of LV integrants (Pan *et al.* 2002) in the spleen over time. However, these studies have not specifically addressed transgene expression in DCs.

It was hypothesised that a reason for this could be the transduction of proliferating cells that give rise to a long term supply of genetically-modified DCs in the spleen. The fact that DCs are the only predominantly BrdU-labelled, transduced population argues for the transduction of a committed DC progenitor that has diverged from common progenitors of macrophages and lymphocytes. Macrophages instead are long-lived, non-dividing cells (Wijffels *et al.* 1994), as reflected by the

absence of BrdU incorporation (Fig. 3.4). Their long life span could explain the persistence of transgene expression in this cell population.

DC precursors are present in the bone marrow (Kang Liu *et al.* 2009) but also locally in the spleen (Naik *et al.* 2006). Long-term DC precursor transduction in the spleen is only observed after intravenous but not after subcutaneous injection. Bone marrow precursors could also be transduced after subcutaneous injection and give rise to transduced DCs in other lymphoid tissues, but this has not been observed (Rowe *et al.* 2006). Furthermore, the fact that other lineages like lymphocytes or macrophages do not incorporate BrdU argue for the transduction of a committed local progenitor in the spleen more than an earlier progenitor in the bone marrow.

Against this argument is the fact that pDCs also incorporate BrdU. No local precursor of these cells has been identified in peripheral tissues, although this remains a possibility. It is generally accepted that pDCs originate from the bone marrow, enter the bloodstream and are distributed to all lymphoid organs. They also have a longer life span than cDCs (Liu *et al.* 2009; O'Keeffe *et al.* 2002). Therefore, incorporation of BrdU in transduced pDCs can only be explained either by transduction of a precursor in the bone marrow, a not yet identified local precursor in the spleen or that, being precursors themselves, LV transduction induces activation and proliferation of these cells.

The presence of divided transduced cells at least 30 days after LV injection suggests either that the splenic DC precursors proliferate for up to 30 days after transduction, or that transduced, dormant precursors are activated to proliferate throughout this period. Part of the BrdU incorporation might also come from the clonal expansion of cDCs themselves, as recent evidence shows that DCs are not necessarily end cells as generally assumed, but retain some capacity for further division (Kabashima *et al.* 2005; Zhang *et al.* 2004; Diao *et al.* 2007).

To confirm transduction of DC precursors, transfer of transduced splenocytes from mice injected with LVs to naïve mice was attempted. However, it was not possible to identify a transduced progeny of DCs in purified $CD11^+$ cells from the recipients' spleens. This can be attributed to their low number and to the purification techniques that were employed. It is thus necessary to validate this hypothesis with further experiments, including reconstitution of bone marrow or precursor assays in

irradiated and non-irradiated mice, as well as *ex vivo* transduction of purified precursors with LVs.

In conclusion, these results suggest that transduction of proliferating DC precursors can give rise to long term supply of these cells in the spleen, allowing a sustained, potentially life-long, transgene expression. This is relevant considering the current interest in using LVs as vaccine vectors or as genetic immunomodulators.

CHAPTER 4

4. PERSISTENCE OF ANTIGEN PRESENTATION AFTER ADMINISTRATION OF LENTIVIRAL VECTORS

4.1. Introduction

The persistence of transgene expression in DCs calls into question whether LV transduction of these cells results in durable antigen processing and presentation. The consequences of persistent antigen presentation have been previously addressed in various models, but they are still not fully understood. Although antigen presentation for several months is beyond the time frame of an optimal primary immune response, it has also been proposed that persistence of antigen is important for the vaccine function of some vectors including adenovirus (Tatsis *et al.* 2007), VSV (Simon *et al.* 2007) and LVs (He *et al.* 2005; Kimura *et al.* 2007).

4.1.1. Regulation of the longevity of antigen presentation

The induction of CTL responses is a complex process triggered by the encounter of antigen presented in APCs and cognate T cells. This can result in full activation of the T cell, with acquisition of effector functions and generation of memory, or in tolerance due to clonal anergy or deletion. The balance between these rather opposite outcomes depends on the conditions in which the antigen is encountered. Several factors, including the activation status of the APC, presence of cytokines, antigen distribution, localisation and amount, as well as duration of antigen presentation, play an important role in defining the fate of the cell (Lanzavecchia & Sallusto 2000; den Boer *et al.* 2001).

In general, the time during which antigen is presented throughout a CTL response is limited by several mechanisms. Firstly, the MHC-I/peptide complexes have a short half life (~10 hours). The maintenance of antigen presentation depends on the continuous synthesis and antigen loading of MHC-I (Lanzavecchia & Sallusto 2000). Rapid clearance of MHC-I/peptide complexes might explain why CTL priming is impaired when antigen supply is restricted to short periods of time (Ludewig *et al.* 2001).

Antigen presentation is also limited by the life span of activated APCs. In the case of DCs, stimulation of TLRs induces accumulation of pro-apoptotic factors (Hou & Van Parijs 2004). Signalling through other receptors results in nuclear translocation of NFAT (Zanoni *et al.* 2009). These two events result in cell death. Activated T cells also deliver pro- and anti-death signals to DCs during the CTL response. $CD8^+$ T cells eliminate DCs via secretion of cytolytic granules (Yang *et al.* 2006), while $CD4^+$ T cells induce DC apoptosis via extrinsic and intrinsic pathways (Matsue *et al.* 1999; Pradhan *et al.* 2006; Wang *et al.* 1999). In addition, $CD4^+$ T cells can also protect DCs from CTL-mediated elimination and prolong class I-restricted antigen presentation by engagement of RANK and CD40 (Chen *et al.* 2004; Mueller *et al.* 2006).

The balance between the mechanisms that prolong or limit the longevity of activated DCs is necessary for optimal $CD8^+$ T cell priming and to prevent aberrant T cell activation and autoimmunity. This is emphasised by the fact that extension of the life span of DCs can result in autoimmunity (Chen *et al.* 2006) and abnormal accumulation of antigen-specific $CD8^+$ T cells in lymphoid organs, which is associated with pathology (Pradhan *et al.* 2006; Ronchese & Hermans 2001).

4.1.2. Effects of persistent antigen presentation on the CTL response

A relatively short-term exposure to peptide/MHC-I complexes is all that is required for commitment of $CD8^+$ T cells to expansion and differentiation. *In vitro*, this initial stimulus can be as short as 2 hours (van Stipdonk *et al.* 2001; Wong & Pamer 2001). Nevertheless, a concerted '3 signal' stimulus for at least 24-40 hours is required for optimal clonal expansion and acquisition of function (Curtsinger *et al.*

2003). This is consistent with *in vivo* data showing that in some acute infections, antigen presentation is limited to ~24-36 hours, after which the immune response carries on independently (Wong & Pamer 2003; Norbury *et al.* 2002; Williams & Bevan 2004). Curtailing the duration of antigen presentation can attenuate the magnitude of the primary CTL response (Prlic *et al.* 2006; Stock *et al.* 2004). However, in some cases it has no effect, probably because of other factors such as ongoing inflammation (Mercado *et al.* 2000; Williams & Bevan 2004).

When antigen presentation persists after the initial stimulation period, it can further modulate—if not determine—the CTL response. This has been extensively studied in chronic bacterial and viral infections. Persistent antigen presentation results in continuous recruitment of naïve $CD8^+$ T cells in chronic lymphocytic choriomeningitis virus (LCMV) and polyoma virus infections (Vezys *et al.* 2006). Similarly, persistence of antigen in cytomegalovirus (CMV) infection drives differentiation of $CD8^+$ T cells and accumulation of memory cells that are functional, albeit not showing a completely activated phenotype (Snyder *et al.* 2008). Furthermore, naïve cells primed later in the immune response by ongoing antigen can differentiate into memory $CD8^+$ T cells (Turner *et al.* 2007).

However, antigen persistence can also result in exhaustion or induce T cell death due to continuous TCR triggering (Bucks *et al.* 2009; Mueller & Ahmed 2009; Wherry *et al.* 2003; Zajac *et al.* 1998). This has been related with the amount and distribution of the antigen. For example, LCMV strains that spread rapidly and systemically lead to a phase of strong $CD8^+$ T cell proliferation followed by clonal exhaustion, while strains that replicate slowly and spread less induce long-lasting CTL memory (Moskophidis *et al.* 1993). In general, antigen that is present systemically and in high amount is more likely to result in $CD8^+$ T cell disfunction, especially when it is presented by non-professional or immature APCs. Instead, localised antigen presentation, in lower amounts and in an environment of APC activation promotes a stronger CTL response (Zinkernagel & Hengartner 2004).

Although most of these studies refer to the priming of naïve $CD8^+$ T cells, the effect of antigen on memory $CD8^+$ T cells might be different. These cells are less dependent on antigen exposure and co-stimulation (Iezzi *et al.* 1998). The dependence of $CD8^+$ T memory cells on antigen presentation is controversial. It has

been reported that maintenance of these cells can be either antigen-dependent (Oehen *et al.* 1992; Kündig *et al.* 1996; Gray & Matzinger 1991) or independent (Müllbacher 1994; Lau *et al.* 1994; Bruno *et al.* 1995).

The longevity of antigen presentation can also modulate the CTL response through its effect on helper T cells. In contrast with $CD8^+$ T cells, $CD4^+$ T cells require antigen stimulation throughout their expansion phase (Obst *et al.* 2005a; Williams & Bevan 2004). It has been shown that prolonged antigen stimulation of $CD4^+$ T cells promotes their proliferation and activation even in the absence of DC maturation (Obst *et al.* 2007). Consequently, durable antigen presentation is necessary for an optimal $CD4^+$ T cell response, which is important for the generation of optimal primary CTL responses and $CD8^+$ T cell memory.

4.1.3. Persistent antigen presentation and vaccination

It has been proposed that persistence of antigen is important for the function of vaccines that aim to protect through cell-mediated immunity (He *et al.* 2006; Simon *et al.* 2007; Tatsis *et al.* 2007). The mechanism is not clear since the role of different $CD8^+$ T cell populations (effector, effector memory and central memory) in providing protection remains controversial.

Some vaccine vectors, such as those derived from adenovirus, have been shown to persist and continue to produce antigen. This contributes to maintaining a cohort of activated effector and memory effector T cells (Tatsis *et al.* 2007). However, it has been argued that these cells have an impaired response. $CD8^+$ T cells undergoing protracted antigen stimulation show a partially exhausted phenotype (Yang *et al.* 2006) and the early termination of antigen expression seems to generate a more robust secondary response (Finn *et al.* 2009). A similar observation has been reported following immunisation with persistent DNA vectors. Although durable antigen expression maintains a bigger pool of cognate $CD8^+$ T cells, a transient burst of antigen results in a better secondary response (Hovav *et al.* 2007; Radcliffe *et al.* 2007).

Part of the efficiency of LVs as vaccine vectors has been attributed to their ability to display a more durable antigen presentation than other vaccination

strategies, *e.g.*, better than peptide pulsed DCs (He *et al.* 2005). However, the duration of antigen presentation and the consequences of the role of long-term transgene expression in DCs have not yet been assessed in the context of LVs.

4.1.4. Aims

- Measure the duration of antigen presentation following LV *in vivo* transduction of professional APCs.

- Determine the effect of protracted antigen presentation on cognate T cells.

- Compare the effects of LVs on the activation status of transduced DCs in the short and the long term.

- Examine the consequences of sustained antigen presentation on the long-term outcome of immunisation with LVs.

4.2. Results

4.2.1. Persistence of Ag presentation in spleen after immunisation with LVs.

To test whether persistence of transduced APCs in the spleen resulted in durable antigen presentation, proliferation of adoptively transferred lymphocytes from TCR-transgenic OT-1 mice was measured. These cells specifically recognise the CD8 epitope $OVA_{257-264}$ (SIINFEKL) in the context of $H2K^b$. Prior to their transfer into naïve or immunised mice, the cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) to monitor their expansion, and then detected by FACS in the spleen 5 days after transferred (Fig. 1). LVs were administered intravenously to keep consistent with the previous experiments.

When transferred five days after immunisation, almost all the OT-1 cells underwent division, resulting in an increase in their absolute number (Fig. 4.1A).

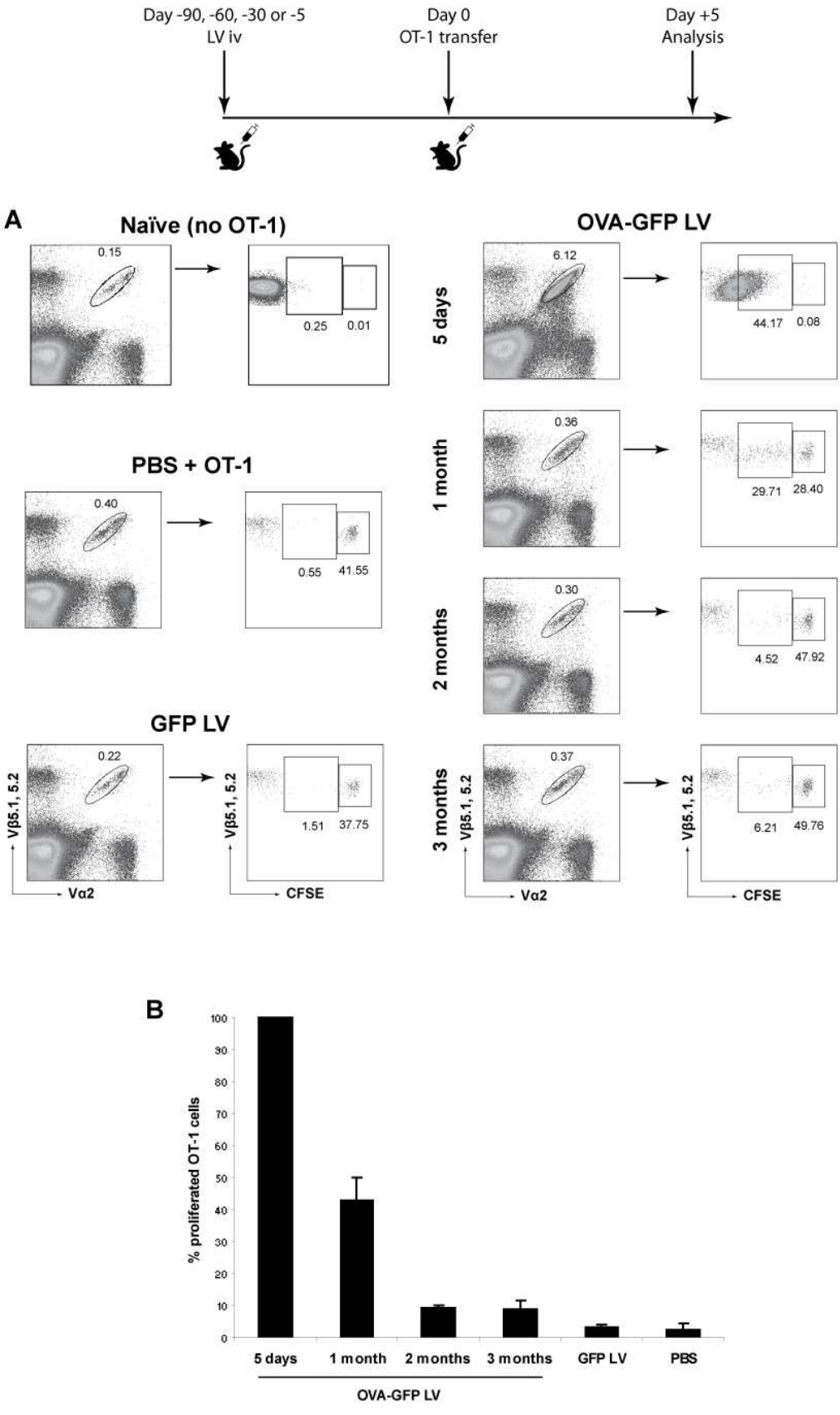
Complete dilution of CFSE in most of the cells denotes that they underwent at least 7-8 divisions. Interestingly, OT-1 cells transferred one month after mice were immunised also proliferated. Antigen presentation could be detected even 2 and 3 months after immunisation (Fig. 4.1A). However, the percentage of divided cells and the number of divisions they underwent progressively decreased in the later time points (Fig. 4.1B). Proliferation of OT-1 cells was not observed in the long term when mice were injected iv with $OVA_{257-264}$ plus monophosphoryl lipid A adjuvant or a vector encoding an irrelevant antigen (GFP LV) (Fig. 4.1C). In summary, antigen presentation is sustained for at least 3 months after systemic administration of lentivectors, leading to proliferation of cognate $CD8^+$ T cells.

4.2.2. Role of the spleen in long-term antigen presentation

To determine the site of prolonged antigen presentation, experiments were repeated in splenectomised mice and OT-1 cell expansion measured in peripheral blood by FACS 5 days after adoptive transfer. As shown in figure 4.2, almost all of the OT-1 cells divided in the mice that were immunised 5 days before OT-1 transfer and removal of the spleen did not affect the number of cells that proliferated. This indicates that following intravenous injection of LVs, antigen presentation in the short term takes place in anatomical sites other than the spleen. In contrast, in the mice transferred with OT-1 cells 30 days after immunisation, there was an approximately 50% reduction in the percentage of OT-1 cells that proliferated when the spleen was removed (Fig. 4.2). It is unlikely that this difference is due to a homing problem of the transferred cells in the absence of spleen, as no differences were observed in the proliferation levels in mice injected after splenectomy (data not shown).

This means that the spleen plays a significant role in long-term antigen presentation in LV-immunised mice, although it is not the exclusive anatomical site where it takes place. Antigen is also presented by cells in other tissues or organs both in the short and the long term.

Figure 4.1


Day -90, -60, -30 or -5
LV iv
Day 0
OT-1 transfer
Day +5
Analysis
A
Naïve (no OT-1)
0.15
0.25
0.01
PBS + OT-1
0.40
0.55
41.55
GFP LV
0.22
1.51
37.75
Vβ5.1, 5.2
Vα2
CFSE
OVA-GFP LV
5 days
6.12
44.17
0.08
1 month
0.36
29.71
28.40
2 months
0.30
4.52
47.92
3 months
0.37
6.21
49.76
B
% proliferated OT-1 cells
100
90
80
70
60
50
40
30
20
10
0
5 days
1 month
2 months
3 months
GFP LV
PBS
OVA-GFP LV

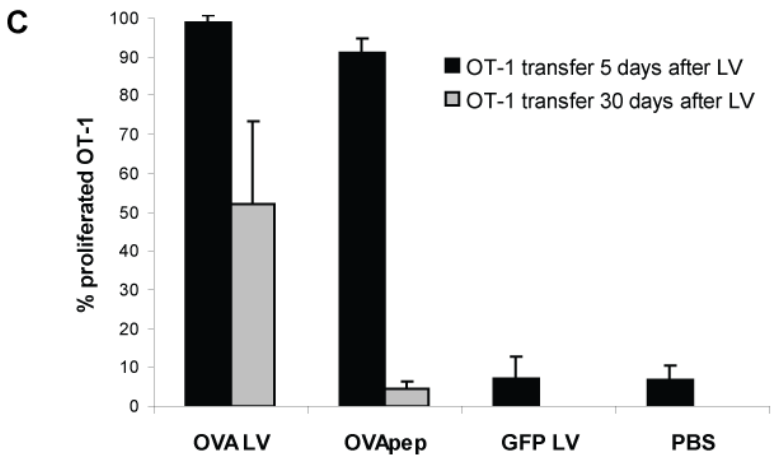


Figure 4.1. Persistence of antigen presentation following administration of LVs. Mice (3-4 per group) received iv 5-10 x 10^6 CFSE-labelled cells from OT-1 mice at different time points after iv injection of PBS, GFP or IiOVA-GFP LVs (500 ng RT). Five days later, transferred cells were tracked in the spleen and their expansion was assessed by dilution of CFSE by flow cytometry. Plots show events gated in $V\alpha2^+$ $V\beta5.1, 5.2^+$ cells from a representative experiment (A). Non-divided cells are labelled as $CFSE^{high}$ and divided cells are $CFSE^{low}$. $CFSE^-$ cells include endogenous $V\alpha2^+$ $V\beta5.1, 5.2^+$ cells and transferred cells that have undergone 7-8 or more divisions. Results from four independent experiments are summarised in the graph (B). As a control, a group of mice injected with $OVA_{257-264}$ peptide (50 µg) plus MPL as adjuvant (30 µg), both iv, either 5 (black bars) or 30 days (grey bars) before transfer of OT-1 cells (C). Bars represent means ± SD.

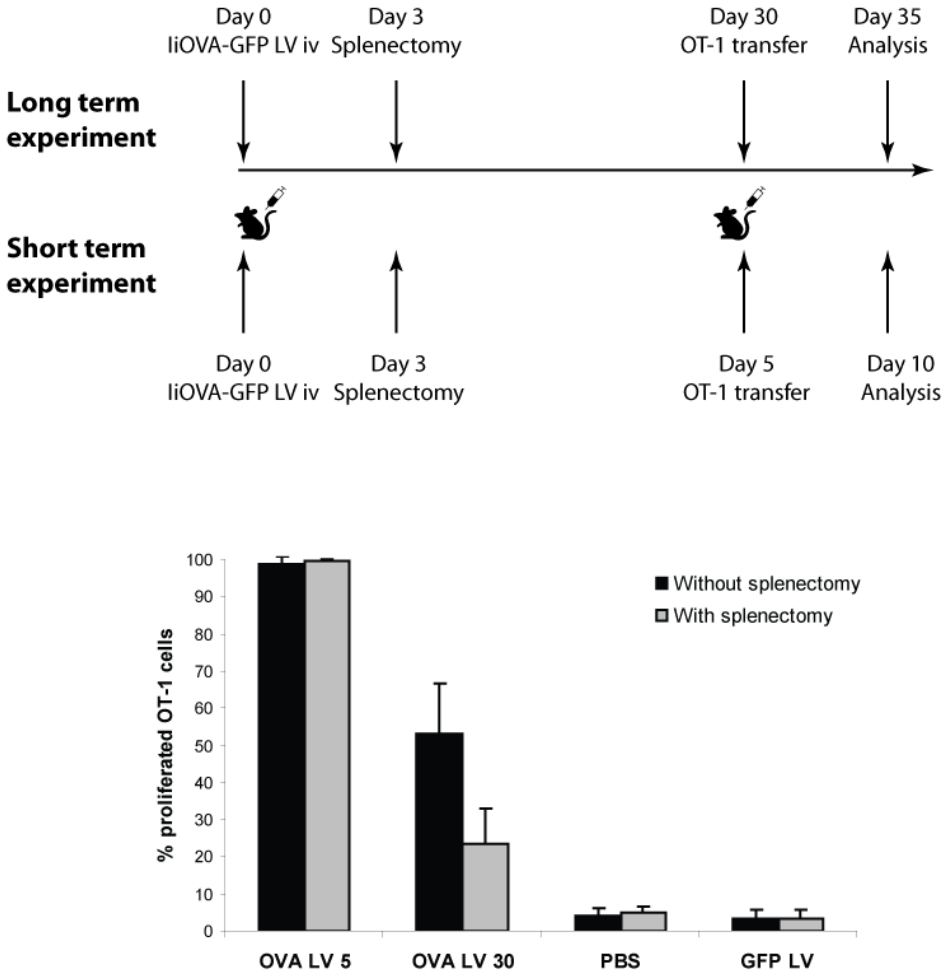


Figure 4.2. Persistence of antigen presentation following splenectomy. Spleens were removed from mice 3 days after intravenous administration of IiOVA-GFP, GFP LVs (500 ng RT) or PBS at different time points. Then CFSE-labelled OT-1 cells were transferred intravenously 5 or 30 days after the LV injection. Expansion of these cells was measured by FACS in peripheral blood 5 days later. Bars represent means ± SD (n = 3-4) of the percentage of proliferated cells; black bars = no splenectomy, grey bars = splenectomy. An equal result to the short term time point was observed in an experiment where the LV was injected 3 days after removing the spleen and the OT-1 cells transferred 5 days later (results not shown).

4.2.3. Activation of transferred OT-1 cells

In order to examine the activation status of the transferred OT-1 cells, expression of the activation marker CD44 and IFN-γ production in response to $OVA_{257-264}$ re-stimulation were measured. Figure 4.3A shows that CD44 was upregulated in the $V\alpha2^+$ $V\beta5.1$, 5.2^+ cell population that contained the transferred OT-1 cells. More activated cells were observed when the OT-1 cells were transferred 5 days after immunisation, but a significant population of activated cells was also detected when transfer was delayed until day 30. In the immunized mice with no OT-1 cells transferred, an average of approximately 200 spots/10^6 cells was observed, which decreased to 100 spots/10^6 cells after 30 days (Fig. 4.3B), corresponding to a long-lived endogenous $CD8^+$ T cell response. Upon OT-1 adoptive transfer, these numbers increased to 950 and 250 respectively, showing that the transferred cells were also functionally activated.

4.2.4. Reverse transcription is necessary for long term antigen presentation

Previous studies have shown that proteins can be co-purified with concentrated retroviral vectors pseudotyped with VSV-G envelope, leading to pseudotransduction by protein instead of gene expression (Gallardo *et al.* 1997; Liu *et al.* 1996). To demonstrate that reverse transcription is necessary for short- and long-term antigen presentation, a control was performed using a packaging plasmid with two mutations (D185A and D186A) in the highly conserved YMDD motif of the catalytic core of the reverse transcriptase (Kaushik *et al.* 1996; Lowe *et al.* 1991). LVs produced with this plasmid (RTmut) contain all the components of the viral particle, but are unable to reverse transcribe the viral RNA because the packaged RT is not functional.

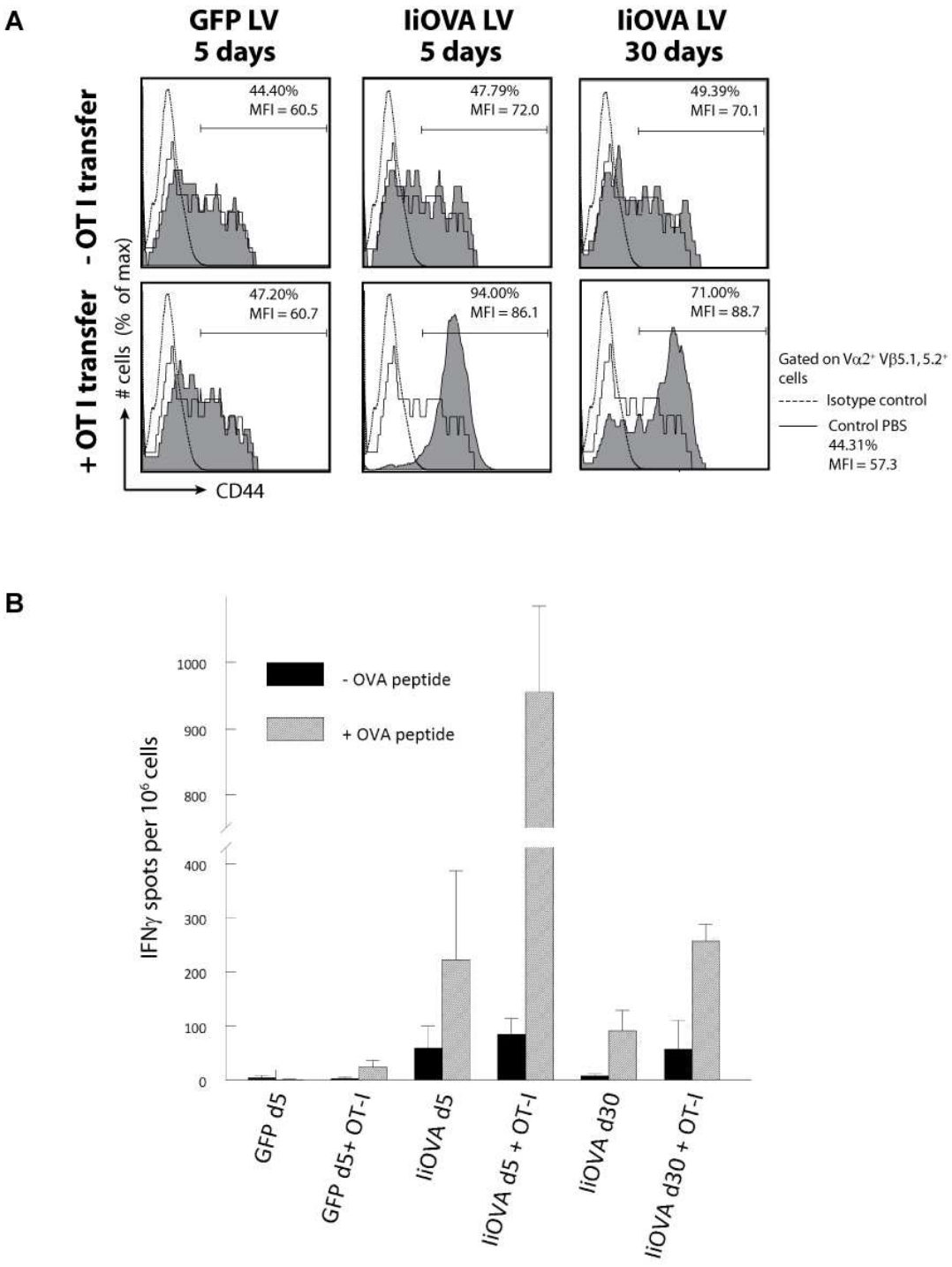
RTmut/LVs were titrated by p24 quantification and tested *in vitro* using GFP as a reporter gene. Low expression of GFP was observed in 293T cells transduced with a high concentration of GFP/RTmut LV (Fig. 4.4A), though not at low

multiplicity of infection. To determine if reverse transcription is necessary for sustained *in vivo* antigen presentation, mice were injected with IiOVA-GFP/RTmut LV and the immune response was measured by ELISpot and OT-1 cell transfer. Figure 4.4B shows that no IFN-γ-producing OVA-specific T cells were measurable by ELISpot. However, a small number of OT-1 cells proliferated in mice injected with IiOVA-GFP/RTmut 5 days earlier, but not when transferred 30 days after immunisation. This means that in the absence of reverse transcription, there is a minor immune activation that could be explained either by co-purified protein. It has also been described the existence of low and transient expression of the transgene by directly from the viral RNA of retroviral vectors (Galla *et al.* 2004) which could also be playing a role in lentiviral transduction. However, for effective antigen presentation and long-term expression, reverse transcription is necessary.

4.2.5. *In vivo* DC activation

To compare activation status of DCs following LV administration, the expression of different co-stimulatory molecules was analysed in these cells in treated and control mice. In these experiments, transduced cells were detected by the expression of the membrane protein Thy1.1 encoded by a LV, and staining with an anti-Thy1.1 antibody. Activation status was assessed at an earlier time point than in previous experiments (day 3 instead of day 5) since some activation markers can be quickly downregulated.

As shown in figure 4.5, CD40, CD80 and CD86 were upregulated in the transduced DCs of mice injected 3 days before. However, the levels of expression 30 days after administration of LVs were at the same level than in control mice injected with PBS (Fig. 4.5B). These results suggest that direct administration of LVs induces DC maturation. However, the transduced cells that persist in the spleen in the long term show a basal expression of co-stimulatory molecules, suggesting they are in an immature state.



IFigure 4.3. *In vivo* activation of transferred OT-1 cells. OT-1 cells were adoptively transferred into mice that had been injected iv with IiOVA-GFP LV (500 ng RT) at different time points. Five days later, the spleen cells from these mice were harvested and analysed by flow cytometry for expression of CD44 in $V\alpha2^{+}V\beta5.1^{+}$ cells (A). Secretion of IFN-γ after re-stimulation with $OVA_{257-264}$ peptide was measured by ELISpot (B). Mice injected with GFP LV or PBS were used as controls. Values were compared with those of mice that did not receive OT-1 cells, showing the response of endogenous lymphocytes.

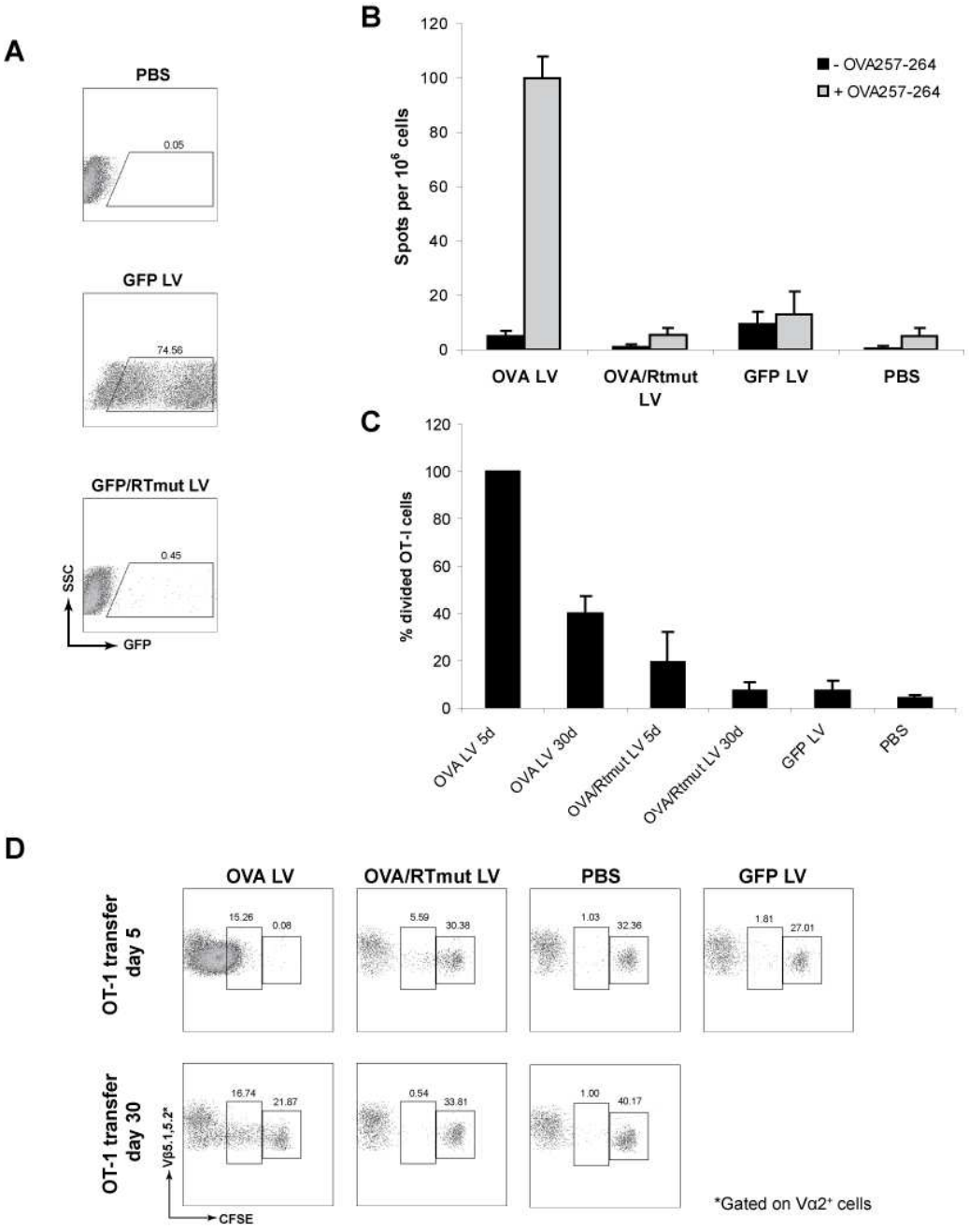


Figure 4.4. Long-term antigen presentation and immunisation dependent on reverse transcription. GFP and IiOVA-GFP LVs were generated using a packaging vector with an inactive mutant of the reverse transcriptase (RTmut). GFP expression was analysed *in vitro* in 293T cells after transduction with GFP/RTmut LV (A). Equal results were observed with IiOVA-GFP/RTmut LV (not shown). Mice (3 per group) were injected iv with equivalent amounts of IiOVA-GFP or IiOVA-GFP/RTmut LVs (500 ng p24) and the immune response measured by ELISpot 10 days later (B). Antigen presentation was assessed by OT-1 expansion in mice injected 5 or 30 days before adoptive cell transfer (C). Results were analysed individually (5 mice per group) and a representative result is shown (D).

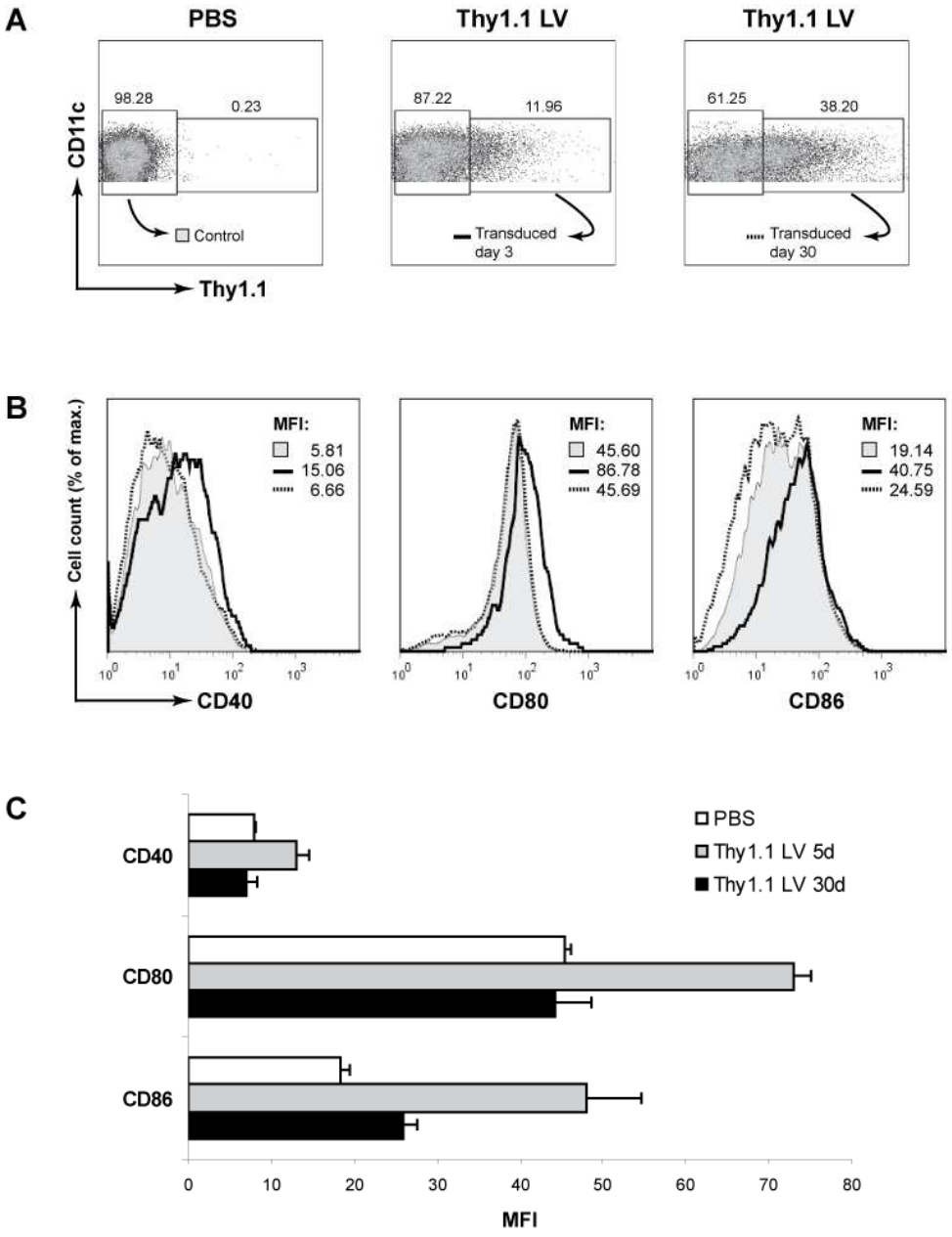


Figure 4.5. *In vivo* activation of transduced DCs. Mice were injected iv with 500 ng RT of Thy1.1 LV (Fig. 2.1). Spleen DCs were purified 3 or 30 days later and stained with antibodies for CD11c, Thy1.1 and the activation markers CD40, CD80 and CD86. Expression of the activation markers in transduced DCs (gated on $CD11c^+/Thy1.1^+$, shown in A) and in control mice was analysed by FACS. A representative result is shown in (B) and results from individually analysed mice (means ± SD, $n = 3$) are represented in (C).

4.2.6. Persistent cytolytic activity after a single injection of LVs

Finally, cytolytic activity was examined in the mice that had been treated with intravenous LVs. Figure 4.6 shows that $OVA_{257-264}$–pulsed targets, injected into mice 10 weeks after LV immunisation were lysed overnight. The efficiency was lower than that observed 10 days after LV administration, but the difference was not statistically significant. This is consistent with previous reports in which mice injected intravenously with LV had a strong T cell memory response when challenged one year later (Rowe *et al.* 2006[a]). Together these data show that following intravenous administration of LV there is a protective memory $CD8^+$ T cell response.

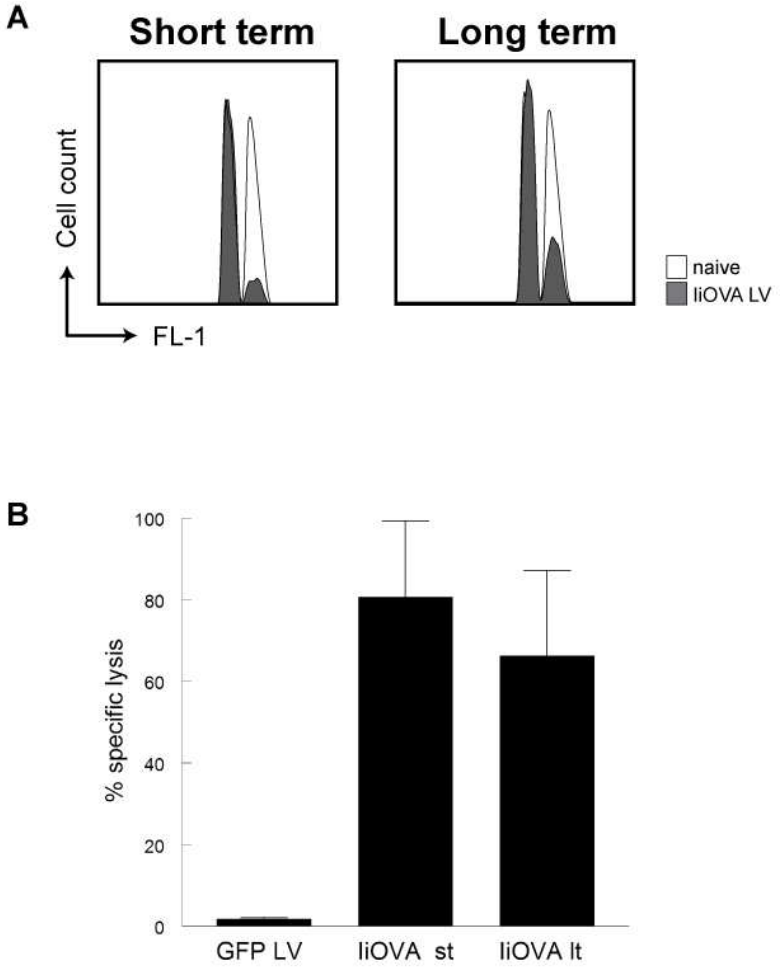


Figure 4.6. *In vivo* killing assay. Mice (6 per group) were injected either with PBS, GFP LV or IiOVA-GFP LV (500 ng RT) iv. After 10 days (short term, st) or 10 weeks (long term, lt), they received a 1:1 mix of unpulsed ($CFSE^{low}$) and $OVA_{257-264}$-pulsed ($CFSE^{high}$) syngeneic splenocytes. The specific lysis of target cells was assessed by FACS in spleens 18 hours later (A). Filled histogram = immunised; unfilled histogram = naïve. The percentage of specific lysis was significantly different between immunised and control mice ($p = 0.03$), but not between the short term and long term groups ($p = 0.38$) (B). Bars represent means ± SD.

4.3. Discussion

The data presented here demonstrate that administration of LVs results in long-term antigen presentation to $CD8^+$ T cells for at least 90 days. This agrees with the persistence of transgene expression in DCs and macrophages shown in chapter 3 and reported in other studies (Kimura *et al.* 2007; VandenDriessche *et al.* 2002). This challenges the concept of antigen presentation as a process restricted in time by the short half life of activated DCs and the elimination of antigen-bearing DCs during the CTL response. Indeed, the latter might be the explanation for the low percentage of transduced DCs observed after administration of OVA-encoding LVs and the reduction in the percentage of proliferated cognate T cells transferred 30 or more days after immunisation (Table 3.4).

The persistence of antigen presentation could be due to a continuous supply of transduced DCs derived from dividing precursors, which could counterbalance their elimination and maintain a population of antigen-expressing cells. DC precursors could act as a reservoir of antigen that is not accessible to CTL elimination and prolong antigen presentation after mature DCs die or are eliminated. The spleen could be the source of these precursors (Naik *et al.* 2006), since long-term antigen presentation was impaired when the spleen was removed, but more experimental work is necessary to prove this hypothesis.

The possibility of antigen presentation by other long-lived cells, including macrophages or non-professional APCs, cannot be discarded. LV-transduced macrophages persist in the spleen of injected mice (Table 3.3); these cells are also competent in stimulating naïve $CD8^+$ T cells to proliferate and differentiate into memory T cells (Pozzi *et al.* 2005). $CD4^+$ T cells could also play a role in the maintenance of long-term antigen presentation by protecting antigen-bearing DCs from elimination by CTLs (Mueller *et al.* 2006).

The transduction of a DC precursor would not necessarily result in effective T cell stimulation by its progeny, as they might not be activated. Furthermore, it is expected that the activation signals delivered by the LV (*e.g.*, viral RNA, DNA from

reverse transcription, components of the viral preparation) will decrease with time. In fact, it was observed that expression of most co-stimulatory molecules that are initially upregulated in DCs following LV transduction return to basal levels after 30 days.

It is widely accepted that antigen presentation by resting DCs can induce peripheral $CD8^+$ T cell tolerance (Bonifaz *et al.* 2002; Probst *et al.* 2003). However, here it was observed that protracted antigen presentation induced proliferation and activation of adoptively transferred OT-1 T cells, as demonstrated by upregulation of CD44 and secretion of IFN-γ. Residual costimulatory activity of DCs could be an explanation for this (Fig. 4.5), although these results must be interpreted carefully. It has been shown that production of other cytokines by $CD8^+$ T cells (*e.g.*, IL-2, TNF-α) can be impaired in the absence of adequate co-stimulation in spite of normal IFN-γ production (Wherry *et al.* 2003; Zajac *et al.* 1998).

It has been argued in other models that prolonged antigen presentation could hinder the immune response. Some recent studies have also associated persistence of antigen with impaired secondary responses (Finn *et al.* 2009; Hovav *et al.* 2007; Radcliffe *et al.* 2007). Results vary depending on the model studied since the outcome of the immune response depends on other factors, such as antigen distribution and amount, inflammatory stimulus and the type of cell presenting antigen (Storni *et al.* 2003).

Here it was observed that LVs induce potent primary and memory CTL responses. A single injection of LVs was sufficient to provide protective immunity 10 weeks later, confirming results obtained previously (Rowe *et al.* 2006). The persistence of antigen can be playing a role in this end result by maintaining a bigger pool of cognate $CD8^+$ T cells through priming of naïve cells (demonstrated by priming of OT-1 cells) or of memory cells, which require less co-stimulation or are more amenable for boosting (Vezys *et al.* 2006). Furthermore, sustained TCR stimulation is important for priming of $CD4^+$ T cells (Obst *et al.* 2005; Obst *et al.* 2007), which are critical for the CTL response induced by LVs (Dullaers *et al.* 2006; Rowe *et al.* 2006).

To further characterise the immune consequences of persistent transduced DCs as well as other APCs, it would be important to identify their effect on different

subpopulations of T cells. This was attempted through *ex vivo* antigen presentation assays and transfer of transduced cells from vaccinated to naïve mice. However, purification of these cells proved to be difficult due to the low frequency of transduced cells. Besides, the purification technique that was employed (antibody labelling and magnetic bead separation) and the disruption of the spleen architecture may affect the ability of transduced DCs to present antigen.

Viral particles void of reverse transcriptase activity were used as controls in these experiments. Surprisingly, some immune response was observed when using adoptive OT-1 transfer. Although this can be due to protein co-purification in the concentrated virus, it has also been reported that direct translation of gammaretroviral RNA can result in low and transient expression of the transgene (Galla *et al.* 2004). This has not been studied in lentiviruses and its immunological consequences remain to be explored.

In conclusion, it has been shown that LVs can transduce and persistently express antigen within DC. Furthermore, these DCs are still functional, in being able to induce proliferation and activate T cells. This contributes to the protective immunity generated by these vectors.

CHAPTER 5

5. REGULATION OF ANTIGEN EXPRESSION WITH LENTIVIRAL VECTORS

5.1. Introduction

The regulation of transgene expression has been a major challenge in gene therapy. For therapeutic genes, modulating their expression would avoid the occurrence of toxicity by overexpression (Stieger *et al.* 2009). In the context of antigen genes, it would allow turning 'off' the gene when the therapeutic effect is achieved, and switching it back 'on' if needed. A regulatable antigen expression model would also be an important tool for basic immunological research and to test the following hypotheses:

(1) A conditional system of antigen expression would help to understand the dynamics of T cell priming induced by LVs. It is still not clear how does the duration of antigen presentation affect the magnitude of the primary response and whether the presence of antigen is important for the maintenance of $CD8^+$ T cell memory. Using vaccine vectors with inducible antigen expression systems, Radcliffe *et al.* (2007) and Finn *et al.* (2009) showed that a prolonged antigen presentation resulted in a bigger pool of cognate T cells, but the secondary responses were better when priming was restricted to shorter periods of time. These conclusions were based in immunisations with a DNA and an adenoviral vector, respectively, both of which lead to long-term expression of the antigen. The outcome could be different with LVs, since a persistent supply of antigen-expressing DCs can be maintained by transduction of dividing precursors. A more detailed study of the consequences of duration of antigen presentation may be achieved with a regulatable antigen expression system in these vectors.

(2) This model would also allow segregating the stimulation of the innate immune response from the actual expression of the antigen. Using an inducible

antigen expression system in transgenic mice, Probst *et al.* (2003) demonstrated that induction of antigen presentation without any further stimulus resulted in a tolerogenic response that could not be broken by subsequent administration of the antigen and adjuvants. In contrast, when accompanied by a stimulatory signal (anti-CD40), it resulted in protective immunity. It has been proposed that the lentiviral particle and other components in the LV preparations activate DCs, contributing to the generation of an immune response to the transgene. The effects of these components could be studied in more detail if the expression of the antigen were segregated from the initial inflammatory signal. In this way, antigen expression could be delayed and induced peripherally with or without different stimulatory signals to study their effects.

(3) The kinetics of precursor transduction could also be further analysed with a regulatable system. Dividing DC precursors may be responsible for the maintenance of transduced DCs in the long term. Antigen-expressing DCs are also eliminated by the CTL response. Therefore, delaying the expression of the antigen could result in a larger accumulation of transduced DCs, deriving from a reservoir of transduced precursors. This could result in a stronger immune response when the antigen expression is switched 'on'.

(4) This model could address whether persistence of transcription is necessary for sustained antigen presentation and not the result of pseudotransduction. The first is supported by the fact that the $CD8^+$ T cell response is curtailed when reverse transcription is absent (Fig. 4.4), but could be confirmed by controlling the duration of antigen expression.

There are currently several systems of regulatable gene expression, some of which have been used with LVs.

5.1.1. Ligand-dependent regulatable systems

The systems for exogenous modulation of gene expression have three components: (1) a regulatable promoter that drives the expression of the gene of interest, (2) a regulatory protein that stimulates or inhibits this promoter and (3) a

ligand that modulates binding of the regulatory protein to the promoter, usually by induction of conformational changes and polymerisation.

Four drug-dependent systems have been used *in vivo* and their components are described in Table 5.1. The tetraycycline-regulatable system has been the best characterised and extensively tested in the context of different vectors. It is regulated by tetracycline (Tet) or its analogue, doxycycline (Dox). Both drugs have a favourable pharmacologic profile for *in vivo* usage (Stieger *et al.* 2009).

Table 5.1. Regulatable gene-expression systems.

System	Ligand	Regulatory protein	Regulatable promoter	Ref.
Tetracycline-dependent	Tetracycline or doxycycline	tTA (TetOff) rtTA (TetOn) KRAB-TetOn rtTA/KRAB	7X TetR binding sites linked to CMV_{min}	See text
Rapamycin-dependent	Rapamycin	Rapamycin-dependent transactivator[a]	12X ZFHD1 binding sites linked to CMV_{min}	Rivera *et al.* 1996
Mammalian steroid receptor	Mifepristone	"Switch" protein[b]	4X GAL4 UAS binding sites linked to TK_{min}	Wang *et al.* 1994
	Tamoxifen	Mutant oestrogen receptor fused with Cre[c]		Feil *et al.* 1997
Insect steroid receptor	Ecdysteroids (*e.g.*, muristerone)	Heterodimer formed by VgEcR[d] and RXR	5X E/GRE binding sites linked to HSP_{min}	No *et al.* 1996

[a] The rapamycin-dependent transactivator is a heterodimer formed by two units that bind in the presence of rapamycin: FRB/p65-HSF1 (fusion of a portion of FKBP-rapamycin complex binding domain and activation domains from p65 subunit of NFκB and human heat shock factor 1) and ZFHD1/3X FKBP (DNA binding domain derived from human transcription factors Zif268 and Oct-1 and 3 copies of FKBP rapamycin-binding protein).
[b] "Switch" is a fusion protein of GAL4 DNA binding domain from *S. cerevisiae*, a truncated binding domain of human progesterone receptor and the activation domain of the p65 subunit of human NFκB
[c] In the absence of tamoxifen, Cre recombinase is associated with heat shock proteins and is cytoplasmatic. After association with tamoxifen, Cre traslocates to the nucleus, excises a loxP-flanked 'stop' cassette and induces gene expression.
[d] VgEcR consists of VP16 transactivation domain from herpes simplex virus fused with a mutated ecdysone receptor of *Drosophila*.
Abbreviations: GAL4 UAS = galactose-induced regulator 4 upstream activation sequence; TK_{min} = minimal thymidine kinase promoter from herpes simplex virus; RXR = retinoid X receptor; E/GRE = ecdysone/glucocorticoid response elements;
HSP_{min} = minimal heat shock protein promoter. The rest of the abbreviations are explained elsewhere in the text.

5.1.1.1. Tetracycline-regulatable system

This system is based on the *E. coli* Tn10 resistance operon, which is composed of the Tet repressor protein (TetR) and a DNA regulatory sequence called the Tet operator (TetO). In the absence of Tet or Dox, the TetR protein binds to the TetO and represses transcription; the process is reverted by the presence of Tet or Dox (Hillen *et al.* 1982).

Five different Tet-responsive regulatory systems have been developed based on the bacterial operon, shown in figure 5.1 (Stieger *et al.* 2009).

5.1.1.1.1. TetOff

In this system, transcription is regulated by tTA (Tet-controlled transcriptional activator), a chimeric protein composed by TetR fused to the transactivator VP16 of herpes simplex virus. Expression of the gene of interest is under control of a promoter that consists of seven repeats of the TetO DNA sequence—also referred as Tet-responsive element (TRE)—and the minimal CMV promoter (CMV_{min}). In the absence of Dox, tTA binds to TRE and activates transcription. Dox induces conformational changes in tTA, which detaches from TRE and gene expression is silenced.

5.1.1.1.2. TetOn

Four point mutations in the TetR domain of tTA result in a reverse Tet repressor (rtTA) that exerts the opposite effect of tTA, *i.e.*, it binds to TRE and induces gene expression in the presence of Dox. Improved versions of rtTA ($rtTA2^S$-M2 and $rtTA^S$-S2) have been generated by mutagenesis and codon optimisation in order to improve the sensitivity to Dox and reduce background activity (Urlinger *et al.* 2000).

5.1.1.1.3. KRAB-TetOn

This system is based on a protein formed by the fusion of TetR with a transinhibitor protein called Krüppel-associated box (KRAB), which is part of the

zink finger protein Kox1 in humans or Kid-1 in mice. The KRAB domain inhibits RNA polymerases II and III within a range of 3 kb of its DNA attachment site (Margolin *et al.* 1994). Detachment of KRAB from the promoter and induction of gene expression is dependent on the presence of Dox.

5.1.1.1.4. Combined TetOn/TetOff

Alternatively, a combination of the systems described before has been developed in order to reduce background expression. rtTA-VP16 and KRAB can be combined in a single cassette separated by an internal ribosomal entry site (IRES). In this system, expression is induced by the presence of Dox (Freundlieb *et al.* 1999). In other cases, the gene of interest can be under control of a separate constitutive promoter, which is inhibited by the presence of KRAB (Szulc *et al.* 2006). Although the background expression is reduced, the size of the coding cassette and the immunogenicity of these molecules restrict the application of this system.

5.1.1.1.5. Autoregulatory loop TetOn/TetOff

Another alternative used to minimise the background expression is the use of a system with an autoregulatory loop, in which the both transactivator and the gene of interest are expressed under control of the Tet-inducible promoter. In this case, expression of the transactivator only takes place in the presence of Dox. This reduces the toxicity of the transactivator and the background expression, although the induction of expression may take longer (Haberman *et al.* 1998). This design can also be used with a TetOff mechanism.

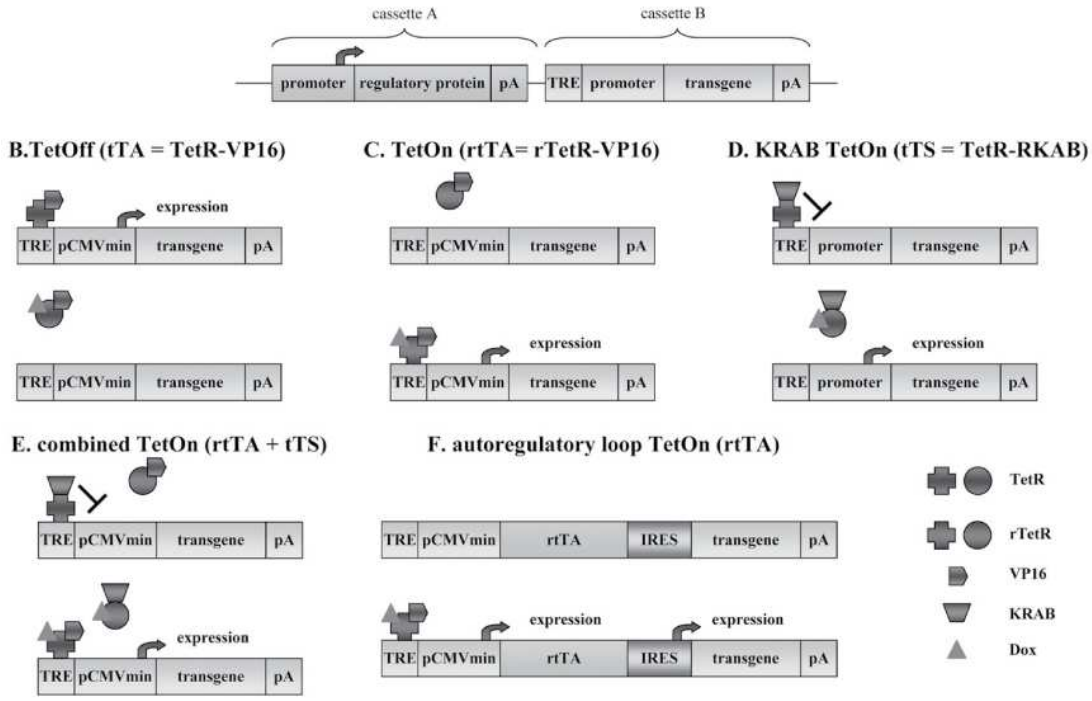


Figure 5.1. Schematic representation of different Tet-regulatable systems. In general, Tet-regulatable systems are composed of two expression cassettes: one containing a regulatory protein under control of a constitutive promoter, and another with the gene of interest under control of a Tet-regulated promoter (A). In the TetOff system, binding of a transactivator to the regulatable promoter and induction of transcription takes place in the absence of doxycycline (Dox) (B). In contrast, gene expression is triggered by the presence of Dox in the TetOn system. In more complex systems, the Tet repressor protein (TetR) has been fused to KRAB, a domain of zinc finger proteins which inhibits RNA polymerases II and III. In the KRAB-TetOn system, Dox releases TetR-KRAB from its binding site and induces gene expression (C). Alternatively, combination of the TetOn/TetOff systems with KRAB (D) or co-expression of the gene of interest and the Dox-dependent regulator in a polycistronic cassette results in reduced background expression (E-F).

Reproduced from Stieger *et al. Advanced Drug Delivery Reviews*, 2009. 61: 527-541, with permission of the publisher.

5.1.1.2. Regulatable expression systems in lentivectors

Most of the conditional expression systems mentioned above have been used in expression plasmids. Some have also been incorporated into viral vectors, especially adenovirus-derived vectors (Lee *et al.* 2005). The main limitations have been background expression, restriction in the use of single vectors because of the size of the regulatory proteins and, in some cases, reduction of the viral titers. Immune responses against the proteins involved in transgene regulation have also been reported in non-human primates, particularly with TetOn systems (Le Guiner *et al.* 2007).

The majority of conditional expression LVs is based on the Tet-dependent system. Early designs employed a binary strategy, which requires co-transduction with one vector driving constitutive expression of the regulatory protein and another with the gene of interest under control of a conditional promoter (Régulier *et al.* 2002; Vigna *et al.* 2002; Johansen *et al.* 2002; Koponen *et al.* 2003). These systems showed variable efficiencies because the rate of transduction of each vector was not the same in every cell. The use of multiple LVs also increases the risk of insertional mutagenesis. To circumvent this problem, inclusion of both components in a single LV construct was achieved, either by using two promoters or polycistronic cassettes.

Both TetOn (Barde *et al.* 2006; Reiser *et al.* 2000) and TetOff (Kafri *et al.* 2000) systems have been employed in single LV constructs. TetOff designs have the disadvantage that they require continuous administration of Dox for as long as the expression needs to be suppressed. Hence, this model is only useful if gene expression is to be maintained for a long time. TetOn systems have been used in several models, but in some cases they appeared to display a poor response to physiological doses of the inducer drug (Vogel *et al.* 2004). Barde *et al.* (2006) compared several TetOn LV designs and showed efficient control of transgene expression *in vitro* and in the haemopoietic system of mice. More recently, combination of a second generation TRE and introduction of an insulator seemed to decrease background expression and achieve good control in regulation of expression in the central nervous system (Xuebi *et al.* 2009).

Based on the existing evidence of improved systems of regulatable antigen expression, it was decided to study an inducible antigen expression model with LVs to further understand the mechanisms involved in the immune response they generate.

5.1.2. Aims

- Test a model of inducible antigen expression in LVs in order to study the effects of the duration of antigen presentation on the immune response.

- Study the effects of LVs on the activation of DCs by delaying antigen expression.

5.2. Results

5.2.1. Inducible transgene expression in DCs *in vitro*

In order to set up a model of regulatable antigen expression, a lentiviral construct described by Barde *et al.* was used. In this TetOn system, expression of the gene of interest is controlled by a Tet-dependent transactivator (rtTA2^{S}-M2), which is constitutively expressed from a second promoter within the same vector (Fig. 2.1C). This vector has been shown to control gene expression in cells of the haemopoietic system, with up to 100-fold induction of expression in the presence of doxycycline and only background levels in the non-induced state (Barde *et al.* 2006).

A cassette encoding GFP was cloned under control of the inducible promoter and tested *in vitro* in BMDCs (Fig. 5.2A-B). Although GFP expression was observed in up to 20% of the cells that were not treated with doxycycline, the presence of the inducer almost doubled the proportion of GFP^{+} cells (Fig. 5.2A). The amount of GFP expressed—as reflected by the mean fluorescence intensity (MFI)—remained almost

at background levels in non-induced cells and increased 3-5 times in the presence of Dox (Fig. 5.2B).

OVA expression was tested by Western blot in 293T cells, since the OVA antibody background in BMDCs difficulted the analysis. Figure 5.2C shows a dose-dependent induction of OVA expression in the presence of Dox. In its absence, expression was only detectable at the highest concentration of LV.

These experiments show that doxycycline effectively induces expression of the gene under control of the regulatable promoter in DCs. However, there is some "leakiness" in the system, resulting in expression of the transgene even in the absence of the inducer.

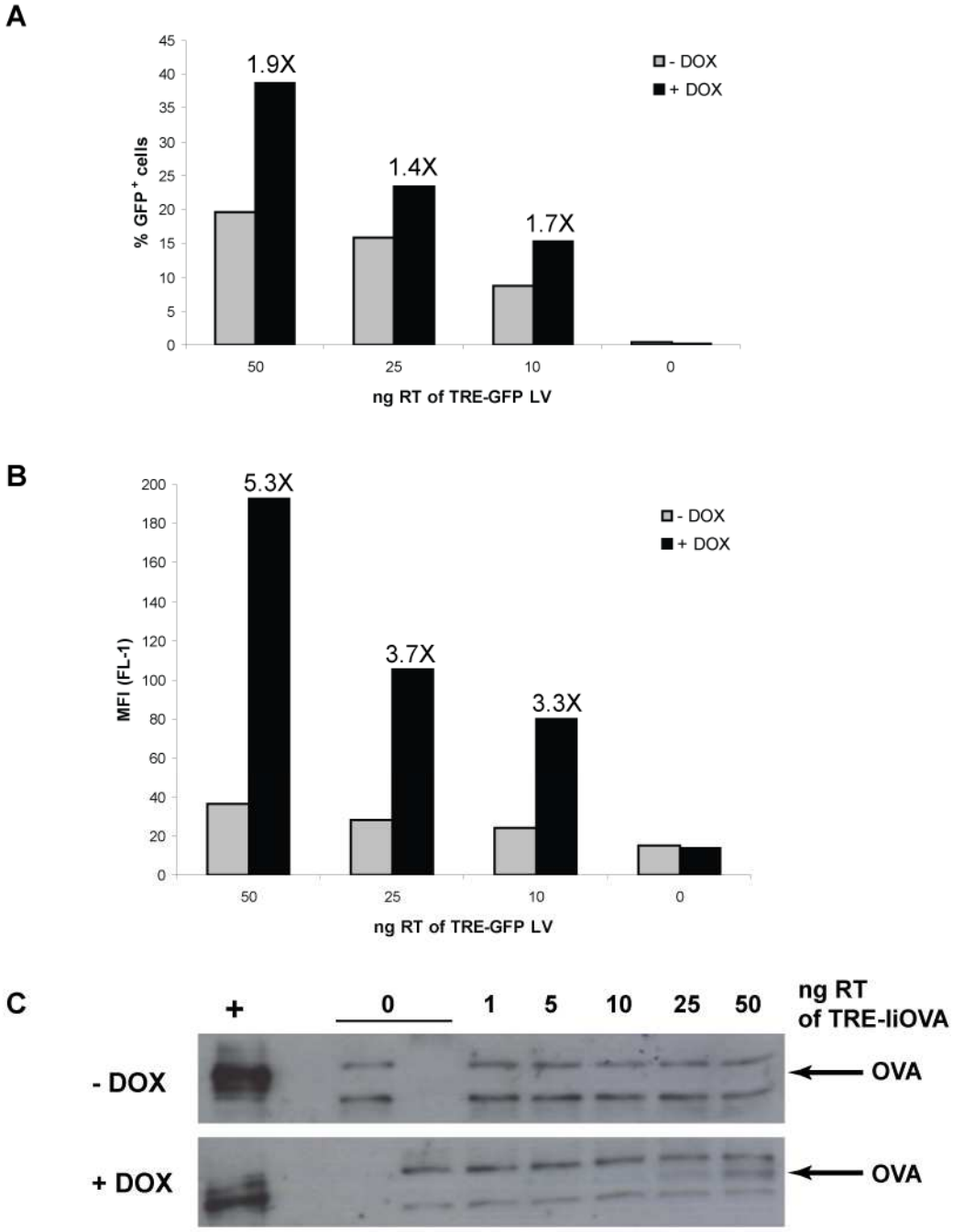


Figure 5.2. Doxycycline-dependent transgene expression after transduction with LVs *in vitro*. BMDCs were transduced with different amounts of TRE-GFP LV (Fig. 2.1C) and treated doxycycline 1 μg/mL (black bars) or medium only (grey bars). Expression of GFP was determined by FACS 72 hours later and shown as percentage of GFP^+ cells (A) or mean fluorescence intensity (MFI) in FL-1 (B). Fold increase is indicated above the bars. Expression of OVA after transduction of 293T cells with TRE-IiOVA LV (Fig. 2.1C) was determined by Western blot (C).

5.2.2. Induction of antigen expression *in vivo*

To determine whether upregulation of antigen expression resulted in a stronger immune response, the OVA-encoding TetOn LV was injected in mice at different doses by subcutaneous and intravenous routes. An IFN-γ ELISPot with $OVA_{257-264}$ re-stimulation was performed 10 days after injection of the vector and continuous administration of Dox.

There was considerable variability among the mice injected intravenously. An OVA-specific $CD8^+$ T cell response was observed both in the groups that received Dox and in controls, probably because of basal OVA expression (Fig. 5.3A). Only a small increase in the number of spots was observed when Dox was provided, which was comparable to that of a constitutively-expressing vector in all groups.

A similar result was observed after subcutaneous injection. In this case, the difference in the response between the induced and non-induced groups was more evident at low doses of LV, but lower than the response observed with a constitutively-expressing vector (Fig. 5.3B).

These results demonstrate that control of the expression of the antigen *in vivo* with this regulatable vector yields variable immune responses that do not necessarily correlate with the administration of doxycycline. This is probably because of the "leakiness" in the expression, also observed *in vitro*.

5.2.3. Delayed antigen expression

The effect of delayed antigen expression was tested *in vivo* by inducing expression of the vector 30 days after the LV was administered. This segregates the DC activation induced by the LVs from the actual antigen presentation. A low dose of LV was used to reduce the effects of basal expression.

An increase in the number of OVA-specific $CD8^+$ T cells was induced by Dox when provided immediately after LV administration, both in groups injected intravenously or subcutaneously (Fig. 5.4A-B). The response in mice injected iv was

higher but still lower than in mice injected with a constitutively-expressing vector, probably because of the difference in the doses used. These results were reflected in the ability to lyse specific cell targets (Fig. 5.4C-D). However, delayed expression of the antigen 30 days after the LV was injected did not result in an immune response in any group (Fig 5.4).

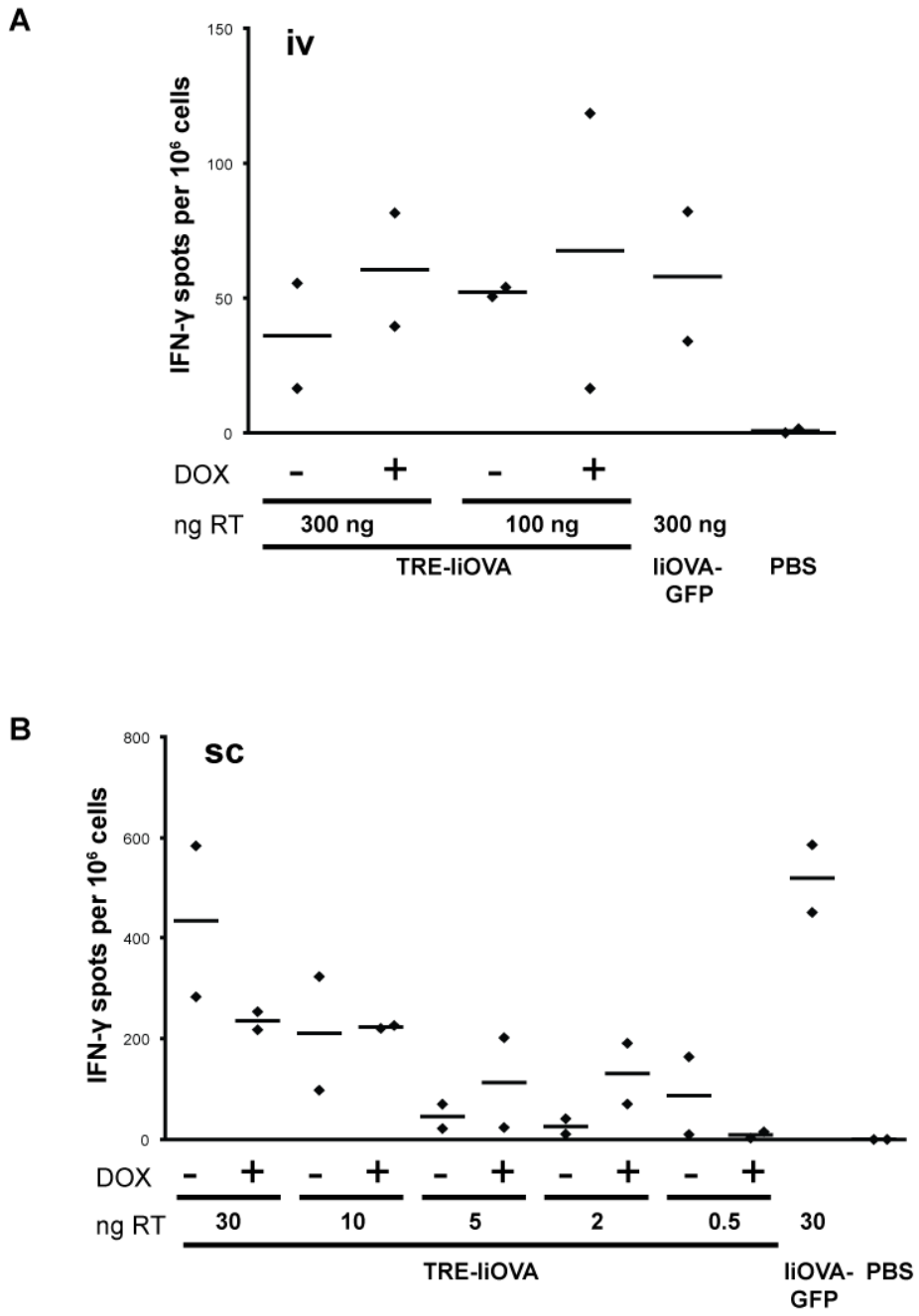
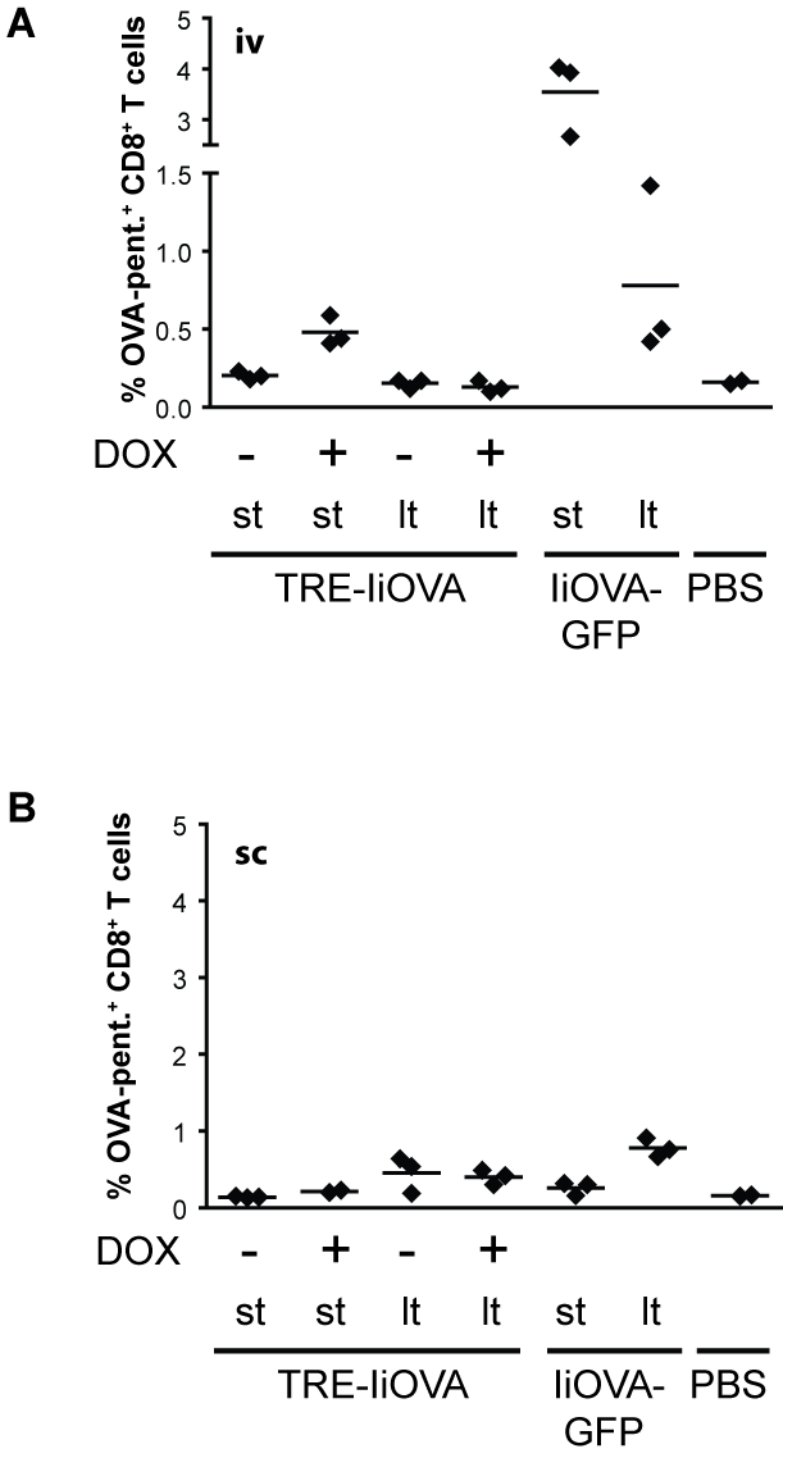


Figure 5.3. Dose-response to inducible antigen expression delivered by LVs. Mice (2 per group) were injected either iv (A) or sc (B) with different amounts of TRE-IiOVA LV. Where indicated, doxycycline (DOX, 200 μg /mL in the drinking water) was started 24 h after injection and continuously provided during 10 days. Then the spleens were harvested and antigen-specific $CD8^+$ T cell response was measured by IFN-γ ELISpot. A vector driving constitutive expression of OVA (IiOVA-GFP) and mice injected with PBS were used as controls. Individual results are represented by dots and means by horizontal bars.

Figure 5.4
OVA-pentamer staining



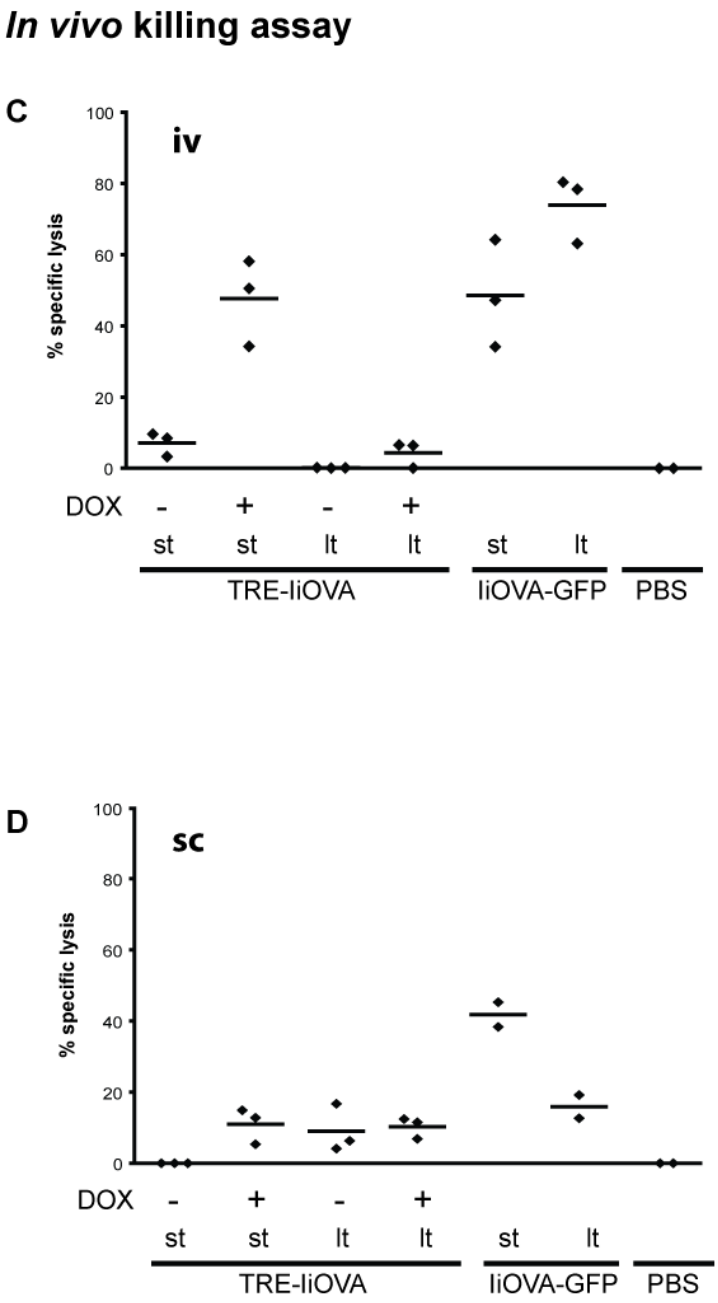


Figure 5.4. Immune response to delayed antigen expression delivered by LVs. Mice (3 per group) were injected either iv (100 ng RT) or sc (1 ng RT) with TRE-IiOVA LV. Where indicated, Dox was provided in the drinking water (200 µg/mL). In the short term groups (st), Dox was started 24 h after injection, while in the long term groups (lt) it was begun 30 days after LV injection. The OVA-specific CTL respons was measured after 10 days of continuous Dox treatment by pentamer staining (A-B) or *in vivo* killing assay (C-D). Mice injected with IiOVA-GFP LV or PBS were used as controls. Individual results are represented by dots and means by horizontal bars.

5.3. Discussion

Regulation of gene expression in a cell-specific and temporally-restricted manner remains a challenge in gene therapy. Many of the approaches used so far have suffered either from unwanted expression or from poor inducibility. Several LV-based systems with regulatable promoters have been described (Stieger *et al.* 2009). In many of them, transduction with two vectors is necessary, resulting in variabile levels of expression. To avoid this problem, a Tet-On inducible system based on a single LV platform was used to study regulated expression of an antigen gene (OVA).

Although the expression inducibility was observed in DCs, basal levels of the transgene were also present in the absence of Dox (Fig. 5.2). Since the amount of antigen required to elicit an immune response is minimal, this 'leakiness' resulted problematic when the vector was used *in vivo*. A very tight regulation is desirable in this case, but tighter systems often result in insufficient expression even in the presence of the inducing agent.

In vivo, basal expression of the antigen resulted in generation of an immune response even in the absence of the inducer. This was more evident at high doses of LV, although there was a high individual variability. As shown in chapter 4, transcription of the transgene is necessary for effective immunisation with LVs. Thus, it is unlikely that the responses observed in non-induced mice were just because of co-purified protein.

To reduce this problem, a lower dose of LV was assayed both in the short and long term. A larger cytolytic response was observed in mice that received doxycycline immediately after injection of LV, in contrast with those where the doxycycline was started 30 days later. Results were more evident when the vector was administered intravenously probably because of the higher dose used.

The absence of immune response after delayed antigen expression could be explained because at this time point, antigen presentation is not accompanied by an innate immune response. Therefore, a lower activation of DCs is expected, agreeing

with the lower expression of co-stimulatory molecules observed in the transduced DCs after 30 days (Fig. 4.5). A way of confirming this hypothesis is by testing if activators of the innate immune system (*e.g.*, poly I:C, CpG) could 'rescue' of the response to protracted antigen presentation.

Another possible explanation is that the antigen expression was insufficient in the long term. However, this is against the previous observation that the proportion of transduced cells increases over time and with the transduction of dividing precursors (Table 3.3). According to the latter, more transduced cells would be expected in the long term, since none or lower antigen expression would protect DCs from CTL elimination. However, the dose of vector used in these cases was lower than the one employed in previous experiments, which means that fewer cells are expected to be transduced. It is also possible that transduction of DC precursors is only achieved at high doses of the vector. Quantification of mRNA would be necessary to clarify these questions and confirm if, even in the presence of transgene expression, activation of DCs is insufficient to generate an immune response in the long term.

An inducible antigen expression system would be a useful tool to study the effect of the duration of antigen presentation on the magnitude and quality of the immune response. LVs with inducible promoters have been used in models of gene therapy and for regulatable central expression of antigens in transgenic mice (Probst *et al.* 2003; Dresch *et al.* 2008), but not for conditional antigen expression in LV-based vaccines. The model described here is limited because of the leakiness of the system. This resulted in a high variability that made interpretation of the results difficult. Further improvements in the design of the vector should be tested to improve this model.

Several alternatives have been evaluated in LVs to minimise background expression of Dox-regulated systems. These include: (1) second generation TREs, in which the distances and positions of consecutive tetO sequence elements have been optimised; (2) separation of expression cassettes with gene insulators, such as the chicken HS4 insulator (cHS4), that prevent positional interference of other promoters (Pluta *et al.* 2005; Xuebi *et al.* 2009); (3) the use of more complex vector designs. For example, Markusic *et al.* used a single LV with a TetOn system and an

autoregulatory loop, achieving high viral titers, improved induction kinetics and increased induction levels (Markusic *et al.* 2005). Szulc *et al.* (2006) have also developed a Dox-controllable LV with a combined tTA/KRAB (TetOff) or rtTA/KRAB (TetOn) complex. Expression from RNA polymerase II or III promoters can be regulated by this system. Combination of a Tet-dependent transactivator (rtTA) and silencer in the same construct also reduces the leakiness, but decreases the level of inducibility (Barde *et al.* 2006; Zhu *et al.* 2001). Alternatively, other regulatable gene expression systems dependent on other drugs or Cre-Lox-based models could be tested.

CHAPTER 6

6. FINAL DISCUSSION AND FUTURE DIRECTIONS

This thesis has explored the possible causes and implications of sustained antigen presentation following antigen gene delivery by LVs to APCs. This work was based on the well established fact that gene transfer using LVs results in long-lasting, stable gene expression, as has been shown in several animal models (Kim *et al.* 2009; Miccio *et al.* 2008; Kang *et al.* 2005b). Long-term gene expression had been reported in DCs in the spleen following systemic administration of LVs (Esslinger *et al.* 2003; Kimura *et al.* 2007), in spite of the short life span of these cells (Kamath *et al.* 2000). Since there is considerable interest in the use of LVs as vaccine vectors, it was important to address the effects of long-term transduction of DCs both as an issue of vaccine efficacy and safety. This chapter summarises the main conclusions reached by this project, their practical implications and directions for future research on the topic.

6.1. Summary and conclusions

The results presented in this thesis have demonstrated that intravenous administration of LVs results in efficient transduction of DCs in the spleen, confirming findings of previous reports (Esslinger *et al.* 2003; Kimura *et al.* 2007; Palmowski *et al.* 2004). Furthermore, there was transduction of all DC subpopulations, B cells and macrophages. Transduced DCs, but not lymphocytes, persist for a longer period of time than the expected half life of these cells. The evidence obtained suggests that this probably results from transduction of proliferating precursors that give rise to a long-term supply of DCs. The fact that DCs are the predominant BrdU-labelled, transduced cell population argues for the

transduction of a committed DC progenitor either in the bone marrow or in the spleen (Naik *et al.* 2006; Liu *et al.* 2009).

The proportion of persistent transduced cells was smaller when the LVs encoded OVA, a stronger immunogen, presumably because of immune killing of the antigen-bearing cells. Despite the elimination of these cells, there was persistence of antigen presentation that stimulated cognate T cells even 3 months later. This suggests that it is possible that the continuous supply of transduced cells from dividing precursors counterbalances the elimination of antigen-expressing DCs by the CTL response. The remaining population of transduced DCs stimulates proliferation and activates antigen-specific $CD8^+$ T cells. Protracted antigen presentation also takes place outside the spleen, suggesting that transduction of DCs or other cell types in other organs also play a role. These include long-lived macrophages and possibly non-professional APCs.

Transduction of DC precursors, however, does not necessarily result in effective T cell stimulation by its progeny, as these cells might not be sufficiently activated. Indeed, it was shown that the expression of costimulatory molecules in transduced DCs was lower in the long term as compared to the activated profile observed immediately after LV administration. Additionally, when the administration of LVs was dissociated from the actual antigen expression using an inducible expression system, the immune response to the antigen was impaired.

However, the long-term consequence of LV administration was immunisation rather than tolerance. This was demonstrated by activation of adoptively transferred transgenic $CD8^+$ T cells and by detection of a sustained endogenous $CD8^+$ T cell response, capable to lyse specific targets. Although more experimental work is necessary to show the effect of protracted antigen presentation on different lymphocyte populations, these data show that a single administration of LVs is sufficient to induce a rapid and protective primary response and to generate effective memory and maintenance of a protective state.

These data emphasise the utility of LVs as tools for immunotherapy. Persistent transduction of APCs also opens the opportunity to employ these vectors for long-term immunomodulation in other scenarios (e.g., autoimmune diseases, gene therapy, transplantation, chronic infections).

6.2. Limitations of the model

This project was performed using OVA as a model antigen in mice as a proof of principle for persistent antigen presentation. Extrapolation of the results must take into account the differences between the mouse and the human DC systems and the possible effects of delivering other type of antigens.

The surface markers of human DC subtypes differ from those in mice, although the distinct DC subsets have been paired with equivalent murine counterparts (Shortman & Liu 2002; Banchereau *et al.* 2009). However, the ontogeny of DCs in humans is poorly understood. Human DCs can be generated from $CD34^+$ $CD38^-$ haemopoietic stem cells or from peripheral blood $CD14^+$ mononuclear cells, but identification of committed DC precursors has been challenging given their low frequency, different markers from those known in mice and the obvious impossibility to perform reconstitution experiments. Local precursors in the spleen or peripheral lymphoid tissues, similar to those in mice, have not been identified yet (Mortellaro *et al.* 2009). It has been shown that LVs efficiently transduce haemopoietic stem cells (Sutton *et al.* 1999), but it is not known if downstream, committed precursors could also be transduced. In spite of these differences, these results suggest the possibility of targeting human DC precursors with LVs.

It would be important to evaluate the persistence of antigen presentation with clinically relevant antigens from pathogens or tumour cells. The latter are particularly important since in many cases TAAs are self-antigens. The consequences of sustained presentation of a self-antigen must be explored, as it could result in autoimmunity.

In this study, the effects of antigen presentation were measured through adoptive transfer of TCR-transgenic cells. With this method, an abnormally high frequency of naïve $CD8^+$ T cells is provided, which can activate DCs or provide their own help (Ruedl *et al.* 1999; Wang *et al.* 2001). Thus, it would be necessary to know the response of endogenous naïve and memory antigen-specific T cells to protracted antigen presentation.

Finally, the route of administration used in these experiments was intravenous. It is more likely that a subcutaneous route will be used for clinical LV immunisation because it is more efficient (Lopes *et al.* 2008). A different route of administration can result in targeting of different cell populations and this could change the duration of antigen presentation. Using the subcutaneous route, expansion of TCR-transgenic T cells specific to the antigen has been shown for 21 days, after which it seems to decline (Dullaers *et al.* 2006; Karwacz *et al.* 2009).

6.3. Considerations for the therapeutic application of LVs

As mentioned before, the possibility of extending antigen presentation for longer periods due to transduction of DC precursors must also be contemplated in humans. LV targeting of an equivalent DC precursor in the human would allow sustained, potentially life-long, expression of the transgene in DCs. In the context of systemic gene delivery for gene therapy, this could be an obstacle since it could result in a sustained or delayed immune response against the therapeutic transgene. In contrast, it could be an advantage when the objective is to generate an immune response against the transgene. Inefficiency of some vaccines has been attributed to short life of antigen presentation. Thus, sustained expression of antigen could improve vaccine efficacy, with a single injection being sufficient to generate protective immunity without the need of boosting.

DCs can also be genetically modified by LVs to become tolerogenic by manipulating intracellular transduction pathways (Escors *et al.* 2008). In this case, delivery of a self-antigen together with tolerogenic signals to appropriate DC precursors could provide life-long therapy for autoimmunity. In this way, LVs offer the unique opportunity to tailor the immune response taking advantage of the ample functional plasticity of the DCs.

6.4. Future perspectives

Several questions arise from the work presented in this thesis that I consider deserve further investigation. In the first place, LV targeting of specific DC subtypes is important as in the past few years distinct functional characteristics have been identified for different DC subpopulations.

Second, it would be important to study the kinetics of DC precursors to determine for how long transduced progenitors can maintain a population of genetically-modified DCs. Further characterisation of these progenitors, including their phenotype and anatomic location, would require better isolation techniques and reconstitution experiments. This would allow identifying which stages of DC development could be targeted by LVs, design vectors to target specific DC subtypes or de-target them from other haemopoietic lineages.

The immune consequences of persistent antigen presentation need to be studied in more detail. The results obtained here show that there is no tolerance to the transgene in the long term. This contrasts with results from other vaccination models where a short presentation of the antigen yields better immune response (Hovav *et al.* 2007; Radcliffe *et al.* 2007; Finn *et al.* 2009; Bergmann-Leitner *et al.* 2009). However, these studies have used DNA or adenoviral vector vaccines, where the mechanism of antigen presentation could be different. To investigate this with LVs, it would be necessary to isolate the persistent transduced DCs and characterise them *ex vivo*. This is a challenging task because of their low frequency. Another possibility could to generate a model where transgene expression in DCs can be controlled in time. This can be done with an improved regulatable vector like the one tested here that would allow a tight control of antigen expression or targeted ablation of transduced DCs.

6.5. Towards a LV-based vaccine

There is considerable interest in the use of LVs as vaccine vectors as they are efficient for delivery and expression of antigen genes in DCs *in vivo*. The evidence

suggests that their potential benefits outweigh the risks, especially for the treatment of serious illnesses like cancer or AIDS. There are still more challenges in the development of LV-based vaccines, including production up-scaling, vector engineering, targeting of specific cells, co-expression of immuno-modulators and use of non-integrating LVs. This thesis has examined the duration and quality of the immune response induced by LVs, highlighting the long-term consequences of transgene expression, which is highly relevant to all fields of therapy with LVs.

BIBLIOGRAPHY

Gene Therapy Clinical Trials Worldwide. Available at: http://www.wiley.co.uk/genetherapy/clinical/

Aicher, A., Westermann, J., Cayeux, S., Willimsky, G., Daemen, K., Blankenstein, T., Uckert, W., Dörken, B. & Pezzutto, A. (1997) Successful retroviral mediated transduction of a reporter gene in human dendritic cells: feasibility of therapy with gene-modified antigen presenting cells. *Experimental Hematology*, 25 (1), 39-44.

Akazawa, T., Shingai, M., Sasai, M., Ebihara, T., Inoue, N., Matsumoto, M. & Seya, T. (2007) Tumor immunotherapy using bone marrow-derived dendritic cells overexpressing Toll-like receptor adaptors. *FEBS Letters*, 581 (18), 3334-3340.

Allan, R.S., Smith, C.M., Belz, G.T., van Lint, A.L., Wakim, L.M., Heath, W.R. & Carbone, F.R. (2003) Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. *Science*, 301 (5641), 1925-1928.

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Brière, F. & Trinchieri, G. (2001) Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nature Immunology*, 2 (12), 1144-1150.

Asselin-Paturel, C., Brizard, G., Pin, J., Brière, F. & Trinchieri, G. (2003) Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *Journal of Immunology*, 171 (12), 6466-6477.

Banchereau, J. & Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature*, 392 (6673), 245-252.

Banchereau, J., Klechevsky, E., Schmitt, N., Morita, R., Palucka, K. & Ueno, H. (2009) Harnessing human dendritic cell subsets to design novel vaccines. *Annals of the New York Academy of Sciences*, 1174, 24-32.

Bao, L., Guo, H., Huang, X., Tammana, S., Wong, M., McIvor, R.S. & Zhou, X. (2009) High-titer lentiviral vectors stimulate fetal calf serum-specific human CD4 T-cell responses: implications in human gene therapy. *Gene Therapy*, 16 (6), 788-795.

Barde, I., Zanta-Boussif, M.A., Paisant, S., Leboeuf, M., Rameau, P., Delenda, C. & Danos, O. (2006) Efficient control of gene expression in the hematopoietic system using a single Tet-on inducible lentiviral vector. *Molecular Therapy*, 13 (2), 382-390.

Basta, S. & Alatery, A. (2007) The cross-priming pathway: a portrait of an intricate immune system. *Scandinavian Journal of Immunology*, 65 (4), 311-319.

Beignon, A., McKenna, K., Skoberne, M., Manches, O., DaSilva, I., Kavanagh, D.G., Larsson, M., Gorelick, R.J., Lifson, J.D. & Bhardwaj, N. (2005) Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. *The Journal of Clinical Investigation*, 115 (11), 3265-3275.

Beignon, A., Mollier, K., Liard, C., Coutant, F., Munier, S., Rivière, J., Souque, P. & Charneau, P. (2009) A Lentiviral Vector-Based Prime/Boost Vaccination Against AIDS: A Pilot Study Shows Protection Against SIVmac251 Challenge in Macaques. *Journal of Virology*, 83(21), 10963-10974.

Belz, G.T., Behrens, G.M.N., Smith, C.M., Miller, J.F.A.P., Jones, C., Lejon, K., Fathman, C.G., Mueller, S.N., Shortman, K., Carbone, F.R. & Heath, W.R. (2002) The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *The Journal of Experimental Medicine*, 196 (8), 1099-1104.

Bennett, C.L., Noordegraaf, M., Martina, C.A.E. & Clausen, B.E. (2007) Langerhans cells are required for efficient presentation of topically applied hapten to T cells. *Journal of Immunology*, 179 (10), 6830-6835.

Bergmann-Leitner, E.S., Leitner, W.W., Duncan, E.H., Savranskaya, T. & Angov, E. (2009) Molecular adjuvants for malaria DNA vaccines based on the modulation of host-cell apoptosis. *Vaccine*, 27 (41), 5700-5708.

Berkhout, B. & Jeang, K.T. (1992) Functional roles for the TATA promoter and enhancers in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. *Journal of Virology*, 66 (1), 139-149.

Björck, P. (2001) Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood*, 98 (13), 3520-3526.

Blander, J.M. & Medzhitov, R. (2006) Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature*, 440 (7085), 808-812.

den Boer, A.T., Diehl, L., van Mierlo, G.J., van der Voort, E.I., Fransen, M.F., Krimpenfort, P., Melief, C.J., Offringa, R. & Toes, R.E. (2001) Longevity of antigen presentation and activation status of APC are decisive factors in the balance between CTL immunity versus tolerance. *Journal of Immunology*, 167 (5), 2522-2528.

Bokhoven, M. (2008) Measurement of insertional mutagenesis by retroviral and lentiviral vectors. PhD Thesis, University College of London, London.

Bonehill, A., Tuyaerts, S., Van Nuffel, A.M.T., Heirman, C., Bos, T.J., Fostier, K., Neyns, B. & Thielemans, K. (2008) Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. *Molecular Therapy*, 16 (6), 1170-1180.

Bonehill, A., Van Nuffel, A.M.T., Corthals, J., Tuyaerts, S., Heirman, C., François, V., Colau, D., van der Bruggen, P., Neyns, B. & Thielemans, K. (2009) Single-step antigen loading and activation of dendritic cells by mRNA electroporation for the purpose of therapeutic vaccination in melanoma patients. *Clinical Cancer Research*, 15 (10), 3366-3375.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C. & Steinman, R.M. (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *The Journal of Experimental Medicine*, 196 (12), 1627-1638.

Borst, J., Hendriks, J. & Xiao, Y. (2005) CD27 and CD70 in T cell and B cell activation. *Current Opinion in Immunology*, 17 (3), 275-281.

Bouard, D., Alazard-Dany, D. & Cosset, F. (2009) Viral vectors: from virology to transgene expression. *British Journal of Pharmacology*, 157 (2), 153-165.

Breckpot, K., Aerts-Toegaert, C., Heirman, C., Peeters, U., Beyaert, R., Aerts, J.L. & Thielemans, K. (2009) Attenuated expression of A20 markedly increases the efficacy of double-stranded RNA-activated dendritic cells as an anti-cancer vaccine. *Journal of Immunology*, 182 (2), 860-870.

Breckpot, K., Emeagi, P., Dullaers, M., Michiels, A., Heirman, C. & Thielemans, K. (2007) Activation of immature monocyte-derived dendritic cells after transduction with high doses of lentiviral vectors. *Human Gene Therapy*, 18 (6), 536-546.

Breckpot, K., Heirman, C., Neyns, B. & Thielemans, K. (2004) Exploiting dendritic cells for cancer immunotherapy: genetic modification of dendritic cells. *The Journal of Gene Medicine*, 6 (11), 1175-1188.

Brewig, N., Kissenpfennig, A., Malissen, B., Veit, A., Bickert, T., Fleischer, B., Mostböck, S. & Ritter, U. (2009) Priming of CD8+ and CD4+ T cells in experimental leishmaniasis is initiated by different dendritic cell subtypes. *Journal of Immunology*, 182 (2), 774-783.

Brockman, M.A. & Knipe, D.M. (2002) Herpes simplex virus vectors elicit durable immune responses in the presence of preexisting host immunity. *Journal of Virology*, 76 (8), 3678-3687.

Brown, B.D., Sitia, G., Annoni, A., Hauben, E., Sergi, L.S., Zingale, A., Roncarolo, M.G., Guidotti, L.G. & Naldini, L. (2007) In vivo administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. *Blood*, 109 (7), 2797-2805.

Bruno, L., Kirberg, J. & von Boehmer, H. (1995) On the cellular basis of immunological T cell memory. *Immunity*, 2 (1), 37-43.

Buchbinder, S.P., Mehrotra, D.V., Duerr, A., Fitzgerald, D.W., Mogg, R., Li, D., Gilbert, P.B., Lama, J.R., Marmor, M., Del Rio, C., McElrath, M.J., Casimiro, D.R., Gottesdiener, K.M., Chodakewitz, J.A., Corey, L. & Robertson, M.N. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet*, 372 (9653), 1881-1893.

Bucks, C.M., Norton, J.A., Boesteanu, A.C., Mueller, Y.M. & Katsikis, P.D. (2009) Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *Journal of Immunology*, 182 (11), 6697-6708.

Buckwalter, M.R. & Albert, M.L. (2009) Orchestration of the immune response by dendritic cells. *Current Biology*, 19 (9), R355-361.

Buffa, V., Negri, D.R.M., Leone, P., Bona, R., Borghi, M., Bacigalupo, I., Carlei, D., Sgadari, C., Ensoli, B. & Cara, A. (2006) A single administration of lentiviral vectors expressing either full-length human immunodeficiency virus 1 (HIV-1)(HXB2) Rev/Env or codon-optimized HIV-1(JR-FL) gp120 generates durable immune responses in mice. *The Journal of General Virology*, 87 (Pt 6), 1625-1634.

Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*, 388 (6644), 782-787.

Center, R.J., Wheatley, A.K., Campbell, S.M., Gaeguta, A.J., Peut, V., Alcantara, S., Siebentritt, C., Kent, S.J. & Purcell, D.F.J. (2009) Induction of HIV-1 subtype B and AE-specific neutralizing antibodies in mice and macaques with DNA prime and recombinant gp140 protein boost regimens. *Vaccine*, 27(47): 6605-6612.

Chapatte, L., Colombetti, S., Cerottini, J. & Lévy, F. (2006) Efficient induction of tumor antigen-specific CD8+ memory T cells by recombinant lentivectors. *Cancer Research*, 66 (2), 1155-1160.

Chen, A., Xu, H., Choi, Y., Wang, B. & Zheng, G. (2004) TRANCE counteracts FasL-mediated apoptosis of murine bone marrow-derived dendritic cells. *Cellular Immunology*, 231 (1-2), 40-48.

Chen, M., Wang, Y., Wang, Y., Huang, L., Sandoval, H., Liu, Y. & Wang, J. (2006) Dendritic cell apoptosis in the maintenance of immune tolerance. *Science*, 311 (5764), 1160-1164.

Chen, X. & Jensen, P.E. (2008) The role of B lymphocytes as antigen-presenting cells. *Archivum Immunologiae Et Therapiae Experimentalis*, 56 (2), 77-83.

Chinnasamy, N., Chinnasamy, D., Toso, J.F., Lapointe, R., Candotti, F., Morgan, R.A. & Hwu, P. (2000) Efficient gene transfer to human peripheral blood monocyte-derived dendritic cells using human immunodeficiency virus type 1-based lentiviral vectors. *Human Gene Therapy*, 11 (13), 1901-1909.

Cockrell, A.S. & Kafri, T. (2007) Gene delivery by lentivirus vectors. *Molecular Biotechnology*, 36 (3), 184-204.

Collins, M.K. & Cerundolo, V. (2004) Gene therapy meets vaccine development. *Trends in Biotechnology*, 22 (12), 623-626.

Cronin, J., Zhang, X. & Reiser, J. (2005) Altering the tropism of lentiviral vectors through pseudotyping. *Current Gene Therapy*, 5 (4), 387-398.

Cui, Y., Golob, J., Kelleher, E., Ye, Z., Pardoll, D. & Cheng, L. (2002) Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood*, 99 (2), 399-408.

Cureton, D.K., Massol, R.H., Saffarian, S., Kirchhausen, T.L. & Whelan, S.P.J. (2009) Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization. *PLoS Pathogens*, 5 (4), e1000394.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K. & Mescher, M.F. (1999) Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *Journal of Immunology*, 162 (6), 3256-3262.

Curtsinger, J.M., Johnson, C.M. & Mescher, M.F. (2003a) CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *Journal of Immunology*, 171 (10), 5165-5171.

Curtsinger, J.M., Lins, D.C. & Mescher, M.F. (2003b) Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *The Journal of Experimental Medicine*, 197 (9), 1141-1151.

Danthinne, X. & Imperiale, M.J. (2000) Production of first generation adenovirus vectors: a review. *Gene Therapy*, 7 (20), 1707-1714.

Daubersies, P., Ollomo, B., Sauzet, J., Brahimi, K., Perlaza, B., Eling, W., Moukana, H., Rouquet, P., de Taisne, C. & Druilhe, P. (2008) Genetic immunisation by liver stage antigen 3 protects chimpanzees against malaria despite low immune responses. *PloS One*, 3 (7), e2659.

Daya, S. & Berns, K.I. (2008) Gene therapy using adeno-associated virus vectors. *Clinical Microbiology Reviews*, 21 (4), 583-593.

D'Costa, J., Mansfield, S.G. & Humeau, L.M. (2009) Lentiviral vectors in clinical trials: Current status. *Current Opinion in Molecular Therapeutics*, 11 (5), 554-564.

Demaison, C., Parsley, K., Brouns, G., Scherr, M., Battmer, K., Kinnon, C., Grez, M. & Thrasher, A.J. (2002) High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Human Gene Therapy*, 13 (7), 803-813.

DePolo, N.J., Reed, J.D., Sheridan, P.L., Townsend, K., Sauter, S.L., Jolly, D.J. & Dubensky, T.W. (2000) VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Molecular Therapy*, 2 (3), 218-222.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C. & Bhardwaj, N. (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *The Journal of Experimental Medicine*, 193 (2), 233-238.

Dhodapkar, M.V., Steinman, R.M., Sapp, M., Desai, H., Fossella, C., Krasovsky, J., Donahoe, S.M., Dunbar, P.R., Cerundolo, V., Nixon, D.F. & Bhardwaj, N. (1999) Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *The Journal of Clinical Investigation*, 104 (2), 173-180.

Dhodapkar, M.V. & Steinman, R.M. (2002) Antigen-bearing immature dendritic cells induce peptide-specific CD8+ regulatory T cells in vivo in humans. *Blood*, 100 (1), 174-177.

Diao, J., Winter, E., Cantin, C., Chen, W., Xu, L., Kelvin, D., Phillips, J. & Cattral, M.S. (2006) In situ replication of immediate dendritic cell precursors contributes to conventional DC homeostasis in lymphoid tissue. *Journal of Immunology*, 176 (12), 7196-7206.

Diao, J., Winter, E., Chen, W., Xu, F. & Cattral, M.S. (2007) Antigen transmission by replicating antigen-bearing dendritic cells. *Journal of Immunology*, 179 (5), 2713-2721.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science*, 303 (5663), 1529-1531.

Dresch, C., Edelmann, S.L., Marconi, P. & Brocker, T. (2008) Lentiviral-mediated transcriptional targeting of dendritic cells for induction of T cell tolerance in vivo. *Journal of Immunology*, 181 (7), 4495-4506.

Drexler, I., Staib, C. & Sutter, G. (2004) Modified vaccinia virus Ankara as antigen delivery system: how can we best use its potential? *Current Opinion in Biotechnology*, 15 (6), 506-512.

DuBridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H. & Calos, M.P. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Molecular and Cellular Biology*, 7 (1), 379-387.

van Duin, D., Medzhitov, R. & Shaw, A.C. (2006) Triggering TLR signaling in vaccination. *Trends in Immunology*, 27 (1), 49-55.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D. & Naldini, L. (1998) A third-generation lentivirus vector with a conditional packaging system. *Journal of Virology*, 72 (11), 8463-8471.

Dullaers, M., Van Meirvenne, S., Heirman, C., Straetman, L., Bonehill, A., Aerts, J.L., Thielemans, K. & Breckpot, K. (2006) Induction of effective therapeutic antitumor immunity by direct in vivo administration of lentiviral vectors. *Gene Therapy*, 13 (7), 630-640.

Dullaers, M., Breckpot, K., Van Meirvenne, S., Bonehill, A., Tuyaerts, S., Michiels, A., Straetman, L., Heirman, C., De Greef, C., Van Der Bruggen, P. & Thielemans, K. (2004) Side-by-side comparison of lentivirally transduced and mRNA-electroporated dendritic cells: implications for cancer immunotherapy protocols. *Molecular Therapy*, 10 (4), 768-779.

Dyall, J., Latouche, J.B., Schnell, S. & Sadelain, M. (2001) Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. *Blood*, 97 (1), 114-121.

Dzionek, A., Sohma, Y., Nagafune, J., Cella, M., Colonna, M., Facchetti, F., Günther, G., Johnston, I., Lanzavecchia, A., Nagasaka, T., Okada, T., Vermi, W., Winkels, G., Yamamoto, T., Zysk, M., Yamaguchi, Y. & Schmitz, J. (2001) BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *The Journal of Experimental Medicine*, 194 (12), 1823-1834.

Ehrhardt, A. & Kay, M.A. (2005) Gutted adenovirus: a rising star on the horizon? *Gene Therapy*, 12 (21), 1540-1541.

Elgueta, R., Benson, M.J., de Vries, V.C., Wasiuk, A., Guo, Y. & Noelle, R.J. (2009) Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunological Reviews*, 229 (1), 152-172.

Engelman, A., Mizuuchi, K. & Craigie, R. (1991) HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell*, 67 (6), 1211-1221.

Escors, D., Lopes, L., Lin, R., Hiscott, J., Akira, S., Davis, R.J. & Collins, M.K. (2008) Targeting dendritic cell signaling to regulate the response to immunization. *Blood*, 111 (6), 3050-3061.

Esslinger, C., Chapatte, L., Finke, D., Miconnet, I., Guillaume, P., Lévy, F. & MacDonald, H.R. (2003) In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *The Journal of Clinical Investigation*, 111 (11), 1673-1681.

Esslinger, C., Romero, P. & MacDonald, H.R. (2002) Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. *Human Gene Therapy*, 13 (9), 1091-1100.

Feil, R., Wagner, J., Metzger, D. & Chambon, P. (1997) Regulation of Cre Recombinase Activity by Mutated Estrogen Receptor Ligand-Binding Domains. *Biochemical and Biophysical Research Communications*, 237 (3), 752-757.

Feng, Y., Broder, C.C., Kennedy, P.E. & Berger, E.A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, 272 (5263), 872-877.

Finn, J.D., Bassett, J., Millar, J.B., Grinshtein, N., Yang, T.C., Parsons, R., Evelegh, C., Wan, Y., Parks, R.J. & Bramson, J.L. (2009) Persistence of transgene expression influences CD8+ T cell expansion and maintenance following immunization with recombinant adenovirus. *Journal of Virology*, 83(23), 12027-12036.

Firat, H., Zennou, V., Garcia-Pons, F., Ginhoux, F., Cochet, M., Danos, O., Lemonnier, F.A., Langlade-Demoyen, P. & Charneau, P. (2002) Use of a lentiviral flap vector for induction of CTL immunity against melanoma. Perspectives for immunotherapy. *The Journal of Gene Medicine*, 4 (1), 38-45.

Fitzgerald-Bocarsly, P. & Feng, D. (2007) The role of type I interferon production by dendritic cells in host defense. *Biochimie*, 89 (6-7), 843-855.

Fonteneau, J., Larsson, M., Beignon, A., McKenna, K., Dasilva, I., Amara, A., Liu, Y., Lifson, J.D., Littman, D.R. & Bhardwaj, N. (2004) Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *Journal of Virology*, 78 (10), 5223-5232.

Frecha, C., Szécsi, J., Cosset, F. & Verhoeyen, E. (2008) Strategies for targeting lentiviral vectors. *Current Gene Therapy*, 8 (6), 449-460.

Freundlieb, S., Schirra-Müller, C. & Bujard, H. (1999) A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. *The Journal of Gene Medicine*, 1 (1), 4-12.

Fritz, J.H., Ferrero, R.L., Philpott, D.J. & Girardin, S.E. (2006) Nod-like proteins in immunity, inflammation and disease. *Nature Immunology*, 7 (12), 1250-1257.

Fuller, D.H., Loudon, P. & Schmaljohn, C. (2006) Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods*, 40 (1), 86-97.

Funke, S., Maisner, A., Mühlebach, M.D., Koehl, U., Grez, M., Cattaneo, R., Cichutek, K. & Buchholz, C.J. (2008) Targeted cell entry of lentiviral vectors. *Molecular Therapy*, 16 (8), 1427-1436.

Galla, M., Will, E., Kraunus, J., Chen, L. & Baum, C. (2004) Retroviral pseudotransduction for targeted cell manipulation. *Molecular Cell*, 16 (2), 309-315.

Gallardo, H.F., Tan, C., Ory, D. & Sadelain, M. (1997) Recombinant retroviruses pseudotyped with the vesicular stomatitis virus G glycoprotein mediate both stable gene transfer and pseudotransduction in human peripheral blood lymphocytes. *Blood*, 90 (3), 952-957.

Garcia Casado, J., Janda, J., Wei, J., Chapatte, L., Colombetti, S., Alves, P., Ritter, G., Ayyoub, M., Valmori, D., Chen, W. & Lévy, F. (2008) Lentivector immunization induces tumor antigen-specific B and T cell responses in vivo. *European Journal of Immunology*, 38 (7), 1867-1876.

Geijtenbeek, T.B.H. & Gringhuis, S.I. (2009) Signalling through C-type lectin receptors: shaping immune responses. *Nature Reviews. Immunology*, 9 (7), 465-479.

Germain, R.N. & Margulies, D.H. (1993) The biochemistry and cell biology of antigen processing and presentation. *Annual Review of Immunology*, 11, 403-450.

Goff, S.P. (2001) Intracellular trafficking of retroviral genomes during the early phase of infection: viral exploitation of cellular pathways. *The Journal of Gene Medicine*, 3 (6), 517-528.

Gómez, C.E., Nájera, J.L., Krupa, M. & Esteban, M. (2008) The poxvirus vectors MVA and NYVAC as gene delivery systems for vaccination against infectious diseases and cancer. *Current Gene Therapy*, 8 (2), 97-120.

Gorski, K.S., Shin, T., Crafton, E., Otsuji, M., Rattis, F.M., Huang, X., Kelleher, E., Francisco, L., Pardoll, D. & Tsuchiya, H. (2003) A set of genes selectively expressed in murine dendritic cells: utility of related cis-acting sequences for lentiviral gene transfer. *Molecular Immunology*, 40 (1), 35-47.

Göttlinger, H.G. (2001) The HIV-1 assembly machine. *AIDS*, 15 Suppl 5, S13-20.

Gray, D. & Matzinger, P. (1991) T cell memory is short-lived in the absence of antigen. *The Journal of Experimental Medicine*, 174 (5), 969-974.

Gregers, T.F., Fleckenstein, B., Vartdal, F., Roepstorff, P., Bakke, O. & Sandlie, I. (2003) MHC class II loading of high or low affinity peptides directed by Ii/peptide fusion constructs: implications for T cell activation. *International Immunology.*, 15 (11), 1291-1299.

Grouard, G., Rissoan, M.C., Filgueira, L., Durand, I., Banchereau, J. & Liu, Y.J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *The Journal of Experimental Medicine*, 185 (6), 1101-1111.

Gruber, A., Kan-Mitchell, J., Kuhen, K.L., Mukai, T. & Wong-Staal, F. (2000) Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro. *Blood*, 96 (4), 1327-1333.

Grünebach, F., Müller, M.R. & Brossart, P. (2005) New developments in dendritic cell-based vaccinations: RNA translated into clinics. *Cancer Immunology, Immunotherapy*, 54 (6), 517-525.

Gulley, J.L., Arlen, P.M., Tsang, K., Yokokawa, J., Palena, C., Poole, D.J., Remondo, C., Cereda, V., Jones, J.L., Pazdur, M.P., Higgins, J.P., Hodge, J.W., Steinberg, S.M., Kotz, H., Dahut, W.L. & Schlom, J. (2008) Pilot study of vaccination with recombinant CEA-MUC-1-TRICOM poxviral-based vaccines in patients with metastatic carcinoma. *Clinical Cancer Research*, 14 (10), 3060-3069.

den Haan, J.M., Lehar, S.M. & Bevan, M.J. (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *The Journal of Experimental Medicine*, 192 (12), 1685-1696.

Haberman, R.P., McCown, T.J. & Samulski, R.J. (1998) Inducible long-term gene expression in brain with adeno-associated virus gene transfer. *Gene Therapy*, 5 (12), 1604-1611.

Haeryfar, S.M.M. & Hoskin, D.W. (2004) Thy-1: More than a mouse pan-T cell marker. *Journal of Immunology*, 173 (6), 3581-3588.

Harman, A.N., Wilkinson, J., Bye, C.R., Bosnjak, L., Stern, J.L., Nicholle, M., Lai, J. & Cunningham, A.L. (2006) HIV induces maturation of monocyte-derived dendritic cells and Langerhans cells. *Journal of Immunology*, 177 (10), 7103-7113.

Harrich, D., Ulich, C., García-Martínez, L.F. & Gaynor, R.B. (1997) Tat is required for efficient HIV-1 reverse transcription. *The EMBO Journal*, 16 (6), 1224-1235.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M. & Nussenzweig, M.C. (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *The Journal of Experimental Medicine*, 194 (6), 769-779.

He, Y., Zhang, J., Donahue, C. & Falo, L.D. (2006) Skin-derived dendritic cells induce potent CD8(+) T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity*, 24 (5), 643-656.

He, Y., Zhang, J., Mi, Z., Robbins, P. & Falo, L.D. (2005) Immunization with lentiviral vector-transduced dendritic cells induces strong and long-lasting T cell responses and therapeutic immunity. *Journal of Immunology*, 174 (6), 3808-3817.

Heath, W.R., Belz, G.T., Behrens, G.M.N., Smith, C.M., Forehan, S.P., Parish, I.A., Davey, G.M., Wilson, N.S., Carbone, F.R. & Villadangos, J.A. (2004) Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunological Reviews*, 199, 9-26.

Heemskerk, M.H., Hooijberg, E., Ruizendaal, J.J., van der Weide, M.M., Kueter, E., Bakker, A.Q., Schumacher, T.N. & Spits, H. (1999) Enrichment of an antigen-specific T cell response by retrovirally transduced human dendritic cells. *Cellular Immunology*, 195 (1), 10-17.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H. & Bauer, S. (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*, 303 (5663), 1526-1529.

Hemmi, H., Yoshino, M., Yamazaki, H., Naito, M., Iyoda, T., Omatsu, Y., Shimoyama, S., Letterio, J.J., Nakabayashi, T., Tagaya, H., Yamane, T., Ogawa, M., Nishikawa, S., Ryoke, K., Inaba, K., Hayashi, S. & Kunisada, T. (2001) Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-beta1-dependent cells. *International Immunology*, 13 (5), 695-704.

Henri, S., Siret, C., Machy, P., Kissenpfennig, A., Malissen, B. & Leserman, L. (2007) Mature DC from skin and skin-draining LN retain the ability to acquire and efficiently present targeted antigen. *European Journal of Immunology*, 37 (5), 1184-1193.

Hillen, W., Klock, G., Kaffenberger, I., Wray, L.V. & Reznikoff, W.S. (1982) Purification of the TET repressor and TET operator from the transposon Tn10 and characterization of their interaction. *The Journal of Biological Chemistry*, 257 (11), 6605-6613.

Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P. & O'Keeffe, M. (2001) Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *Journal of Immunology*, 166 (9), 5448-5455.

Hou, W. & Van Parijs, L. (2004) A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. *Nature Immunology*, 5 (6), 583-589.

Hovav, A., Panas, M.W., Rahman, S., Sircar, P., Gillard, G., Cayabyab, M.J. & Letvin, N.L. (2007) Duration of antigen expression in vivo following DNA immunization modifies the magnitude, contraction, and secondary responses of CD8+ T lymphocytes. *Journal of Immunology*, 179 (10), 6725-6733.

del Hoyo, G.M., Martín, P., Vargas, H.H., Ruiz, S., Arias, C.F. & Ardavín, C. (2002) Characterization of a common precursor population for dendritic cells. *Nature*, 415 (6875), 1043-1047.

Iezzi, G., Karjalainen, K. & Lanzavecchia, A. (1998) The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity*, 8 (1), 89-95.

Iglesias, M.C., Frenkiel, M., Mollier, K., Souque, P., Despres, P. & Charneau, P. (2006) A single immunization with a minute dose of a lentiviral vector-based vaccine is highly effective at eliciting protective humoral immunity against West Nile virus. *The Journal of Gene Medicine*, 8 (3), 265-274.

Iglesias, M.C., Mollier, K., Beignon, A., Souque, P., Adotevi, O., Lemonnier, F. & Charneau, P. (2007) Lentiviral vectors encoding HIV-1 polyepitopes induce broad CTL responses in vivo. *Molecular Therapy*, 15 (6), 1203-1210.

Ikeda, Y., Yonemitsu, Y., Miyazaki, M., Kohno, R., Murakami, Y., Murata, T., Tabata, T., Ueda, Y., Ono, F., Suzuki, T., Ageyama, N., Terao, K., Hasegawa, M., Sueishi, K. & Ishibashi, T. (2009) Stable retinal gene expression in nonhuman primates via subretinal injection of SIVagm-based lentiviral vectors. *Human Gene Therapy*, 20 (6), 573-579.

Iwasaki, A. & Medzhitov, R. (2004) Toll-like receptor control of the adaptive immune responses. *Nature Immunology*, 5 (10), 987-995.

Janeway, C.A. & Bottomly, K. (1994) Signals and signs for lymphocyte responses. *Cell*, 76 (2), 275-285.

Jensen, P.E. (2007) Recent advances in antigen processing and presentation. *Nature Immunology*, 8 (10), 1041-1048.

Johansen, J., Rosenblad, C., Andsberg, K., Møller, A., Lundberg, C., Björlund, A. & Johansen, T.E. (2002) Evaluation of Tet-on system to avoid transgene down-regulation in ex vivo gene transfer to the CNS. *Gene Therapy*, 9 (19), 1291-1301.

Jonuleit, H., Schmitt, E., Steinbrink, K. & Enk, A.H. (2001) Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends in Immunology*, 22 (7), 394-400.

Kabashima, K., Banks, T.A., Ansel, K.M., Lu, T.T., Ware, C.F. & Cyster, J.G. (2005) Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells. *Immunity*, 22 (4), 439-450.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F. & Liu, Y.J. (2001) Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *The Journal of Experimental Medicine*, 194 (6), 863-869.

Kafri, T., van Praag, H., Gage, F.H. & Verma, I.M. (2000) Lentiviral vectors: regulated gene expression. *Molecular Therapy*, 1 (6), 516-521.

Kaliński, P., Hilkens, C.M., Wierenga, E.A. & Kapsenberg, M.L. (1999) T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunology Today*, 20 (12), 561-567.

Kamath, A.T., Pooley, J., O'Keeffe, M.A., Vremec, D., Zhan, Y., Lew, A.M., D'Amico, A., Wu, L., Tough, D.F. & Shortman, K. (2000) The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *Journal of Immunology*, 165 (12), 6762-6770.

Kamath, A.T., Henri, S., Battye, F., Tough, D.F. & Shortman, K. (2002) Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood*, 100 (5), 1734-1741.

Kang, Y., Xie, L., Tran, D.T., Stein, C.S., Hickey, M., Davidson, B.L. & McCray, P.B. (2005) Persistent expression of factor VIII in vivo following nonprimate lentiviral gene transfer. *Blood*, 106 (5), 1552-1558.

Karsunky, H., Merad, M., Cozzio, A., Weissman, I.L. & Manz, M.G. (2003) Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. *The Journal of Experimental Medicine*, 198 (2), 305-313.

Karwacz, K., Mukherjee, S., Apolonia, L., Blundell, M.P., Bouma, G., Escors, D., Collins, M.K. & Thrasher, A.J. (2009) Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy. *Journal of Virology*, 83 (7), 3094-3103.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O. & Akira, S. (2005) Cell type-specific involvement of RIG-I in antiviral response. *Immunity*, 23 (1), 19-28.

Kaushik, N., Rege, N., Yadav, P.N., Sarafianos, S.G., Modak, M.J. & Pandey, V.N. (1996) Biochemical analysis of catalytically crucial aspartate mutants of human immunodeficiency virus type 1 reverse transcriptase. *Biochemistry*, 35 (36), 11536-11546.

Keller, A.M., Schildknecht, A., Xiao, Y., van den Broek, M. & Borst, J. (2008) Expression of costimulatory ligand CD70 on steady-state dendritic cells breaks CD8+ T cell tolerance and permits effective immunity. *Immunity*, 29 (6), 934-946.

Keller, A.M., Xiao, Y., Peperzak, V., Naik, S.H. & Borst, J. (2009) Costimulatory ligand CD70 allows induction of CD8+ T-cell immunity by immature dendritic cells in a vaccination setting. *Blood*, 113 (21), 5167-5175.

Kim, J.H., Majumder, N., Lin, H., Watkins, S., Falo, L.D. & You, Z. (2005) Induction of therapeutic antitumor immunity by in vivo administration of a lentiviral vaccine. *Human Gene Therapy*, 16 (11), 1255-1266.

Kim, Y., Kim, Y., Larochelle, A., Renaud, G., Wolfsberg, T.G., Adler, R., Donahue, R.E., Hematti, P., Hong, B., Roayaei, J., Akagi, K., Riberdy, J.M., Nienhuis, A.W., Dunbar, C.E. & Persons, D.A. (2009) Sustained high-level polyclonal hematopoietic marking and transgene expression 4 years after autologous transplantation of rhesus macaques with SIV lentiviral vector-transduced CD34+ cells. *Blood*, 113 (22), 5434-5443.

Kimura, T., Koya, R.C., Anselmi, L., Sternini, C., Wang, H., Comin-Anduix, B., Prins, R.M., Faure-Kumar, E., Rozengurt, N., Cui, Y., Kasahara, N. & Stripecke, R. (2007) Lentiviral vectors with CMV or MHCII promoters administered in vivo: immune reactivity versus persistence of expression. *Molecular Therapy*, 15 (7), 1390-1399.

Kingsman, S.M., Mitrophanous, K. & Olsen, J.C. (2005) Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). *Gene Therapy*, 12 (1), 3-4.

Klinman, D.M. (2006) Adjuvant activity of CpG oligodeoxynucleotides. *International Reviews of Immunology*, 25 (3-4), 135-154.

Koponen, J.K., Kankkonen, H., Kannasto, J., Wirth, T., Hillen, W., Bujard, H. & Ylä-Herttuala, S. (2003) Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression in vitro and in vivo. *Gene Therapy*, 10 (6), 459-466.

Koya, R.C., Kasahara, N., Favaro, P.M.B., Lau, R., Ta, H.Q., Weber, J.S. & Stripecke, R. (2003) Potent maturation of monocyte-derived dendritic cells after CD40L lentiviral gene delivery. *Journal of Immunotherapy*, 26 (5), 451-460.

Koyama, S., Coban, C., Aoshi, T., Horii, T., Akira, S. & Ishii, K.J. (2009) Innate immune control of nucleic acid-based vaccine immunogenicity. *Expert Review of Vaccines*, 8 (8), 1099-1107.

Kündig, T.M., Bachmann, M.F., Oehen, S., Hoffmann, U.W., Simard, J.J., Kalberer, C.P., Pircher, H., Ohashi, P.S., Hengartner, H. & Zinkernagel, R.M. (1996) On the role of antigen in maintaining cytotoxic T-cell memory. *Proceedings of the National Academy of Sciences of the United States of America*, 93 (18), 9716-9723.

Kutzler, M.A. & Weiner, D.B. (2008) DNA vaccines: ready for prime time? *Nature Reviews. Genetics*, 9 (10), 776-788.

Lang, K.S., Recher, M., Junt, T., Navarini, A.A., Harris, N.L., Freigang, S., Odermatt, B., Conrad, C., Ittner, L.M., Bauer, S., Luther, S.A., Uematsu, S., Akira, S., Hengartner, H. & Zinkernagel, R.M. (2005) Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nature Medicine*, 11 (2), 138-145.

Lanzavecchia, A. & Sallusto, F. (2000) Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science*, 290 (5489), 92-97.

Lasaro, M.O. & Ertl, H.C.J. (2009) New insights on adenovirus as vaccine vectors. *Molecular Therapy*, 17 (8), 1333-1339.

Lau, L.L., Jamieson, B.D., Somasundaram, T. & Ahmed, R. (1994) Cytotoxic T-cell memory without antigen. *Nature*, 369 (6482), 648-652.

Lauterbach, H., Ried, C., Epstein, A.L., Marconi, P. & Brocker, T. (2005) Reduced immune responses after vaccination with a recombinant herpes simplex virus type 1 vector in the presence of antiviral immunity. *The Journal of General Virology*, 86 (Pt 9), 2401-2410.

Le Guiner, C., Stieger, K., Snyder, R.O., Rolling, F. & Moullier, P. (2007) Immune responses to gene product of inducible promoters. *Current Gene Therapy*, 7 (5), 334-346.

Lechleider, R.J., Arlen, P.M., Tsang, K., Steinberg, S.M., Yokokawa, J., Cereda, V., Camphausen, K., Schlom, J., Dahut, W.L. & Gulley, J.L. (2008) Safety and immunologic response of a viral vaccine to prostate-specific antigen in combination with radiation therapy when metronomic-dose interleukin 2 is used as an adjuvant. *Clinical Cancer Research*, 14 (16), 5284-5291.

Lee, Y., Glover, C.P.J., Cosgrave, A.S., Bienemann, A. & Uney, J.B. (2005) Optimizing regulatable gene expression using adenoviral vectors. *Experimental Physiology*, 90 (1), 33-37.

Levine, B.L., Humeau, L.M., Boyer, J., MacGregor, R., Rebello, T., Lu, X., Binder, G.K., Slepushkin, V., Lemiale, F., Mascola, J.R., Bushman, F.D., Dropulic, B. & June, C.H. (2006) Gene transfer in humans using a conditionally replicating lentiviral vector. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (46), 17372-17377.

Liu, J., Yu, Q., Stone, G.W., Yue, F.Y., Ngai, N., Jones, R.B., Kornbluth, R.S. & Ostrowski, M.A. (2008) CD40L expressed from the canarypox vector, ALVAC, can boost immunogenicity of HIV-1 canarypox vaccine in mice and enhance the in vitro expansion of viral specific CD8+ T cell memory responses from HIV-1-infected and HIV-1-uninfected individuals. *Vaccine*, 26 (32), 4062-4072.

Liu, K., Victora, G.D., Schwickert, T.A., Guermonprez, P., Meredith, M.M., Yao, K., Chu, F., Randolph, G.J., Rudensky, A.Y. & Nussenzweig, M. (2009) In vivo analysis of dendritic cell development and homeostasis. *Science*, 324 (5925), 392-397.

Liu, K., Waskow, C., Liu, X., Yao, K., Hoh, J. & Nussenzweig, M. (2007) Origin of dendritic cells in peripheral lymphoid organs of mice. *Nature Immunology*, 8 (6), 578-583.

Liu, M.L., Winther, B.L. & Kay, M.A. (1996) Pseudotransduction of hepatocytes by using concentrated pseudotyped vesicular stomatitis virus G glycoprotein (VSV-G)-Moloney murine leukemia virus-derived retrovirus vectors: comparison of VSV-G and amphotropic vectors for hepatic gene transfer. *Journal of Virology*, 70 (4), 2497-2502.

Liu, Y., Peng, Y., Mi, M., Guevara-Patino, J., Munn, D.H., Fu, N. & He, Y. (2009) Lentivector immunization stimulates potent CD8 T cell responses against melanoma self-antigen tyrosinase-related protein 1 and generates antitumor immunity in mice. *Journal of Immunology*, 182 (10), 5960-5969.

Loisel-Meyer, S., Felizardo, T., Mariotti, J., Mossoba, M.E., Foley, J.E., Kammerer, R., Mizue, N., Keefe, R., McCart, J.A., Zimmermann, W., Dropulic, B., Fowler, D.H. & Medin, J.A. (2009) Potent induction of B- and T-cell immunity against human carcinoembryonic antigen-expressing tumors in human carcinoembryonic antigen transgenic mice mediated by direct lentivector injection. *Molecular Cancer Therapeutics*, 8 (3), 692-702.

Lopes, L., Dewannieux, M., Gileadi, U., Bailey, R., Ikeda, Y., Whittaker, C., Collin, M.P., Cerundolo, V., Tomihari, M., Ariizumi, K. & Collins, M.K. (2008) Immunization with a lentivector that targets tumor antigen expression to dendritic cells induces potent CD8+ and CD4+ T-cell responses. *Journal of Virology*, 82 (1), 86-95.

Lopes, L., Fletcher, K., Ikeda, Y. & Collins, M. (2006) Lentiviral vector expression of tumour antigens in dendritic cells as an immunotherapeutic strategy. *Cancer Immunology, Immunotherapy: CII*, 55 (8), 1011-1016.

Lotem, M., Zhao, Y., Riley, J., Hwu, P., Morgan, R.A., Rosenberg, S.A. & Parkhurst, M.R. (2006) Presentation of tumor antigens by dendritic cells genetically modified with viral and nonviral vectors. *Journal of Immunotherapy*, 29 (6), 616-627.

Lowe, D.M., Parmar, V., Kemp, S.D. & Larder, B.A. (1991) Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. *FEBS Letters*, 282 (2), 231-234.

Ludewig, B., McCoy, K., Pericin, M., Ochsenbein, A.F., Dumrese, T., Odermatt, B., Toes, R.E., Melief, C.J., Hengartner, H. & Zinkernagel, R.M. (2001) Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells. *Journal of Immunology*, 166 (6), 3678-3687.

Macatonia, S.E., Gompels, M., Pinching, A.J., Patterson, S. & Knight, S.C. (1992) Antigen-presentation by macrophages but not by dendritic cells in human immunodeficiency virus (HIV) infection. *Immunology*, 75 (4), 576-581.

Mahadevan, M., Liu, Y., You, C., Luo, R., You, H., Mehta, J.L. & Hermonat, P.L. (2007) Generation of robust cytotoxic T lymphocytes against prostate specific antigen by transduction of dendritic cells using protein and recombinant adeno-associated virus. *Cancer Immunology, Immunotherapy*, 56 (10), 1615-1624.

Maldonado-López, R., De Smedt, T., Michel, P., Godfroid, J., Pajak, B., Heirman, C., Thielemans, K., Leo, O., Urbain, J. & Moser, M. (1999) CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *The Journal of Experimental Medicine*, 189 (3), 587-592.

Malim, M.H. & Emerman, M. (2008) HIV-1 accessory proteins--ensuring viral survival in a hostile environment. *Cell Host & Microbe*, 3 (6), 388-398.

Manickasingham, S.P., Edwards, A., Schulz, O. & Sousa, C. (2003) The ability of murine dendritic cell subsets to direct T helper cell differentiation is dependent on microbial signals. *European Journal of Immunology*, 33 (1), 101-107.

Marangoni, F., Bosticardo, M., Charrier, S., Draghici, E., Locci, M., Scaramuzza, S., Panaroni, C., Ponzoni, M., Sanvito, F., Doglioni, C., Liabeuf, M., Gjata, B., Montus, M., Siminovitch, K., Aiuti, A., Naldini, L., Dupré, L., Roncarolo, M.G., Galy, A. & Villa, A. (2009) Evidence for long-term efficacy and safety of gene therapy for Wiskott-Aldrich syndrome in preclinical models. *Molecular Therapy*, 17 (6), 1073-1082.

Marconi, P., Argnani, R., Berto, E., Epstein, A.L. & Manservigi, R. (2008) HSV as a vector in vaccine development and gene therapy. *Human Vaccines*, 4 (2), 91-105.

Margolin, J.F., Friedman, J.R., Meyer, W.K., Vissing, H., Thiesen, H.J. & Rauscher, F.J. (1994) Krüppel-associated boxes are potent transcriptional repression domains. *Proceedings of the National Academy of Sciences of the United States of America*, 91 (10), 4509-4513.

Markusic, D., Oude-Elferink, R., Das, A.T., Berkhout, B. & Seppen, J. (2005) Comparison of single regulated lentiviral vectors with rtTA expression driven by an autoregulatory loop or a constitutive promoter. *Nucleic Acids Research*, 33 (6), p.e63.

Martin-Serrano, J. (2007) The role of ubiquitin in retroviral egress. *Traffic*, 8 (10), 1297-1303.

Matsue, H., Edelbaum, D., Hartmann, A.C., Morita, A., Bergstresser, P.R., Yagita, H., Okumura, K. & Takashima, A. (1999) Dendritic cells undergo rapid apoptosis in vitro during antigen-specific interaction with CD4+ T cells. *Journal of Immunology*, 162 (9), 5287-5298.

McCarty, D.M. (2008) Self-complementary AAV vectors; advances and applications. *Molecular Therapy*, 16 (10), 1648-1656.

McLellan, A.D., Kapp, M., Eggert, A., Linden, C., Bommhardt, U., Brocker, E., Kammerer, U. & Kampgen, E. (2002) Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression. *Blood*, 99 (6), 2084-2093.

Means, T.K., Hayashi, F., Smith, K.D., Aderem, A. & Luster, A.D. (2003) The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *Journal of Immunology*, 170 (10), 5165-5175.

Mercado, R., Vijh, S., Allen, S.E., Kerksiek, K., Pilip, I.M. & Pamer, E.G. (2000) Early programming of T cell populations responding to bacterial infection. *Journal of Immunology*, 165 (12), 6833-6839.

Metharom, P., Ellem, K.A., Schmidt, C. & Wei, M.Q. (2001) Lentiviral vector-mediated tyrosinase-related protein 2 gene transfer to dendritic cells for the therapy of melanoma. *Human Gene Therapy*, 12 (18), 2203-2213.

Miccio, A., Cesari, R., Lotti, F., Rossi, C., Sanvito, F., Ponzoni, M., Routledge, S.J.E., Chow, C., Antoniou, M.N. & Ferrari, G. (2008) In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia. *Proceedings of the National Academy of Sciences of the United States of America*, 105 (30), 10547-10552.

Mitchell, T.C., Hildeman, D., Kedl, R.M., Teague, T.K., Schaefer, B.C., White, J., Zhu, Y., Kappler, J. & Marrack, P. (2001) Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nature Immunology*, 2 (5), 397-402.

Modlich, U., Navarro, S., Zychlinski, D., Maetzig, T., Knoess, S., Brugman, M.H., Schambach, A., Charrier, S., Galy, A., Thrasher, A.J., Bueren, J. & Baum, C. (2009) Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Molecular Therapy*, 17 (11), 1919-1928.

Mooij, P., Balla-Jhagjhoorsingh, S.S., Koopman, G., Beenhakker, N., van Haaften, P., Baak, I., Nieuwenhuis, I.G., Kondova, I., Wagner, R., Wolf, H., Gómez, C.E., Nájera, J.L., Jiménez, V., Esteban, M. & Heeney, J.L. (2008) Differential CD4+ versus CD8+ T-cell responses elicited by different poxvirus-based human immunodeficiency virus type 1 vaccine candidates provide comparable efficacies in primates. *Journal of Virology*, 82 (6), 2975-2988.

Mortellaro, A., Wong, S.C., Fric, J. & Ricciardi-Castagnoli, P. (2010) The need to identify myeloid dendritic cell progenitors in human blood. *Trends in Immunology,* 31 (1), 18-23.

Moskophidis, D., Lechner, F., Pircher, H. & Zinkernagel, R.M. (1993) Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature*, 362 (6422), 758-761.

Movassagh, M., Baillou, C., Cosset, F.L., Klatzmann, D., Guigon, M. & Lemoine, F.M. (1999) High level of retrovirus-mediated gene transfer into dendritic cells derived from cord blood and mobilized peripheral blood CD34+ cells. *Human Gene Therapy*, 10 (2), 175-187.

Müllbacher, A. (1994) The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen. *The Journal of Experimental Medicine*, 179 (1), 317-321.

Mueller, S.N. & Ahmed, R. (2009) High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (21), 8623-8628.

Mueller, S.N., Jones, C.M., Stock, A.T., Suter, M., Heath, W.R. & Carbone, F.R. (2006) CD4+ T cells can protect APC from CTL-mediated elimination. *Journal of Immunology*, 176 (12), 7379-7384.

Murphy, C.G., Lucas, W.T., Means, R.E., Czajak, S., Hale, C.L., Lifson, J.D., Kaur, A., Johnson, R.P., Knipe, D.M. & Desrosiers, R.C. (2000) Vaccine protection against simian immunodeficiency virus by recombinant strains of herpes simplex virus. *Journal of Virology*, 74 (17), 7745-7754.

Naik, S.H. (2008) Demystifying the development of dendritic cell subtypes, a little. *Immunology and Cell Biology*, 86 (5), 439-452.

Naik, S.H., Metcalf, D., van Nieuwenhuijze, A., Wicks, I., Wu, L., O'Keeffe, M. & Shortman, K. (2006) Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nature Immunology*, 7 (6), 663-671.

Nakamura, T., Peng, K., Harvey, M., Greiner, S., Lorimer, I.A.J., James, C.D. & Russell, S.J. (2005) Rescue and propagation of fully retargeted oncolytic measles viruses. *Nature Biotechnology*, 23 (2), 209-214.

Nakano, H., Lin, K.L., Yanagita, M., Charbonneau, C., Cook, D.N., Kakiuchi, T. & Gunn, M.D. (2009) Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nature Immunology*, 10 (4), 394-402.

Naldini, L., Blömer, U., Gage, F.H., Trono, D. & Verma, I.M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proceedings of the National Academy of Sciences of the United States of America*, 93 (21), 11382-11388.

Naldini, L., Blömer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M. & Trono, D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, 272 (5259), 263-267.

Negri, D.R.M., Michelini, Z., Baroncelli, S., Spada, M., Vendetti, S., Buffa, V., Bona, R., Leone, P., Klotman, M.E. & Cara, A. (2007) Successful immunization with a single injection of non-integrating lentiviral vector. *Molecular Therapy*, 15 (9), 1716-1723.

Neil, S.J.D., Zang, T. & Bieniasz, P.D. (2008) Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature*, 451 (7177), 425-430.

Nisole, S. & Saïb, A. (2004) Early steps of retrovirus replicative cycle. *Retrovirology*, 1, p.9.

No, D., Yao, T.P. & Evans, R.M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, 93 (8), 3346-3351.

Norbury, C.C., Malide, D., Gibbs, J.S., Bennink, J.R. & Yewdell, J.W. (2002) Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nature Immunology*, 3 (3), 265-271.

Obst, R., van Santen, H., Mathis, D. & Benoist, C. (2005) Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *The Journal of Experimental Medicine*, 201 (10), 1555-1565.

Obst, R., van Santen, H., Melamed, R., Kamphorst, A.O., Benoist, C. & Mathis, D. (2007) Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation. *Proceedings of the National Academy of Sciences of the United States of America*, 104 (39), 15460-15465.

Oehen, S., Waldner, H., Kündig, T.M., Hengartner, H. & Zinkernagel, R.M. (1992) Antivirally protective cytotoxic T cell memory to lymphocytic choriomeningitis virus is governed by persisting antigen. *The Journal of Experimental Medicine*, 176 (5), 1273-1281.

O'Keeffe, M., Hochrein, H., Vremec, D., Caminschi, I., Miller, J.L., Anders, E.M., Wu, L., Lahoud, M.H., Henri, S., Scott, B., Hertzog, P., Tatarczuch, L. & Shortman, K. (2002) Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8+ dendritic cells only after microbial stimulus. *The Journal of Experimental Medicine*, 196 (10), 1307-1319.

O'Keeffe, M., Hochrein, H., Vremec, D., Scott, B., Hertzog, P., Tatarczuch, L. & Shortman, K. (2003) Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood*, 101 (4), 1453-1459.

Oki, M., Ando, K., Hagihara, M., Miyatake, H., Shimizu, T., Miyoshi, H., Nakamura, Y., Matsuzawa, H., Sato, T., Ueda, Y., Gansuvd, B., Kato, S. & Hotta, T. (2001) Efficient lentiviral transduction of human cord blood CD34(+) cells followed by their expansion and differentiation into dendritic cells. *Experimental Hematology*, 29 (10), 1210-1217.

Olsen, J.C. (1998) Gene transfer vectors derived from equine infectious anemia virus. *Gene Therapy*, 5 (11), 1481-1487.

Onai, N., Obata-Onai, A., Schmid, M.A., Ohteki, T., Jarrossay, D. & Manz, M.G. (2007) Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nature Immunology*, 8 (11), 1207-1216.

Palmowski, M.J., Lopes, L., Ikeda, Y., Salio, M., Cerundolo, V. & Collins, M.K. (2004) Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. *Journal of Immunology*, 172 (3), 1582-1587.

Paludan, C., Schmid, D., Landthaler, M., Vockerodt, M., Kube, D., Tuschl, T. & Münz, C. (2005) Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science*, 307 (5709), 593-596.

Pan, D., Gunther, R., Duan, W., Wendell, S., Kaemmerer, W., Kafri, T., Verma, I.M. & Whitley, C.B. (2002) Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. *Molecular Therapy*, 6 (1), 19-29.

Pang, B., Neijssen, J., Qiao, X., Janssen, L., Janssen, H., Lippuner, C. & Neefjes, J. (2009) Direct antigen presentation and gap junction mediated cross-presentation during apoptosis. *Journal of Immunology*, 183 (2), 1083-1090.

Pascolo, S. (2004) Messenger RNA-based vaccines. *Expert Opinion on Biological Therapy*, 4 (8), 1285-1294.

Pear, W.S., Nolan, G.P., Scott, M.L. & Baltimore, D. (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proceedings of the National Academy of Sciences of the United States of America*, 90 (18), 8392-8396.

Peters, B.S., Jaoko, W., Vardas, E., Panayotakopoulos, G., Fast, P., Schmidt, C., Gilmour, J., Bogoshi, M., Omosa-Manyonyi, G., Dally, L., Klavinskis, L., Farah, B., Tarragona, T., Bart, P., Robinson, A., Pieterse, C., Stevens, W., Thomas, R., Barin, B., McMichael, A.J., McIntyre, J.A., Pantaleo, G., Hanke, T. & Bwayo, J. (2007) Studies of a prophylactic HIV-1 vaccine candidate based on modified vaccinia virus Ankara (MVA) with and without DNA priming: effects of dosage and route on safety and immunogenicity. *Vaccine*, 25 (11), 2120-2127.

Pichlmair, A., Diebold, S.S., Gschmeissner, S., Takeuchi, Y., Ikeda, Y., Collins, M.K. & Reis e Sousa, C. (2007) Tubulovesicular structures within vesicular stomatitis virus G protein-pseudotyped lentiviral vector preparations carry DNA and stimulate antiviral responses via Toll-like receptor 9. *Journal of Virology*, 81 (2), 539-547.

Pluta, K., Luce, M.J., Bao, L., Agha-Mohammadi, S. & Reiser, J. (2005) Tight control of transgene expression by lentivirus vectors containing second-generation tetracycline-responsive promoters. *The Journal of Gene Medicine*, 7 (6), 803-817.

Pollard, V.W. & Malim, M.H. (1998) The HIV-1 Rev protein. *Annual Review of Microbiology*, 52, 491-532.

Pooley, J.L., Heath, W.R. & Shortman, K. (2001) Cutting Edge: Intravenous soluble antigen is presented to CD4+ T cells by CD8- dendritic cells, but cross-presented to CD8+ T cells by CD8+ dendritic cells. *Journal of Immunology*, 166 (9), 5327-5330.

Pozzi, L.M., Maciaszek, J.W. & Rock, K.L. (2005) Both dendritic cells and macrophages can stimulate naive CD8+ T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. *Journal of Immunology*, 175 (4), 2071-2081.

Pradhan, S., Genebriera, J., Denning, W.L., Felix, K., Elmets, C.A. & Timares, L. (2006) CD4+ T cell-induced, bid-dependent apoptosis of cutaneous dendritic cells regulates T cell expansion and immune responses. *Journal of Immunology*, 177 (9), 5956-5967.

Prlic, M., Hernandez-Hoyos, G. & Bevan, M.J. (2006) Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. *The Journal of Experimental Medicine*, 203 (9), 2135-2143.

Probst, H.C., Lagnel, J., Kollias, G. & van den Broek, M. (2003) Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance. *Immunity*, 18 (5), 713-720.

Quaranta, M.G., Mattioli, B., Giordani, L. & Viora, M. (2009) Immunoregulatory effects of HIV-1 Nef protein. *BioFactors*, 35 (2), 169-174.

Radcliffe, J.N., Roddick, J.S., Stevenson, F.K. & Thirdborough, S.M. (2007) Prolonged antigen expression following DNA vaccination impairs effector CD8+ T cell function and memory development. *Journal of Immunology*, 179 (12), 8313-8321.

Randolph, G.J., Inaba, K., Robbiani, D.F., Steinman, R.M. & Muller, W.A. (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity*, 11 (6), 753-761.

Rege, T.A. & Hagood, J.S. (2006) Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J.*, 20 (8), 1045-1054.

Régulier, E., Pereira de Almeida, L., Sommer, B., Aebischer, P. & Déglon, N. (2002) Dose-dependent neuroprotective effect of ciliary neurotrophic factor delivered via tetracycline-regulated lentiviral vectors in the quinolinic acid rat model of Huntington's disease. *Human Gene Therapy*, 13 (16), 1981-1990.

Reis e Sousa, C. (2006) Dendritic cells in a mature age. *Nature Reviews. Immunology*, 6 (6), 476-483.

Reis e Sousa, C. (2004) Toll-like receptors and dendritic cells: for whom the bug tolls. *Seminars in Immunology*, 16 (1), 27-34.

Reiser, J., Lai, Z., Zhang, X.Y. & Brady, R.O. (2000) Development of multigene and regulated lentivirus vectors. *Journal of Virology*, 74 (22), 10589-10599.

Rice, J., Ottensmeier, C.H. & Stevenson, F.K. (2008) DNA vaccines: precision tools for activating effective immunity against cancer. *Nature Reviews. Cancer*, 8 (2), 108-120.

Richter, S., Ping, Y. & Rana, T.M. (2002) TAR RNA loop: a scaffold for the assembly of a regulatory switch in HIV replication. *Proceedings of the National Academy of Sciences of the United States of America*, 99 (12), 7928-7933.

Rivera, V.M., Clackson, T., Natesan, S., Pollock, R., Amara, J.F., Keenan, T., Magari, S.R., Phillips, T., Courage, N.L., Cerasoli, F., Holt, D.A. & Gilman, M. (1996) A humanized system for pharmacologic control of gene expression. *Nature Medicine*, 2 (9), 1028-1032.

Rizzitelli, A., Vremec, D., Villadangos, J., Mavaddat, N., Wright, M. & Shortman, K. (2005) Switching from a restricted to an effective CD4 T cell response by activating CD8+ murine dendritic cells with a Toll-like receptor 9 ligand. *European Journal of Immunology*, 35 (11), 3209-3220.

Roe, T., Reynolds, T.C., Yu, G. & Brown, P.O. (1993) Integration of murine leukemia virus DNA depends on mitosis. *The EMBO Journal*, 12 (5), 2099-2108.

Ronchese, F. & Hermans, I.F. (2001) Killing of dendritic cells: a life cut short or a purposeful death? *The Journal of Experimental Medicine*, 194 (5), F23-26.

Rouas, R., Uch, R., Cleuter, Y., Jordier, F., Bagnis, C., Mannoni, P., Lewalle, P., Martiat, P. & Van den Broeke, A. (2002) Lentiviral-mediated gene delivery in human monocyte-derived dendritic cells: optimized design and procedures for highly efficient transduction compatible with clinical constraints. *Cancer Gene Therapy*, 9 (9), 715-724.

Rowe, H.M., Lopes, L., Brown, N., Efklidou, S., Smallie, T., Karrar, S., Kaye, P.M. & Collins, M.K. (2009) Expression of vFLIP in a lentiviral vaccine vector activates NFκB, matures dendritic cells, and increases CD8+ T-cell responses. *Journal of Virology*, 83 (4), 1555-1562.

Rowe, H.M., Lopes, L., Ikeda, Y., Bailey, R., Barde, I., Zenke, M., Chain, B.M. & Collins, M.K. (2006) Immunization with a lentiviral vector stimulates both CD4 and CD8 T cell responses to an ovalbumin transgene. *Molecular Therapy*, 13 (2), 310-319.

Rowe, H.M. (2006[a]) Cancer vaccines: Using lentiviral vectors to deliver antigens to dendritic cells *in vivo*. PhD Thesis, University College of London, London.

Ruedl, C., Koebel, P., Bachmann, M., Hess, M. & Karjalainen, K. (2000) Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes. *Journal of Immunology*, 165 (9), 4910-4916.

Ruedl, C., Kopf, M. & Bachmann, M.F. (1999) CD8(+) T cells mediate CD40-independent maturation of dendritic cells in vivo. *The Journal of Experimental Medicine*, 189 (12), 1875-1884.

Russell, N.D., Graham, B.S., Keefer, M.C., McElrath, M.J., Self, S.G., Weinhold, K.J., Montefiori, D.C., Ferrari, G., Horton, H., Tomaras, G.D., Gurunathan, S., Baglyos, L., Frey, S.E., Mulligan, M.J., Harro, C.D., Buchbinder, S.P., Baden, L.R., Blattner, W.A., Koblin, B.A. & Corey, L. (2007) Phase 2 study of an HIV-1 canarypox vaccine (vCP1452) alone and in combination with rgp120: negative results fail to trigger a phase 3 correlates trial. *Journal of Acquired Immune Deficiency Syndromes (1999)*, 44 (2), 203-212.

Sakurai, H., Kawabata, K., Sakurai, F., Nakagawa, S. & Mizuguchi, H. (2008) Innate immune response induced by gene delivery vectors. *International Journal of Pharmaceutics*, 354 (1-2), 9-15.

Sanderson, S., Frauwirth, K. & Shastri, N. (1995) Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 92 (16), 7217-7221.

Schildknecht, A., Miescher, I., Yagita, H. & van den Broek, M. (2007) Priming of CD8+ T cell responses by pathogens typically depends on CD70-mediated interactions with dendritic cells. *European Journal of Immunology*, 37 (3), 716-728.

Schröder, A.R.W., Shinn, P., Chen, H., Berry, C., Ecker, J.R. & Bushman, F. (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell*, 110 (4), 521-529.

Schroers, R., Sinha, I., Segall, H., Schmidt-Wolf, I.G., Rooney, C.M., Brenner, M.K., Sutton, R.E. & Chen, S.Y. (2000) Transduction of human PBMC-derived dendritic cells and macrophages by an HIV-1-based lentiviral vector system. *Molecular Therapy*, 1 (2), 171-179.

Schubert, U., Bour, S., Ferrer-Montiel, A.V., Montal, M., Maldarell, F. & Strebel, K. (1996) The two biological activities of human immunodeficiency virus type 1 Vpu protein involve two separable structural domains. *Journal of Virology*, 70 (2), 809-819.

Schultz, B.R. & Chamberlain, J.S. (2008) Recombinant adeno-associated virus transduction and integration. *Molecular Therapy*, 16 (7), 1189-1199.

Schulz, O., Diebold, S.S., Chen, M., Näslund, T.I., Nolte, M.A., Alexopoulou, L., Azuma, Y., Flavell, R.A., Liljeström, P. & Reis e Sousa, C. (2005) Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature*, 433 (7028), 887-892.

Seliger, B., Marincola, F.M., Ferrone, S. & Abken, H. (2008) The complex role of B7 molecules in tumor immunology. *Trends in Molecular Medicine*, 14 (12), 550-559.

Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., Kuziel, W.A. & Pamer, E.G. (2003) TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity*, 19 (1), 59-70.

Sheehy, A.M., Gaddis, N.C. & Malim, M.H. (2003) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nature Medicine*, 9 (11), 1404-1407.

Shortman, K. & Liu, Y. (2002) Mouse and human dendritic cell subtypes. *Nature Reviews. Immunology*, 2 (3), 151-161.

Shortman, K. & Naik, S.H. (2007) Steady-state and inflammatory dendritic-cell development. *Nature Reviews. Immunology*, 7 (1), 19-30.

Sierra, S., Kupfer, B. & Kaiser, R. (2005) Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology*, 34 (4), 233-244.

Simon, I.D., Publicover, J. & Rose, J.K. (2007) Replication and propagation of attenuated vesicular stomatitis virus vectors *in vivo*: vector spread correlates with induction of immune responses and persistence of genomic RNA. *Journal of Virology*, 81 (4), 2078-2082.

Smith, C.L., Mirza, F., Pasquetto, V., Tscharke, D.C., Palmowski, M.J., Dunbar, P.R., Sette, A., Harris, A.L. & Cerundolo, V. (2005) Immunodominance of poxviral-specific CTL in a human trial of recombinant-modified vaccinia Ankara. *Journal of Immunology*, 175 (12), 8431-8437.

Smith, C.M., Belz, G.T., Wilson, N.S., Villadangos, J.A., Shortman, K., Carbone, F.R. & Heath, W.R. (2003) Cutting edge: conventional CD8α+ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. *Journal of Immunology*, 170 (9), 4437-4440.

Snyder, C.M., Cho, K.S., Bonnett, E.L., van Dommelen, S., Shellam, G.R. & Hill, A.B. (2008) Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity*, 29 (4), 650-659.

Song, X., Evel-Kabler, K., Shen, L., Rollins, L., Huang, X.F. & Chen, S. (2008) A20 is an antigen presentation attenuator, and its inhibition overcomes regulatory T cell-mediated suppression. *Nature Medicine*, 14 (3), 258-265.

Spaner, D.E., Astsaturov, I., Vogel, T., Petrella, T., Elias, I., Burdett-Radoux, S., Verma, S., Iscoe, N., Hamilton, P. & Berinstein, N.L. (2006) Enhanced viral and tumor immunity with intranodal injection of canary pox viruses expressing the melanoma antigen, gp100. *Cancer*, 106 (4), 890-899.

Spies, B., Hochrein, H., Vabulas, M., Huster, K., Busch, D.H., Schmitz, F., Heit, A. & Wagner, H. (2003) Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *Journal of Immunology*, 171 (11), 5908-5912.

von Stebut, E., Belkaid, Y., Nguyen, B.V., Cushing, M., Sacks, D.L. & Udey, M.C. (2000) Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis. *European Journal of Immunology*, 30 (12), 3498-3506.

Steinman, R.M. & Banchereau, J. (2007) Taking dendritic cells into medicine. *Nature*, 449 (7161), 419-426.

Stieger, K., Belbellaa, B., Le Guiner, C., Moullier, P. & Rolling, F. (2009) *In vivo* gene regulation using tetracycline-regulatable systems. *Advanced Drug Delivery Reviews*, 61 (7-8), 527-541.

van Stipdonk, M.J., Lemmens, E.E. & Schoenberger, S.P. (2001) Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nature Immunology*, 2 (5), 423-429.

Stitz, J., Mühlebach, M.D., Blömer, U., Scherr, M., Selbert, M., Wehner, P., Steidl, S., Schmitt, I., König, R., Schweizer, M. & Cichutek, K. (2001) A novel lentivirus vector derived from apathogenic simian immunodeficiency virus. *Virology*, 291 (2), 191-197.

Stock, A.T., Mueller, S.N., van Lint, A.L., Heath, W.R. & Carbone, F.R. (2004) Cutting edge: prolonged antigen presentation after herpes simplex virus-1 skin infection. *Journal of Immunology*, 173 (4), 2241-2244.

Storni, T., Ruedl, C., Renner, W.A. & Bachmann, M.F. (2003) Innate immunity together with duration of antigen persistence regulate effector T cell induction. *Journal of Immunology*, 171 (2), 795-801.

Sutton, R.E., Reitsma, M.J., Uchida, N. & Brown, P.O. (1999) Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *Journal of Virology*, 73 (5), 3649-3660.

Suzuki, Y. & Craigie, R. (2007) The road to chromatin - nuclear entry of retroviruses. *Nature Reviews. Microbiology*, 5 (3), 187-196.

Szécsi, J., Gabriel, G., Edfeldt, G., Michelet, M., Klenk, H.D. & Cosset, F. (2009) DNA vaccination with a single-plasmid construct coding for viruslike particles protects mice against infection with a highly pathogenic avian influenza A virus. *The Journal of Infectious Diseases*, 200 (2), 181-190.

Szulc, J., Wiznerowicz, M., Sauvain, M., Trono, D. & Aebischer, P. (2006) A versatile tool for conditional gene expression and knockdown. *Nature Methods*, 3 (2), 109-116.

Talmor, M., Mirza, A., Turley, S., Mellman, I., Hoffman, L.A. & Steinman, R.M. (1998) Generation or large numbers of immature and mature dendritic cells from rat bone marrow cultures. *European Journal of Immunology*, 28 (3), 811-817.

Tan, J.K.H. & O'Neill, H.C. (2007) Concise review: Dendritic cell development in the context of the spleen microenvironment. *Stem Cells*, 25 (9), 2139-2145.

Tatsis, N., Fitzgerald, J.C., Reyes-Sandoval, A., Harris-McCoy, K.C., Hensley, S.E., Zhou, D., Lin, S., Bian, A., Xiang, Z.Q., Iparraguirre, A., Lopez-Camacho, C., Wherry, E.J. & Ertl, H.C.J. (2007) Adenoviral vectors persist *in vivo* and maintain activated CD8+ T cells: implications for their use as vaccines. *Blood*, 110 (6), 1916-1923.

Thacker, E.E., Nakayama, M., Smith, B.F., Bird, R.C., Muminova, Z., Strong, T., Timares, L., Korokhov, N., O'Neill, A.M., de Gruijl, T.D., Glasgow, J.N., Tani, K. & Curiel, D.T. (2009) A genetically engineered adenovirus vector targeted to CD40 mediates transduction of canine dendritic cells and promotes antigen-specific immune responses in vivo. *Vaccine*, 27(50): 7116-7124.

Thacker, E.E., Timares, L. & Matthews, Q.L. (2009) Strategies to overcome host immunity to adenovirus vectors in vaccine development. *Expert Review of Vaccines*, 8 (6), 761-777.

Turner, D.L., Cauley, L.S., Khanna, K.M. & Lefrançois, L. (2007) Persistent antigen presentation after acute vesicular stomatitis virus infection. *Journal of Virology*, 81 (4), 2039-2046.

Unutmaz, D., KewalRamani, V.N., Marmon, S. & Littman, D.R. (1999) Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *The Journal of Experimental Medicine*, 189 (11), 1735-1746.

Urlinger, S., Baron, U., Thellmann, M., Hasan, M.T., Bujard, H. & Hillen, W. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proceedings of the National Academy of Sciences of the United States of America*, 97 (14), 7963-7968.

Van Kaer, L. (2002) Major histocompatibility complex class I-restricted antigen processing and presentation. *Tissue Antigens*, 60 (1), 1-9.

Van Tendeloo, V.F., Snoeck, H.W., Lardon, F., Vanham, G.L., Nijs, G., Lenjou, M., Hendriks, L., Van Broeckhoven, C., Moulijn, A., Rodrigus, I., Verdonk, P., Van Bockstaele, D.R. & Berneman, Z.N. (1998) Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. *Gene Therapy*, 5 (5), 700-707.

VandenDriessche, T., Thorrez, L., Naldini, L., Follenzi, A., Moons, L., Berneman, Z., Collen, D. & Chuah, M.K.L. (2002) Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood*, 100 (3), 813-822.

Varol, C., Landsman, L., Fogg, D.K., Greenshtein, L., Gildor, B., Margalit, R., Kalchenko, V., Geissmann, F. & Jung, S. (2007) Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *The Journal of Experimental Medicine*, 204 (1), 171-180.

Veron, P., Allo, V., Rivière, C., Bernard, J., Douar, A. & Masurier, C. (2007) Major subsets of human dendritic cells are efficiently transduced by self-complementary adeno-associated virus vectors 1 and 2. *Journal of Virology*, 81 (10), 5385-5394.

Veron, P., Boutin, S., Martin, S., Chaperot, L., Plumas, J., Davoust, J. & Masurier, C. (2009) Highly efficient transduction of human plasmacytoid dendritic cells without phenotypic and functional maturation. *Journal of Translational Medicine*, 7, 10.

Vezys, V., Masopust, D., Kemball, C.C., Barber, D.L., O'Mara, L.A., Larsen, C.P., Pearson, T.C., Ahmed, R. & Lukacher, A.E. (2006) Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *The Journal of Experimental Medicine*, 203 (10), 2263-2269.

Vigna, E., Cavalieri, S., Ailles, L., Geuna, M., Loew, R., Bujard, H. & Naldini, L. (2002) Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. *Molecular Therapy*, 5 (3), 252-261.

Villadangos, J.A. & Schnorrer, P. (2007) Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nature Reviews. Immunology*, 7 (7), 543-555.

Villadangos, J.A. & Young, L. (2008) Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity*, 29 (3), 352-361.

Vogel, R., Amar, L., Thi, A.D., Saillour, P. & Mallet, J. (2004) A single lentivirus vector mediates doxycycline-regulated expression of transgenes in the brain. *Human Gene Therapy*, 15 (2), 157-165.

Vyas, J.M., Van der Veen, A.G. & Ploegh, H.L. (2008) The known unknowns of antigen processing and presentation. *Nature Reviews. Immunology*, 8 (8), 607-618.

Wagner, R., Graf, M., Bieler, K., Wolf, H., Grunwald, T., Foley, P. & Uberla, K. (2000) Rev-independent expression of synthetic gag-pol genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors. *Human Gene Therapy*, 11 (17), 2403-2413.

Wang, B., Norbury, C.C., Greenwood, R., Bennink, J.R., Yewdell, J.W. & Frelinger, J.A. (2001) Multiple paths for activation of naive CD8+ T cells: CD4-independent help. *Journal of Immunology*, 167 (3), 1283-1289.

Wang, J., Zheng, L., Lobito, A., Chan, F.K., Dale, J., Sneller, M., Yao, X., Puck, J.M., Straus, S.E. & Lenardo, M.J. (1999) Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*, 98 (1), 47-58.

Wang, Y., O'Malley, B.W., Tsai, S.Y. & O'Malley, B.W. (1994) A regulatory system for use in gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 91 (17), 8180-8184.

Waskow, C., Liu, K., Darrasse-Jèze, G., Guermonprez, P., Ginhoux, F., Merad, M., Shengelia, T., Yao, K. & Nussenzweig, M. (2008) The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nature Immunology*, 9 (6), 676-683.

Wherry, E.J., Blattman, J.N., Murali-Krishna, K., van der Most, R. & Ahmed, R. (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *Journal of Virology*, 77 (8), 4911-4927.

Wijffels, J.F., de Rover, Z., Beelen, R.H., Kraal, G. & van Rooijen, N. (1994) Macrophage subpopulations in the mouse spleen renewed by local proliferation. *Immunobiology*, 191 (1), 52-64.

Williams, M.A. & Bevan, M.J. (2004) Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells. *Journal of Immunology*, 173 (11), 6694-6702.

Wong, P. & Pamer, E.G. (2001) Cutting edge: antigen-independent CD8 T cell proliferation. *Journal of Immunology*, 166 (10), 5864-5868.

Wong, P. & Pamer, E.G. (2003) Feedback regulation of pathogen-specific T cell priming. *Immunity*, 18 (4), 499-511.

Wu, T.C., Guarnieri, F.G., Staveley-O'Carroll, K.F., Viscidi, R.P., Levitsky, H.I., Hedrick, L., Cho, K.R., August, J.T. & Pardoll, D.M. (1995) Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. *Proceedings of the National Academy of Sciences of the United States of America*, 92 (25), 11671-11675.

Wu, Y. (2004) HIV-1 gene expression: lessons from provirus and non-integrated DNA. *Retrovirology*, 1, 13.

Xuebi, T., Gongming, W., Ying, X., Ping, W., Shasha, C., Hui, Y., Feng, G., Aijun, X., Fei, C., Xiaogao, J., Anne, M. & Yuke, T. (2008) An improved Tet-on system for expression in neurons delivered by a single lentiviral vector. *Human Gene Therapy*, 20(2), 113-123.

Yamazaki, S., Dudziak, D., Heidkamp, G.F., Fiorese, C., Bonito, A.J., Inaba, K., Nussenzweig, M.C. & Steinman, R.M. (2008) CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *Journal of Immunology*, 181 (10), 6923-6933.

Yang, J., Huck, S.P., McHugh, R.S., Hermans, I.F. & Ronchese, F. (2006) Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (1), 147-152.

Yang, L., Yang, H., Rideout, K., Cho, T., Joo, K.I., Ziegler, L., Elliot, A., Walls, A., Yu, D., Baltimore, D. & Wang, P. (2008) Engineered lentivector targeting of dendritic cells for in vivo immunization. *Nature Biotechnology*, 26 (3), 326-334.

Yang, T., Millar, J., Groves, T., Grinshtein, N., Parsons, R., Takenaka, S., Wan, Y. & Bramson, J.L. (2006) The CD8+ T cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with prolonged antigen presentation that nonetheless provides long-term immunity. *Journal of Immunology*, 176 (1), 200-210.

Yang, Y., Huang, C., Huang, X. & Pardoll, D.M. (2004) Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nature Immunology*, 5 (5), 508-515.

Yewdell, J.W., Snyder, H.L., Bacik, I., Antón, L.C., Deng, Y., Behrens, T.W., Bachi, T. & Bennink, J.R. (1998) TAP-independent delivery of antigenic peptides to the endoplasmic reticulum: therapeutic potential and insights into TAP-dependent antigen processing. *Journal of Immunotherapy*, 21 (2), 127-131.

Yu, Y., Pilgrim, P., Yan, J., Zhou, W., Jenkins, M., Gagliano, N., Bumm, K., Cannon, M., Milzani, A., Dalle-Donne, I., Kast, W.M., Cobos, E. & Chiriva-Internati, M. (2008) Protective CD8+ T-cell responses to cytomegalovirus driven by rAAV/GFP/IE1 loading of dendritic cells. *Journal of Translational Medicine*, 6, 56.

Zajac, A.J., Blattman, J.N., Murali-Krishna, K., Sourdive, D.J., Suresh, M., Altman, J.D. & Ahmed, R. (1998) Viral immune evasion due to persistence of activated T cells without effector function. *The Journal of Experimental Medicine*, 188 (12), 2205-2213.

Zanoni, I., Ostuni, R., Capuano, G., Collini, M., Caccia, M., Ronchi, A.E., Rocchetti, M., Mingozzi, F., Foti, M., Chirico, G., Costa, B., Zaza, A., Ricciardi-Castagnoli, P. & Granucci, F. (2009) CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. *Nature*, 460 (7252), 264-268.

Zanta-Boussif, M.A., Charrier, S., Brice-Ouzet, A., Martin, S., Opolon, P., Thrasher, A.J., Hope, T.J. & Galy, A. (2009) Validation of a mutated PRE sequence allowing high and sustained transgene expression while abrogating WHV-X protein synthesis: application to the gene therapy of WAS. *Gene Therapy*, 16 (5), 605-619.

Zarei, S., Abraham, S., Arrighi, J., Haller, O., Calzascia, T., Walker, P.R., Kündig, T.M., Hauser, C. & Piguet, V. (2004) Lentiviral transduction of dendritic cells confers protective antiviral immunity in vivo. *Journal of Virology*, 78 (14), 7843-7845.

Zarei, S., Leuba, F., Arrighi, J., Hauser, C. & Piguet, V. (2002) Transduction of dendritic cells by antigen-encoding lentiviral vectors permits antigen processing and MHC class I-dependent presentation. *The Journal of Allergy and Clinical Immunology*, 109 (6), 988-994.

Zennou, V., Serguera, C., Sarkis, C., Colin, P., Perret, E., Mallet, J. & Charneau, P. (2001) The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nature Biotechnology*, 19 (5), 446-450.

Zhang, M., Tang, H., Guo, Z., An, H., Zhu, X., Song, W., Guo, J., Huang, X., Chen, T., Wang, J. & Cao, X. (2004) Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. *Nature Immunology*, 5 (11), 1124-1133.

Zhao, X., Deak, E., Soderberg, K., Linehan, M., Spezzano, D., Zhu, J., Knipe, D.M. & Iwasaki, A. (2003) Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *The Journal of Experimental Medicine*, 197 (2), 153-162.

Zhu, Z., Ma, B., Homer, R.J., Zheng, T. & Elias, J.A. (2001) Use of the tetracycline-controlled transcriptional silencer (tTS) to eliminate transgene leak in inducible overexpression transgenic mice. *The Journal of Biological Chemistry*, 276 (27), 25222-25229.

Zinkernagel, R.M. & Hengartner, H. (2004) On immunity against infections and vaccines: credo 2004. *Scandinavian Journal of Immunology*, 60 (1-2), 9-13.

Zufferey, R., Donello, J.E., Trono, D. & Hope, T.J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *Journal of Virology*, 73 (4), 2886-2892.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L. & Trono, D. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *Journal of Virology*, 72 (12), 9873-9880.

Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L. & Trono, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotechnology*, 15 (9), 871-875.