Lentivector based gene transfer for immunotherapy – application of integration deficient vectors and PDL1 knockdown as tools to manipulate immune responses

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DECLARATION

I, Katarzyna Karwacz, confirm that 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 this thesis

ABSTRACT

Lentiviral-based vectors are effective and promising tools for the generation of cell mediated immunity. Multiple studies have demonstrated that subcutaneous injection of lentivectors encoding tumour antigens results in induction of strong CTL responses and often in tumour killing. However, integration of lentivectors into human genomic DNA poses a risk of insertional mutagenesis. Indeed, this possibility has been highlighted by gene therapy trials that resulted in the development of T cell leukaemia in several patients. For this reason, non-integrating lentiviral vectors (NILVs) have been developed as a safer alternative for gene delivery.

The first part of this thesis demonstrates that lentivectors carrying multiple mutations preventing integration are effective vaccines. Subcutaneous injection of these vectors resulted in induction of systemic dose-dependant CD8+ T-cell responses to the encoded antigen. The duration of the persistence of antigen presentation was measured using transfer of OT1 transgenic T cells into previously immunized mice. Measuring expansion of those cells revealed that the antigen was present and presented for at least 30 days. CD8+ T-cell responses were further enhanced by addition of dendritic cell (DC) stimulators: p38 MAP kinase and NF-κB stimulators. These activators led to a more rapid response peaking at day 7. Finally, NILVs expressing the antigen and DC activators were tested in a tumour therapy model and were found to be effective.

The second part of this thesis focused on altering DC-T cell interactions to enhance responses to immunization by lentivector-mediated knockdown of PDL1 on DCs. The analysis of DCs infected with anti-PDL1 shRNA showed that knocking down this molecule drives DCs towards a mature phenotype. The influence of PDL1 knockdown was assessed on co-cultured T cells. The absence of PDL1 enhanced their proliferation and reduced antigenic stimulation induced TCR complex degradation. DCs transduced with lentivectors expressing PDL1 shRNA were also tested in vaccination and tumour therapy.

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ABBREVIATIONS

AAV	Adeno-associated virus
APC	Antigen presenting cell
BCG	Bacille Calmette-Guerin
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BMDC	Bone-marrow derived dendritic cell
CA	Capsid
CCR7	Chemokine receptor type 7
CD40L	CD40 ligand
CD62L	CD62 ligand
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's djuvant
CFSE	Carboxyfluorescein succinimidyl ester
cPPT	Central polypurine tract
CSK	C-src tyrosine kinase
CTL	Cytotoxic T lymphocyte
CypA	cyclophilin A
DC	Dendritic cell
DC-SIGN	Dendritic cell- specific intercellular adhesion molecule 3-grabbing non- integrin
DC-SIGNR	DC-SIGN related
DMEM	Dulbecco´s Modified Eagle Medium
dsDNA	Double stranded Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISpot	Enzyme-linked immunosorbent spot assay
ERK	Extracellular-signal-regulated kinase
FACS	Fluorescence activated cell storting
FasL	Fas ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's Buffered Salt Solution
hCMV	Human cytomegalovirus
HIV-1	Human immunodeficiency virus 1
HS	Heparan sulfate
HSV	Herpes simplex virus
ICAM-I	Inter-Cellular Adhesion Molecule 1
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFA	Incomplete Freund's Adjuvant
IFN –γ	Interferon gamma
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
IN	Integrase

ITIM	Immunoreceptor tyrosine-based inhibition motif
iTregs	Inducible Tregs
ITRs	Inverted terminal repeats
ITSM	Immunoreceptor tyrosine based switch motif
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
KLH	Keyhole limpet hemocyanin
KSHV	Kaposi's sarcoma- associated herpesvirus
LB	Luria-Bertani
LCK	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic Choriomeningitis Virus
LTR	Long terminal repeat
MA	Matrix
MAPK	Mitogen activated protein kinase
MDCS	Monocyte-derived myeloid dendritic cells
MFI	Mean fluorescence intensity
mGM-CSF	Mouse growth monocyte – colony stimulating factor
MHC	Major histocompability complex
MKK6	Mitogen-activated protein kinase kinase 6
MLV	Moloney murine leukemia virus
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MVA	Modified vaccinia virus Ankara
NC	Nucleocapsid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NILV	Non-integrating lentiviral vector
ORF	Open Reading Frame
OVA	Ovalbumin (chicken)
PBS	Phosphate-buffered saline
PCR	Polymerase chain re action
PD1	Programmed Death 1
PDL1	Programmed Death Ligand-1
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIC	preintegration complex
PKC-γ	Protein kinase C gamma type
pre-DCs	Precursor dendritic cells
RCRs	Replication-competent recombinants
RRE	Rev response element
RT	Reverse transcriptase
SA	Splice akceptor
scAAV	Self-complementary AAV
scFv	Single-chain antibodies
SCID-X1	X linked severe combined immunodeficiency
SD	Splice donor
SFFV	Spleen focus-forming virus promoter

SHP-1	Src homology region 2 domain-containing phosphatase-1
SHP-2	Src homology region 2 domain-containing phosphatase-2
shRNA	Short hairpin RNA
SIN	Self-inactivating vectors
SINs	Self-inactivating lentivirus vectors
SIV	Simian immunodeficiency virus
TAAs	Tumour associated antigens
T-ALL	T cell acute lymphoblastic leukaemia
TAP1	Antigen peptide transporter 1
TCR	T cell receptor
TGF-β	Transforming growth factor β
TLR	Toll like receptor
TNF-α	Tumor necrosis factors α
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF -related activation-induced cytokinement
TVS	Tubovesicular structures
vFLIP	Viral FADD-like interleukin-1-β-converting enzyme/ caspase-8-inhibitory protein
VSV-G	Vesicular stomatitis virus G protein
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
ZAP70	Zeta-chain-associated protein kinase 70

CHAPTER 1. INTRODUCTION

Cancer vaccines based on targeting of the adaptive immune system responses are an attractive treatment because of their specificity and potential avoidance of collateral tissue damage. They are based on induction of specific effector T cells against tumour associated antigens (TAAs), often at the same time aiming at overcoming peripheral tolerance and tumour immunosurveillance.

The design of an effective vaccine is challenging. Mobilization of dendritic cells (DCs) to present antigens to T lymphocytes is crucial for effective immunization (Collins and Cerundolo 2004). For this reason, the ideal vaccine should be able to efficiently deliver antigen to antigen presenting cells (APCs) in the right activation context. More specifically, in the case of cancer vaccines, dendritic cells (DCs) should be targeted to elicit CTL responses. In recent years viral vectors have been used to express antigens in activated DCs. Upon gene delivery into APCs, the antigen is processed, presented on major histocompatibility complex (MHC) molecules and recognized by T cells (Steinman and Banchereau 2007).

This chapter describes both the relationship between cancer and the immune system as well as some viral and non-viral methods used to deliver antigen to dendritic cells (DCs) to initiate immune responses.

1.1. THE IMMUNE SYSTEM AND CANCER

To treat cancer successfully one needs to understand the relationship between tumours and the immune system. There is evidence pointing at both similarities and differences between responses to tumoral antigens and self antigens. This should not be surprising as tumours are derived from normal tissues. However, some of these differences in antigen composition and biologic behaviour significantly alter interactions of tumoral cells and the cells of the immune system (table 1.1). Genetic and epigenetic instability result in a constantly new repertoire of antigens and

expressed genes. Transformed malignant cells invade across natural tissue barriers disrupting tissue architecture and causing the elaboration of proinflammatory signals. Thus, cancers are constantly under siege by the inflammatory responses. There is evidence that the interplay of tumours and host immune system involves both immune surveillance and immune tolerance of cancers.

Table 1.1. Differences between normal and cancer cells

	Normal cell	**Cancer cell**	**Effect on cancer immunity**
Genome	Stable	Altered	Multiple neoantigens arising
Transcriptome	Stable	Instable	Altered levels of antigen density
Tissue invasion	None	Yes + metastasis	Induction of inflammation
Cytokine/growth factor expression	Stable	Abnormal	Local inhibitory effects on immunity

Adapted from (Pardoll 2003)

1.1. CANCER IMMUNOSURVEILLANCE

The prediction that the immune system has the ability to repress growth of carcinomas was stated for the first time already in 1909 by Paul Ehrlich and revisited fifty years later by F. Macfarlane Burnet and Lewis Thomas when it was formally introduced as cancer immunosurveillance (Dunn, Old et al. 2004).

Thomas and Burnet predicted that continuously arising transformed cells were eliminated by lymphocytes. At that time the idea was put to test using nude mice and was soon disproved after studies by Stutman and Rygaard and Povlsen showed no difference in cancer incidence between nude and wild-type mice (Rygaard and Povlsen 1974; Rygaard and Povlsen 1974). However, there were a few flaws in this study: nude mice, even though with no thymus, have a population of functional T cells and other cells of the immune system such as NK cells. It was the new studies in 1990 that ultimately validated the cancer immunosurveillance concept. However, there has been growing evidence that immunosurveillance represents only one dimension of the

complex relationship between the immune system and cancer. For this reason, a new hypothesis termed immunoediting has been developed (Dunn, Old et al. 2004).

1.2. CANCER IMMUNOEDITING

Cancer immunoediting is a dynamic process consisting of three phases: elimination, equilibrium, and escape (Fig. 1.1.). Elimination is equivalent to the classical concept of cancer immunosurveillance. Equilibrium is a result of incomplete tumor destruction and is characterized by immune-mediated latency. Escape is the final outgrowth of tumors that have escaped the equilibrium phase (Dunn, Old et al. 2004).

1.2.1. ELIMINATION

The elimination phase represents the original concept of cancer immunosurveillance. Immunoediting will only progress to the subsequent phases if this phase does not lead to a complete eradication of a developing tumour. Elimination can be divided into 4 stages: initiation of the antitumor immune response, amplification of innate responses, development of adaptive immune responses, and tumour killing.

In the first phase, two of the six “hallmarks of cancer”, angiogenesis and tissue-invasive growth, cause stromal remodelling and local tissue disruption which result in initiation of anti-tumour responses (Hanahan and Weinberg 2000). Stromal remodelling causes secretion of proinflammatory molecules which, in combination with chemokines produced by cancer cells, sends a local “danger” signal and attracts NKT cells, γδ T cells, NK cells and macrophages to the cancer cells (Matzinger 1994; Wrenshall, Stevens et al. 1999). These attracted cells recognize molecules induced on cancer cells, such as the ligands for NKG2D. Those events lead to production of IFN-γ.

In the second step, because of the initial amount of IFN-γ released at the site of the tumour, locally produced chemokines recruit more cells of the innate system thus amplifying the innate response. A positive feedback system is created between

tumour-infiltrating macrophages producing IL-12 and tumour-infiltrating NK cells producing IFN-γ. NK cells activate macrophages to produce more IL-12, which in turn leads to increased IFN-γ production by NK cells. As a result a number of antiproliferative, proapoptotic and angiostatic IFN-γ-dependent processes are initiated resulting in killing of the tumour (Kumar, Commane et al. 1997). In addition, macrophages can kill tumours via a TRAIL-mediated mechanism while NK cells kill via perforin-dependent mechanisms (Smyth, Cretney et al. 2001; Takeda, Hayakawa et al. 2001; Hayakawa, Kelly et al. 2002).

In the third step, as a result of tumour killing, dead tumour cells become a source of antigens which results in development of adaptive immune responses. Dendritic cells recruited to the site acquire tumour antigens by direct ingestion of cell debris. Next, activated DCs migrate to the tumour draining lymph node and activate tumour specific Th1 responses. Those, via cross-presentation of antigenic tumour peptides on MHC class I molecules, facilitate the development of tumour-specific $CD8^+$ CTLs (Huang, Golumbek et al. 1994; Yu, Spiotto et al. 2003)

Cross-presentation allows the acquisition of antigens from infected and abnormal cells by specific APCs and display of those exogenous antigens in the context of MHC class I molecules.

Both dendritic cells and macrophages are thought to cross-present antigens from apoptotic cells, but only the former have the ability to induce responses from naïve CTLs in this process probably as only DCs can supply co-stimulatory signals (Heath and Carbone 2001).

Cross-presentation of both class I– or class II–restricted antigens is possible. The result of cross-presentation can be T cell cross-priming or T cell cross-tolerance. One of the aspects influencing the outcome of cross-presentation is the level of antigen expression. The level of antigen expressed by a cell must be above a certain threshold for cross-presentation to occur (Kurts, Miller et al. 1998). Antigen expression needs to be higher for cross-presentation than for direct presentation.

Three main areas in which cross-presentation plays an important role are DNA vaccination, tumor immunity and peripheral tolerance. There is a strong evidence that tumor antigens can induce responses by cross-priming but only if the level of antigen expression is sufficiently high (Huang, Golumbek et al. 1994).

Apart from APCs and CTL precursors, the cell type appearing to be essential for cross-priming is a CD4+ T helper cell. It is not exactly known if cross-priming always requires CD4+ T cell help. However, so far all examples of cross-priming analyzed for this requirement seem to be CD4 T cell dependent (Bennett, Carbone et al. 1997).

In the last step, both tumour-specific $CD4^+$ and $CD8^+$ T cells migrate to the site of the tumour and mediate killing of antigen-positive tumour cells through different mechanisms.

There are two pathways of CTL killing - one mediated by perforin and granzymes, and the second one mediated by death receptor.

Once CTL recognizes its target it synthesizes granules containing mainly perforin and granzymes (Cullen and Martin 2008). This triggers polarization of the microtubule-organizing center (MTOC) toward the immunological synapse (Geiger, Rosen et al. 1982). Granules use the microtubules to move towards the plasma membrane (Kupfer, Dennert et al. 1983). After they polarize at the cell membrane, they are secreted into the immunological synapse and cause rapid cell death within as little as 20 minutes (Cullen and Martin 2008).

It is though that perforin forms pores in the target cell membrane thus allowing the granzymes to pass directly into the cytosol. Alternatively, granzymes may be taken up into target cell endosomes where perforin is required for their release into the cytosol.

Granzymes belong to the family of serine proteases. Granzymes A, B, C, D, E, F, G, K, L, M and N are found in the mouse, while only A, B, H, K and M in the humans. The most abundant ones are granzymes A and B (Cullen and Martin 2008).

Fas ligand (FasL/CD95-L) is a cell surface molecule which is a member of the

tumor necrosis factor family (that also include TNF-a among others) . FasL binds to its receptor Fas and in this way induces apoptosis of Fas-bearing cells (Nagata and Golstein 1995).

Fas is expressed by various cells and in addition becomes upregulated in rapidly proliferating cells such as lymphocytes and tumor cells, while Fas ligand is expressed mostly on activated T cells after antigen recognition through either *de novo* synthesis or transformation from an inactive to active form (Groscurth and Filgueira 1998).

Fas and FasL have two main roles in the immune system: they are involved in down-regulation of immune reactions (as *malfunction of the Fas system causes autoimmune diseases or tissue destruction*) and they mediate T cell cytotoxicity (Nagata and Golstein 1995).

In vivo the system is triggered by cross-linking of Fas with cells expressing FasL, which leads to apoptotic cell death (Trauth, Klas et al. 1989). Target cell death signal is generated by a cytoplasmic 65-amino acid domain of Fas called “death domain”, which is highly homologous to the death domain of the TNF-a receptor. The death domain is thought to have no catalytic function, thus it is likely that ligand binding induces recrutation of cytoplasmic proteins which are used as downstream messengers for death, several of which have been identified. The death induced by Fas-FasL interaction is morphologically indistinguishable from granzyme-mediated cell death (Groscurth and Filgueira 1998).

Overall, Fas-mediated cytotoxicity by CTLs consists of two processes: induction of FasL expression in the cytotoxic cell upon recognition of the target cell followed by engagement of FasL on CTL and Fas on the target cell triggering suicide program in the target cell.

During generation of an anti-tumour immune response, CD4 T cells play a pivotal role in the activation and expansion of CD8 T cells, generation of CD8 T cell memory cells and are required for reactivation of memory CTL (Gerloni and Zanetti 2005; Knutson and Disis 2005).

T cell help is provided by either direct or indirect activation of tumour antigen-specific CTL (Gerloni and Zanetti 2005). The former involves direct interaction between Th cells and CTL. It has indeed been demonstrated that antigen-primed Th cells can directly activate tumor antigen-specific CTL as infusion of Th cells into tumor-bearing mice is able to activate a CTL-mediated anti-tumor response. Both Th1 and Th2 cells were able to initiate a CTL-based immune response, which implies that there are multiple mechanisms boosting CTL immunity. One of those shows that Th cells enhance the function of tumor-specific CTL through co-stimulatory molecules present on the surface of the CTL, such as CD27, CD134, and MHC class II (Knutson and Disis 2005).

There are several mechanisms of indirect CTL activation. For example, activated CD4 T cells could induce delayed type hypersensitivity (DTH)-like reactions and in this way attract inflammatory cells like macrophages, granulocytes, eosinophils, or natural killer (NK) cells to the tumour site (Gerloni and Zanetti 2005)

IFN-γ secreted by Th1 cells plays an important role in tumor rejection. It activates APCs to upregulate molecules involved in increased antigen presentation, such as LMP2, LMP7, MECL, PA28, and MHC class I which leads to increased tumor cell recognition and elimination (Fruh and Yang 1999) . However, it could also have more direct effects such as cytotoxic activity on tumor cells, alteration of the endogenous antigen-processing machinery, and inhibition of angiogenesis by tumor cells (Gerloni and Zanetti 2005).

Also secretion of IL2 by T helper cells plays an important role in tumour rejection as IL-2 can directly stimulate growth of CTLs (Cheever and Chen 1997).

It is also possible, however, that tumour antigen specific - CD4 T cells exert their effector function via direct killing of tumor targets. There is scarce evidence supporting this thesis coming from experimental models of tumours where mice were able to reject the tumour in the absence of CD8 T cells. Even though MHC class II-restricted killing has been rarely documented in the mouse, it is commonly observed in humans (Gerloni and Zanetti 2005).

1.2.2. EQUILIBRIUM

This is a phase of equilibrium between the host immune system and any tumour cells that have survived the elimination phase. Lymphocytes still secrete IFN-γ but at a level that is not able to fully extinguish the tumour. New mutations in the tumour are created. Those give tumour cells extra resistance to the attack mediated by the immune system (Dunn, Old et al. 2004). Equilibrium is most likely the longest of the three phases of cancer immunoediting. It may occur over a period of many years in humans.

1.2.3. ESCAPE

In this phase, genetic and epigenetic changes, accumulated in cancer cells during the equilibrium phase, confer resistance to immune elimination allowing the tumours to expand. Multiple immunoevasive strategies are employed by the tumours to escape detection and elimination by both the innate and adaptive immune responses.

One of the mechanisms used by tumours to escape recognition and destruction by the immune system is loss of tumors antigens, as the amount of tumors antigen may play a role in recognition. Indeed, there are some examples confirming it in the clinics: decrease in expression of melanoma differentiation antigens such as gp100, MART1 and tyrosinase promotes disease progression (Khong and Restifo 2002). Epitope immunodominance could explain propagation of such antigen loss variants (Schreiber, Wu et al. 2002). This theory states that parental tumours cells possess the immunodominant epitope, which diverts the attack of the immune system from other tumour variants. As the parental cells get eliminated, the initially immunorecessive epitopes become dominant (Schreiber, Wu et al. 2002).

Another common strategy used by tumors to escape detection by T cells is downregulation of antigen presentation through decreased or lost MHC class I

expression and defects in the antigen processing machinery, such as mutations in TAP and LMP2 and LMP7 (Rabinovich, Gabrilovich et al. 2007).

Another mechanism contributing to tumour escape, demonstrated in both mice and patients with advanced tumours, is impaired TCR signaling which inhibits CTL lytic functions and leads to inactivation of the effector phase of antitumour responses. Tumour infiltrating lymphocytes (TILs) are often characterized by decreased expression of several molecules critical for signaling events promoting T cell activation, including the CD3ζ chain and the p56lck and p59fyn tyrosine kinases (Khong and Restifo 2002).

Tumour cells as well as stromal cells synthesize immunosuppressive factors that contribute to tumour escape from the immune system. TGF-β and IL-10 are two of such cytokines. TGF-β is responsible for inhibition of T cell activation, proliferation, and differentiation and has been linked to cancer progression as there is a correlation between high serum levels of TGF-β and poor prognosis in some cancers (Montini, Cesana et al. 2006). Also, interestingly it has been shown that highly immunogenic tumors transfected with TGF-β1 cDNA escape destruction by the immune system (Torre-Amione, Beauchamp et al. 1990). Specific action of TGF-β on the function of CTLs has been demonstrated: it represses production of perforin, granzyme A, granzyme B, Fas ligand (FasL), and IFN-γ. These effects are restored by antibody-mediated neutralization of TGF-β (Thomas and Massague 2005). Just like with TGF-β, elevated levels of IL-10 are often observed in the serum of cancer patients. IL-10 can exert inhibitory effects on DCs, it inhibits their differentiation from stem cell precursors, blocks DC maturation and functionality (IL-10 impairs DC functionality) as well as enhances spontaneous apoptosis of DCs (Ludewig, Graf et al. 1995; Girolomoni and Ricciardi-Castagnoli 1997). The *in vivo* effects of IL-10 have been investigated and include inhibition of antigen presentation, IL-12 production and blockade of Th1 responses (De Smedt, Van Mechelen et al. 1997). Finally, it has been demonstrated that IL-10 may down-regulate expression of HLA classes I,II and ICAM-1 molecules in cancer cells (Yue, Dummer et al. 1997).

1.3. DENDRITIC CELLS

Dendritic cells were first described almost thirty years ago by Ralph Steinman. They are widely distributed cells of haematopoietic origin. Their primary function is to capture, process and present antigens to T cells. Only DCs have the ability to induce a primary immune response in resting naïve T cells . For these reasons they are referred to as professional APCs.

The interactions of DCs with naïve T cells influence the type of T cell-mediated response (Fig. 1.2.A). They may also induce tolerance depending on the type of DC and its activation state (Shortman and Liu 2002).

1.3.1. TYPES OF DENDRITIC CELLS

DCs in steady state are often divided into conventional DCs (cDCs) and precursors of DCs (pre-DCs) (Fig. 1.2.B). Conventional DCs have a dendritic form and functions, while pre-DCs require further development (Naik 2008).

1.3.2. CONVENTIONAL DCS

Conventional DCs are divided into migratory DCs and lymphoid-tissue-resident DCs:

- **Migratory DCs** - sample antigens in peripheral tissues, then migrate through the lymph to lymph nodes in response to danger signals. At the time when they reach a lymph node, they already have a mature phenotype and stop antigen uptake. Migratory DCs can be further divided according to the tissue of origin. Both the Langerhans cells (LCs) and interstitial DCs belong to this group.
- **Lymphoid-tissue-resident DCs** - collect and present antigens in the lymphoid organ itself. This group consists of most of the DCs in the thymus and spleen as well as around half the DCs in the lymph nodes. Those DCs have an immature phenotype and actively uptake and process the antigen. In mice lymphoid-tissue-resident cDCs are further separated into $CD8^+$ cDCs expressing CD8α on the cell surface, and $CD8^-$ cells. These two groups produce different cytokines and differ in their presentation of antigens on MHC class I molecules.

Figure 1.1.

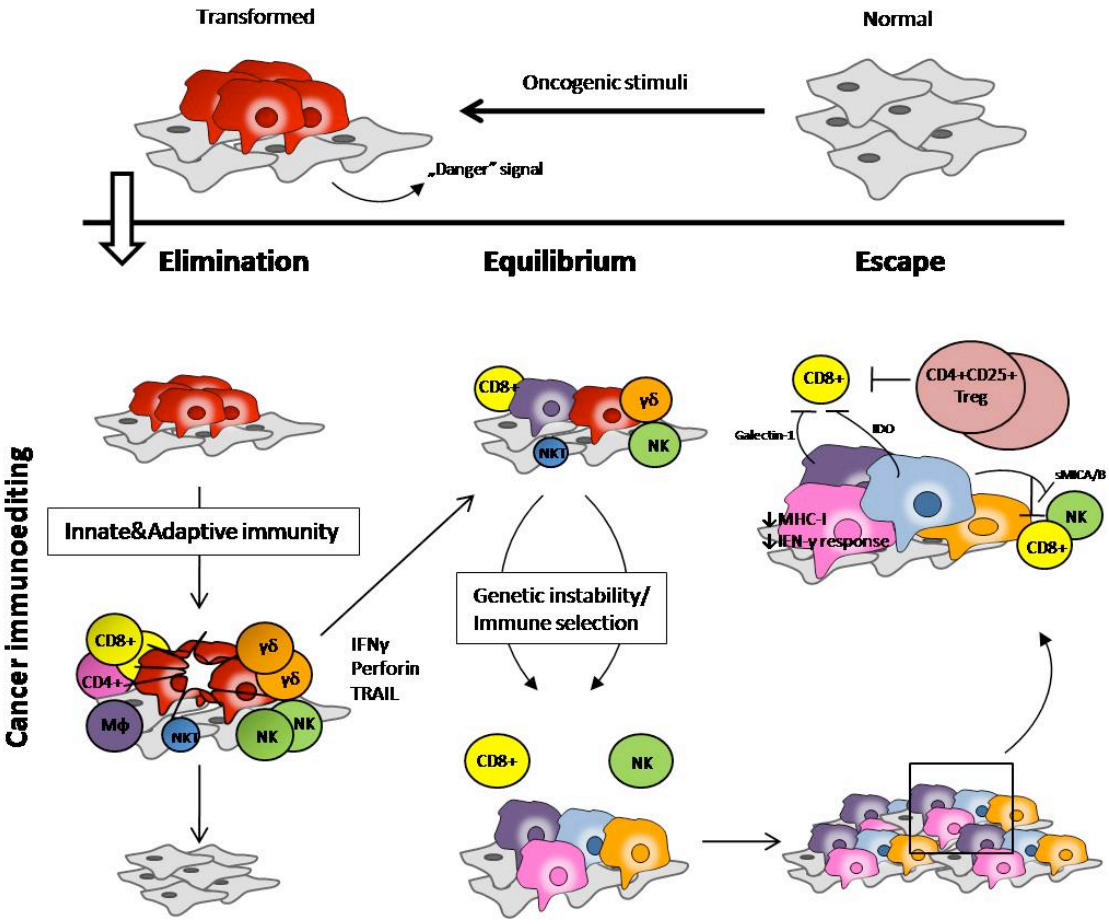

Figure 1.1. Phases of cancer immunoediting process

Normal cells, under the influence of an oncogenic stimuli, become tumour cells. These cells express distinct tumor-specific markers and generate a "danger" signal that initiates the immunoediting process.

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1.3.3. PRE-DCS

Pre-DCs don't have a full DC function. Those DC precursors differentiate into resident DCs with little or no cell division.

Plasmacytoid DCs – relatively long-lived circulating cells which produce large quantities of type I interferons when stimulated by viral or other microbial infections. Type I interferons also initiate pDCs conversion into a dendritic form and makes them acquire some DC antigen-processing and antigen-presentation properties.

Inflammatory DCs - Inflammatory DCs appear as a consequence of infection or inflammation. One example is the DCs produced *in vivo* when pDCs are stimulated by the influenza virus.

Figure 1.2.

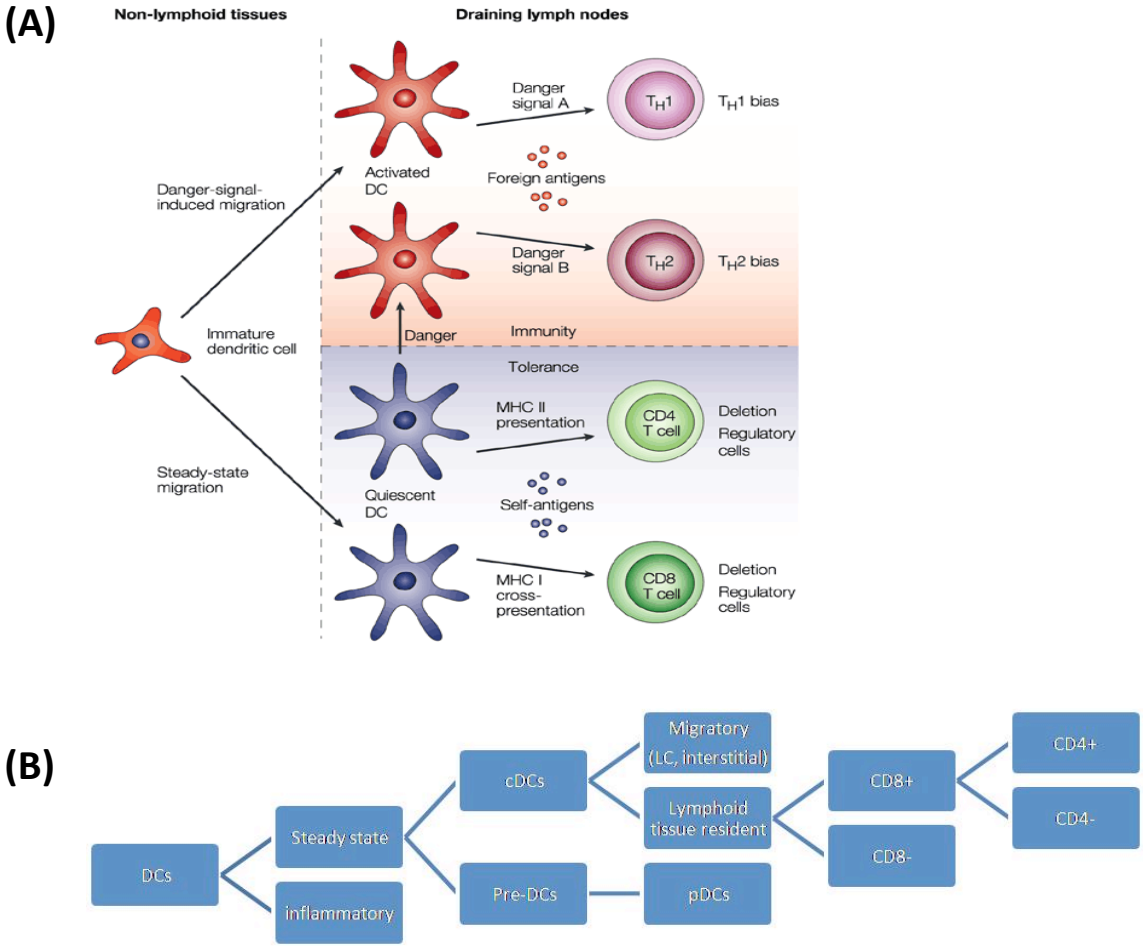

Figure 1.2. Dendritic cells and the immune system *(A)The influence of DC activation status on the type of generated response. When no "danger" signals are present, there is a constant steady-state flow of dendritic cells into lymphoid tissues. Those quiescent DCs contribute to the maintenance of peripheral tolerance to self-antigens. Dendritic cells become activated as a result of infection, inflammation or tissue damage. This increases the rate of DC migration into lymphoid tissues and leads to initiation of immune responses. (B) Mouse DC classification. Several phenotypically and functionally different DC subpopulations constitute the mouse DC network. They are classified based on different markers, location and presence in the steady state or inflammatory conditions. cDCs-conventional DCs, pDCs-plasmacytoid DCs, preDCs-precursor of cDCs, iDCs-inflammatory DCs.*

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1.4. CANCER IMMUNOTHERAPY

Several efforts have been put into development of effective cancer immunotherapies. The methods can be divided into a few categories: non-specific immunotherapies and adjuvants, active immunotherapies which stimulate the body's own immune system, e.g. cancer vaccines, and passive immunotherapies - usually involving transfer of manufactured antibodies. Forms of the latter – monoclonal antibodies attaching to specific cancer antigens – are currently the most commonly used form of cancer immunotherapy and are being tested in clinical trials for almost every type of cancer owing to the fact that a vast number of tumour associated antigens have been identified. Monoclonal antibodies can act in several different ways. For example naked MAbs attach themselves to specific antigens and act as a marker for destruction by the host's immune system. Others – named activation blockers – attach to antigens that are functional in the cancer cell or other cells that help the cancer to grow and block their activity. Finally, conjugated MAbs can act as homing devices and carry drugs, toxin or radioactive substances directly to the cancer.

Many compounds have been used as adjuvants to boost the activity of the immune system. Bacille Calmette-Guerin (BCG) was one of the earliest forms of immunotherapy and is still used for treating early stages of bladder cancer (Bevers, Kurth et al. 2004). Other examples include Keyhole limpet hemocyanin (KLH) and incomplete Freund's adjuvant (IFA). Cytokines have also been used to boost patient's immune systems. IL2 has been successfully used to treat advanced kidney cancer and metastatic melanoma (McDermott, Regan et al. 2005). However, when administered in high doses for several weeks it becomes toxic and costly (McDermott, Regan et al. 2005). Also treatment with IFN-α has improved survival of cancer patients (Pfeffer, Dinarello et al. 1998). Other promising cytokines include IL-7, IL-12, IL-21 and GM-CSF (Richtig, Hofmann-Wellenhof et al. 2004).

Active forms of immunotherapy include tumour cell-, antigen- and dendritic cell -vaccines.

1.4.1. ADOPTIVE T CELL TRANSFER

T cell transfer therapy involves administration of a numbers of selected cells characterized by high avidity for specific tumor antigens. Before the transfer, cells are activated *ex vivo* thus they exhibit strong anti-tumour effector functions. Infusion of those cells is facilitated by conditioning of the patient which involves treatment with lympho-depleting chemotherapy or irradiation (Schumacher and Restifo 2009). Two sources of T cells can be used: naturally-occurring T cells (which can only reliably be grown from patients with melanoma) and genetically engineered T cells. In most patients with cancer, tumour-reactive T cells are not usually present, and therefore they have to be engineered (Schumacher 2002).

There are several advantages of using T cells over other cytolytic cells for cell transfer. First of all, they are able to recognize tumour proteins expressed on the surface of cancer cells and in this way specifically target the tumour cells. Furthermore, T cells are characterized by long clonal life span which results not only in therapeutic treatment but also in immunoprophylaxis . Last, but not least, T cells can be genetically manipulated and thus can be genetically enhanced (June 2007).

It has been demonstrated that CD8+ T cell memory cells require the presence of CD4+ T cell help during the process of their generation and maintenance (Bevan 2004). CD4+ T cells use several mechanisms to enhance survival and function of CD8+ T cells upon adoptive transfer. Those mechanisms include secretion of cytokines like IL-2 and IL-21 and expression of CD40L (June 2007). Studies show that administration of IL-2 or CD4+ T cells at the same time as transfer of CD8+ T cells, enhances persistence of the latter (Yee, Thompson et al. 2002). Additionally, administration of a population containing both specific CD8+ and CD4+ T cells results in establishment of central memory component (Rapoport, Stadtmauer et al. 2005). However, CD4+ T cells can also recruit and activate macrophages and eosinophils to enhance antiumour effects (June 2007).

Several strategies improving the function of adoptively transferred T cells are used. These include administration of cytokines (IL-2, IL-7, IL-12, IL-15, IL-21),

chemotherapy , administration of antibodies blocking CTLA4 and PD1/PDL1 pathways, using cytokine antagonists (for example blocking TGF-β), host Treg depletion or inhibition among the others (June 2007).

So far, clinical trials using adoptive T cell transfer have been performed for gastric cancer, hepatocellular carcinoma, renal cancer and melanoma and lung cancer (June 2007).

1.4.2. WHOLE TUMOUR CELL AND ANTIGEN VACCINES

Tumour cell vaccines are made up of cancer removed during surgery which is usually treated with radiation and injected back into the patient. Often, they are injected along with adjuvants to boost the response. Recently, tumour cells fused to dendritic cells have been used (Koido, Hara et al. 2005; Weigel, Panoskaltsis-Mortari et al. 2006). This approach is meant to further stimulate the response. The main advantage of using whole tumour cell vaccines is the exposure of the immune system to a whole range of antigens.

Antigen vaccines, in contrast to the whole tumour cell vaccines, boost the immune system by using only one or a few antigens. This strategy often gives a stronger response against the antigens and additionally allows for modification of the antigen.

1.4.3. DENDRITIC CELLS IN CANCER IMMUNOTHERAPY

Dendritic cells are the most important cell type involved in initiation and regulation of immune responses. For this reason, a lot of efforts have been put into fully exploiting DCs for cancer immunotherapy. It is the dendritic cells that uptake, process and present antigens to cognate T cells. In cancer settings, the important mode of antigen presentation is cross-presentation resulting from uptake and processing of apoptotic or necrotic cancer matter. The outcome of cross-presentation is highly dependent on the activation status of the involved DC. Despite the fact that many cancer cells secrete endogenous danger signals, such as heat shock proteins

(HSPs), the extent of tumour-associated DCs maturation is usually low. Cross-presentation of tumour associated antigens (TAAs) tends to be effective if the tumour associated protein is stable and highly expressed or in the case of a high spontaneous rate of apoptosis and necrosis of tumour cells. If this is not the case, therapies promoting tumour cell death can be used. Those include chemotherapy, hyperthermia, and irradiation.

1.5. VACCINES & DENDRITIC CELL MODIFICATION METHODS

Several methods of loading DC with antigens have been studied. These approaches can be divided into viral and non-viral methods. In recent years, gene delivery methods have not only been used to deliver antigens, but also to simultaneously deliver signals modifying DC, through inclusion of activation-inducing molecules for cancer immunotherapy. Below is a summary of methods used to genetically modify DCs.

1.5.1. DNA VACCINES

DNA vaccines are a conceptually safe approach shown to induce both humoral and cellular immunity (Kutzler and Weiner 2008). The first experiments to deliver DNA into the skin of mice using a gene gun were conducted already in the early 1990s.

The target cells for DNA plasmids are cells like myocytes, keratinocytes and some resident APCs. Once the plasmid enters the nucleus of those cells, gene transcription is initiated. This generates foreign antigens which are shed from live or dying cells in the form of proteins or peptide strings and can become the subject of immune surveillance by MHC class I and class II molecules on APCs. APCs loaded with antigens travel to the draining lymph nodes, present foreign antigens and initiate immune responses. Both humoral and cellular immune responses are induced.

DNA vaccination offers several advantages over other gene delivery methods, starting with the ease of design and production. Also, the safety profile looks very promising. So far, there have been no adverse effects in any of the clinical trials using DNA plasmids.

However, potential concerns regarding DNA vaccination exists. Optimized expression plasmids pose a risk of integration into cellular DNA and causing insertional mutagenesis and chromosomal instability. There is a possibility that antibiotic resistance from a plasmid is transferred to patients. Finally, since first generation plasmids yielded low levels of immunogenicity, adjuvants and novel delivery systems have to be used (Kutzler and Weiner 2008).

In anti-cancer therapies DNA plasmids gave good results when tested in animals. However, once applied to humans, they showed very little response (Buchan, Gronevik et al. 2005; Rice, Ottensmeier et al. 2008). Direct comparison of CTL induced in humans by DNA plasmid and an Ad5 recombinant vector showed that the latter is at least 4 times more potent, which highlights the need to improve the potency of DNA vaccination (Kutzler and Weiner 2008). Several efforts are taken to achieve this. One of them is a prime-boost strategy, where priming with DNA containing several HIV-1 antigens is followed by modified vaccinia virus Ankara (MVA) boost (Dorrell, Williams et al. 2007; Peters, Jaoko et al. 2007).

Alum, microsphere, nanoparticles, liposomes and polymers can all be used as formulation adjuvants, while cytokines (e.g. IL-12), chemokines and Toll receptor ligands (TLRs) constitute immune plasmids adjuvants (Kutzler and Weiner 2008). Last, but not least, currently there is an array of methods used to deliver DNA plasmids including electroporation, transcutaneous microneedle injections, skin abrasion, gene gun, ultrasound, tattoo perforating needle, jet-injector and topical patch.

Currently, there are about 70 phase I and several phase II and phase III clinical trials focusing mostly on cancer, but also cardiovascular and infectious disease, and healthy volunteers are being tested for HIV-1 vaccine safety.

Heterologous prime-boost vaccination is amongst the most effective ways to improve DNA vaccine platform. Upon primary immunization, a portion of antigen-specific T cells transforms into antigen-specific memory T cells. Those cells have the ability to greatly and rapidly expand upon encountering the same antigen for the second time and using different vectors for priming and boosting allows for greater expansion (Fioretti, Iurescia et al. 2010).

When compared to homologous prime-boost regime using the same DNA vaccine, heterologous vaccination protocols yields 4 to 10 times higher cellular responses (Schneider, Gilbert et al. 1998).

Several viral vectors have been used as a boost to DNA vaccine. Those include adenoviral vectors, vaccinia virus based recombinant vectors, as well as vectors based on fowlpox and recombinant vesicular stomatitis viruses (Fioretti, Iurescia et al. 2010).

Also recombinant protein matching the antigens included in the DNA prime vaccine has been used as a boost (Wang, Kennedy et al. 2008). This approach is especially useful for eliciting protective antibody responses

The following combination of prime/boost regime have been used in the clinics: plasmid DNA followed by plasmid DNA + EP for prostate and colon cancer; plasmid DNA boosted by recombinant protein for prostate carcinomas and breast cancer; plasmid DNA/viral vector in liver cancer, melanoma and prostate carcinoma; viral vector prime followed by plasmid DNA boost for prostate cancer (Fioretti, Iurescia et al. 2010).

Despite the fact that prime/boost strategies as mostly used to enhance responses to DNA vaccination, this strategy has also been used with other vectors, including lentivectors. Palmowski et al showed that direct i.v. injection of a lentivector endocind NY-ESO-1 generates specific CD8+ T cells that are expanded by boosting with a vaccinia vector encoding the same antigen (Palmowski, Lopes et al. 2004).

More recently, lentivector system was used to target human a commonly expressed tumour antigen - human telomerase reverse transcriptase. The study found that the additional use of a heterologous boosted vaccination drastically improves self/TERT-specific CD8 responses in lv-hTERT primed mice (Adotevi, Mollier et al. 2010).

1.5.2. mRNA VACCINES

Another effective tool to stimulate CTL responses *in vitro* and *in vivo* is transfection of dendritic cells with mRNA. This method is more efficient than DNA transfection and mRNAs can be isolated directly from tumour cells (Breckpot, Heirman

et al. 2004). Several methods can be used to deliver mRNA to dendritic cells, including cationic lipids, incubation of mRNA with DCs, lipofection, transferrin receptor (CD71)–based endocytosis and electroporation (Breckpot, Heirman et al. 2004) (Grunebach, Muller et al. 2005). Electroporation is highly reproducible and easy to perform with an efficiency of up to 90% in CD14+ and CD34+ DCs without affecting their phenotype and viability. Furthermore, mRNA activates APCs through recognition by TLR-7 in mice and TLR-8 in humans (Heil, Hemmi et al. 2004; Diebold 2008).

The mRNA approach has been tested using several tumour antigens, such as CEA and PSA, papillomavirus oncoproteins, human telomerase reverse transcriptase, Mucin 1, HER-2/neu and melanoma antigens (Grunebach, Kayser et al. 2005). The elicited response was both CD8+ and CD4+ mediated with all reported clinical trials well tolerated with no major toxicity observed in any of the patients.

Several strategies are used to enhance CTL responses elicited with this method. For example Bonehill et al. use transfection of CD40, CD70 and TLR-4 expressing mRNA along with mRNA for melanoma antigens (Bonehill, Tuyaerts et al. 2008).

Despite the promising outcomes of clinical trials, there is a concern that mRNA molecules will only give transient expression as they are degraded by cellular ribonucleases.

1.6. VIRAL VECTORS

Currently, more than half of vectors tested in gene therapy trials are derived from viruses. Those are produced by disabling the viruses and turning them into gene delivery vehicles. Viral vectors can be live attenuated (e.g. MVA) or replication deficient (AdV, AAV, retroviruses). They display a number of advantages over non-viral gene delivery methods, such as more efficient delivery and improved transgene expression levels. In addition, accessory molecules can be inserted (e.g. co-stimulatory molecules).

Viruses most commonly turned into viral vectors are: adeno-associated virus, adenovirus, poxvirus, herpes simplex virus, oncoretrovirus and lentivirus. Table 1.2. presents a summary of their characteristics.

Table 1.2. Viruses most commonly used as viral vectors

Vaccine based on	Genome	Advantages	Disadvantages
Adeno-associated virus (AAV)	ssDNA	– Parental virus non-pathogenic	– Low packaging capacity – Stable infection is helper-dependant
Adenovirus (AdV)	dsDNA	– Infects non-dividing cells – Does not block maturation, activates DCs – Produced in high titers	– Episomal genome leads to transient gene expression – Anti-vector immunity
Poxvirus	dsDNA	– Large insertional capacity – Produced in high titers – Infects and matures DCs	– Transient gene expression – Anti-vector immunity – Immune responses dominated by viral epitopes
Herpes simplex virus (HSV)	dsDNA	– Large insertional capacity – High titers – Infects DCs	– Blocks DC maturation – Encodes immunodimant viral epitopes
Oncoretroviruses	ssRNA+	– Stable gene expression – No viral genes encoded	– Risk of insertional mutagenesis – Only infects dividing cells
Lentivirus	ssRNA+	– Stable gene expression – Infects non-dividing cells	– Risk of insertional mutagenesis

1.6.1. ADENOVIRUSES

Adenoviruses are non-enveloped double-stranded DNA viruses with a small genome of ~30–35 kilobases consisting of five segments encoding early gene products (E1a, E1b, E2a, E2b, E3, and E4) and five segments coding for late gene products (L1–L5). While E1, E2, and E4 gene products are indispensable for viral replication, E3 gene products are not needed for the process; their role is subversion of immune responses (antigen presentation, cytokine production and apoptosis) (Tatsis and Ertl 2004).

Most vaccine constructs are generated by introduction of deletions in E1 or E1/E3 transcription units. E3-deleted adenoviral vectors are replication-competent and can accommodate an additional 3.5 kb of foreign sequence (Tatsis and Ertl 2004). E1-deleted vectors require the E1 gene provided *in trans* to replicate. Several advantageous characteristics make adenoviral vectors popular. They have the ability to transduce both dividing and non-dividing cells, a large insertional capacity and a high titer during production. Nevertheless, the episomal viral DNA is unable to replicate in infected cells and becomes degraded. In addition to that, adenoviral particles are immunogenic (Breckpot, Heirman et al. 2004). For this reason new serotypes are being explored to circumvent problems of pre-existing neutralizing antibodies in humans.

Adenoviral vectors are very efficient *in vitro* – up to 100% of CD38+ DCs can be transduced (Dietz and Vuk-Pavlovic 1998; Frey, Hackett et al. 1998; Mulders, Pang et al. 1998) and very high titers can be well tolerated by DCs (Arthur, Butterfield et al. 1997).

Preclinical models showed induction of potent specific T and B cells responses, with T cells being predominantly CD8+ (Xiang, Yang et al. 1996). Repeated immunizations were not effective.

At the moment adenoviral vectors are being tested for cancer, degenerative diseases (Alzheimer) and pathogens like Plasmodium falciparum, leishmania, Mycobacterium tuberculosis, dengue virus, Japanese and Venezuelan encephalitis

virus, rabies virus, influenza virus, and hepatitis viruses (Lasaro and Ertl 2009). Furthermore, the effect of vaccination with adenovectors on the rate of HIV-1 acquisition was tested in two clinical trials: STEP and Phambili. The STEP trial was halter in its early phase as it showed lack of efficacy and a trend toward increased HIV-1 acquisition in vaccine recipients (Buchbinder, Mehrotra et al. 2008; McElrath, De Rosa et al. 2008).

1.6.2. ADENO-ASSOCIATED VECTORS (AAVs)

Adeno-associated virus (AAV) belongs to the *Parvoviridae* family. They are nonenveloped viruses with a linear single-stranded DNA genome spanning roughly 4.7 kilobases (kb) (Daya and Berns 2008). They contain two open reading frames: the rep region which codes for replication-related proteins and the cap region coding for the three proteins (VP1, VP2, and VP3) forming the viral capsid. AAV genome is flanked by inverted terminal repeats (ITRs) (Schultz and Chamberlain 2008). Cellular receptor heparan sulfate proteoglycan serves as a receptor for AAV-2 and allows it to enter the target cells. AAVs require the presence of a helper virus (adenovirus or herpesvirus) for infection. AAV (serotype 2) is characterized by site-specific integration into chromosome 19q13.4 where it can set up latency by integrating (Daya and Berns 2008).

In the absence of helper virus, AAV replication and viral gene expression are repressed. Both adenovirus and herpesvirus can act as helper virus. Their function is to *regulate cellular gene expression to provide a permissive intracellular environment for a productive infection by AAV.* Each of these viruses provides different sets of genes for helper function. The adenoviral genes that provide helper functions regarding AAV gene expression have been identified and include E1a, E1b, E2a, E4, and VA RNA. Herpesvirus aids in AAV gene expression by providing viral DNA polymerase and helicase as well as the early functions necessary for HSV transcription (Daya and Berns 2008).

The first protein identified to mediate recombination between the genome of AAV virus and the AAVS1 chromosomal target was the Rep protein. Rep binds to Rep-

binding elements (RBEs) that are situated within both the AAV genome and the AAVS1 site. The complex of Rep/AAV-DNA gets localized to the AAVS1 site where recombination takes place through nonhomologous deletion-insertion resulting in AAV integration.

Recently, a cis sequence domain p5IEE – an AAV integration efficiency element- has been discovered to mediate site-specific interaction and enhance it by 10-100 fold. P5IEE element is 138-bp long and is both necessary for efficient site-specific integration and sufficient (Philpott, Giraud-Wali et al. 2002).

The disadvantages of recombinant AAV vectors include limited capacity for insertion of foreign genes (up to 4.5 kb), low-titer virus stocks and the need for intracellular conversion of single-stranded provirus into a transcriptionaly active double-stranded DNA viral template. The latter has been bypassed by the design of self-complementary AAV (scAAV) vectors (McCarty, Monahan et al. 2001). However, as a consequence the maximal size of the transgene had to be reduced by 50%.

Trans-splicing AAV vectors have been developed to increase AAV vector capacity (Yan, Zhang et al. 2000). In this approach the transgene is distributed between two rAAV vectors containing splice donor and splice acceptor sites.

The use of different AAV serotypes in a pseudotyping approach has allowed broader tissue tropisms. However, some tissues remain refractory to transduction using available serotypes. This presents a major challenge for AAV-based gene therapy for clinically relevant tissues (Daya and Berns 2008).

Transduction of DCs with AAVs is strongly DC culture-dependent and varies from 2 to 55% (Ponnazhagan, Curiel et al. 2001; Ponnazhagan, Mahendra et al. 2001).

1.6.3. POXVIRUSES

The poxvirus family are viruses with large double-stranded DNA genomes (167 to 224 Kb) encoding several hundred proteins; they replicate in the cytoplasm of the target cell. They are attractive vaccine candidates because of their packaging flexibility (at least 25 kb), the ability to induce both antibody and cytotoxic T cell

responses, and finally their stability, low cost and the ease of manufacture (Gomez, Najera et al. 2008).

Highly attenuated strains of poxvirus vaccines with enhanced safety profile have been developed, with MVA or NYVAC being most popular.

The MVA virus was derived from chorioallantoid vaccinia Ankara (CVA), a Turkish smallpox vaccine strain that lost nearly 30 Kb of its genome (Antoine, Scheiflinger et al. 1998). The attenuated NYVAC strain was derived from an isolate of the Copenhagen vaccine strain (VACV-COP) through deletion of 18 Open Reading Frames (ORFs) implicated in pathogenicity and virulence (Tartaglia, Perkus et al. 1992).

A variety of recombinant poxvirus vectors have been extensively used to express a multitude of foreign genes in preclinical studies and in clinical trials of cancer immunotherapy. Among them, attenuated strains of vaccinia virus MVA and NYVAC have been demonstrated as excellent vaccine candidates because of the high levels of recombinant protein expression, strong immunogenicity and their safety profile. Moreover, administration of VACV vectors provides a "danger signal" to the host that helps to prime effective T cell responses and break immune tolerance to tumor associated antigens (Overwijk, Lee et al. 1999; Gallucci and Matzinger 2001).

Several MVA and NYVAC vectors are being tested in clinical trials against disorders such as AIDS, malaria, tuberculosis and cancer. They have demonstrated a degree of immunogenicity. However, the CTL responses did not match the levels observed in preclinical studies as immunodominant vaccinia-specific CTL responses limit the effectiveness of poxviruses in recombinant vaccination strategies (Smith, Mirza et al. 2005).

The prime/boost strategy has also been used by combining recombinants based on MVA or NYVAC with DNA or other recombinant viruses. This approach elicited antigen-specific cellular immune responses which in some cases correlate with protection (Zavala, Rodrigues et al. 2001).

Vaccinia-derived vectors are capable of transducing DCs differentiated from CD34+ precursors (but not the precursors themselves) even at low MOIs (Di Nicola, Siena et al. 1998). For example at an MOI of 2.5 up to 60% of DCs are transduced (Sutter and Moss 1992; Drillien, Spehner et al. 2000). However, those vectors have been demonstrated to affect DC maturation and lead to a low expression of co-stimulatory molecules, HLA molecules, and low T cell activation capacity (Engelmayer, Larsson et al. 1999; Drillien, Spehner et al. 2000; Jenne, Hauser et al. 2000).

Another member of Orthpox family – the canarypox virus – has also been used to design gene transfer vectors resulting in generation of the ALVAC system (Aventis Pasteur) (Chaux, Luiten et al. 1999; Motta, Andre et al. 2001; Tsang, Zhu et al. 2001; Marovich, Mascola et al. 2002). ALVAC is relatively efficient at transducing immature DCs and infection is accompanied by TNFα-mediated maturation (Ignatius, Marovich et al. 2000; Tsang, Zhu et al. 2001; Marovich, Mascola et al. 2002).

1.6.4. HERPSE SIMPLEX VIRUS (HSV)

Herpes simplex virus (HSV) is a linear double stranded DNA virus with a 152 kb long genome encoding at least 80 gene products. Almost half of them are nonessential for growth in *vitro* and may be deleted. This creates space for transgenes and at the same time removes functions essential for virulence and toxicity *in vivo* (Argnani, *Lufino et al. 2005)*.

Several aspects of HSV-derived vectors make them attractive. HSV is highly infectious and its cellular receptors - heparan sulfate (HS), herpes virus entry mediator (HVEM), and nectin-1 and 2 – are broadly expressed which allows the virus to infect a range of cells. HSV efficiently transduces and infects nondividing cells. Last, but not least, these vectors are easily produced and reach high titers (Argnani, Lufino et al. 2005).

HSV type 1 - based vectors can be divided into 3 different categories: amplicon vectors, replication-defective viruses and genetically engineered replication-competent viruses with restricted host range.

HSV-1 amplicon is a helper-dependent plasmid-based vector enclosed within enveloped HSV-1 capsids (Geller and Breakefield; Spaete and Frenkel). This system transduces a broad range of cells without introducing viral coding sequences. Each amplicon contains 10 to 15 plasmid copies. Up to 70% transduced DCs, with MOIs as low as 1, can be obtained by using the HSV-1 amplicon. This is accompanied by a certain level of activation. Dendritic cells transduced with HSV-1 amplicons expressing prostate-specific antigen were shown to process and present TAAs in the context of MHC class I and generate antitumor immunity in mice (Willis, Bowers et al.).

Some concerns regarding the safety of HSV-1 derived vectors remain. Undesirable viral spreading is a problem so it would be advantageous to introduce a secondary mechanism to inactivate the vector (Argnani, Lufino et al. 2005).

HSV vectors were initially designed for gene delivery to the brain, but currently they are also used for immunotherapy against tumors and prophylaxis against infectious diseases including HSV (Argnani, Lufino et al. 2005; Marconi, Argnani et al. 2008).

1.6.5. RETROVIRUS

Retroviruses belong to the RNA virus family and replicate through a double stranded DNA intermediate that integrates into the host genome, which provides long term expression. The most commonly used retroviral vectors are based on Moloney murine leukemia virus (MLV).

In addition to a long-term gene expression, another advantage of retroviral vectors is that they are not immunogenic as genes encoding viral proteins can be removed. However, they are difficult to produce at high titers, and even more importantly, they require disruption of the nuclear membrane to integrate into host's DNA and thus do not transduce non-dividing cells which limits their capability to

transduce monocyte-derived DCs (Roe et al. 1993). Therefore, they can only be used for transduction of $CD34^+$-derived DC precursors.

Another issue associated with using retroviruses is their safety. In two clinical trials against X-linked severe combined immunodeficiency (SCID-X1), some patients went on to develop leukaemia as a result of vector integration near the LMO2 oncogene (Hacein-Bey-Abina, Garrigue et al. 2008; Howe, Mansour et al. 2008).

The use of retroviral vectors has been followed by development of lentivectors based on HIV-1 genome which have the ability to transduce non-dividing cells.

1.6.6. LENTIVIRUS

Lentiviruses, just like retroviruses, belong to the Retroviridiae family with simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) being the most extensively studied. They also share the same genomic organization as oncoretroviruses, but they encode some accessory genes (*vif, vpr, vpu, nef, tat, ref*) ().

Engineered lentivirus-based vectors have same advantages over other vectors. To start with, they transduce non-dividing cells which makes them interesting for vaccination purpose. All non-essential viral proteins have been removed from the vector, which minimizes cellular toxicity and immunogenicity, and most people will not have pre-existing antibodies to the vector. Finally, they can be pseudotyped with different viral envelopes which allows for specific targeting of several cell types (Kim, Majumder et al. 2005; Loisel-Meyer, Felizardo et al. 2009). They are a powerful tool used for gene transfer into $CD34^+$- and $CD14^+$-derived DCs.

1.7. HIV-1 STRUCTURE AND LIFE CYCLE

The HIV-1 virion consists of a nucleocapsid containing 2 identical 9.2 kb single stranded RNA molecules surrounded by a lipid bilayer incorporating envelope glycoprotein spikes. Structural proteins forming the core are: MA (matrix/p17), CA (capsid/p24), NC (nucleocapsid/p7) and p6 (Fig. 1.3.A).

1.7.1. GENOME

Four main regions can be distinguished in the HIV-1 genome (Berkhout and Jeang 1992; Richter, Ping et al. 2002):

- LTR - regulatory regions at both DNA ends created during reverse transcription of viral RNA and flanking the coding region, each of them contains 3 elements: U3 region (contains binding sites for cellular transcription factors), the R region (contains the trans-activation response element (TAR) implicated in Tat-mediated trans-activation) and U5 region
- The *gag-pol* gene - encodes two polyprotein precursors which includes the proteins of the nucleocapsid, some structural proteins and three viral enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN).
- The *env* gene - encodes the exterior gp120 and the transmembrane gp41 in the form of a gp160 polypeptide precursor
- HIV accessory genes: *tat, rev, nef, vif, vpr* and *vpu* (for HIV-1) or *vpx* (for HIV-2).

A schematic representation of HIV-1 genome is presented in Fig. 1.3.B.

Figure 1.3.

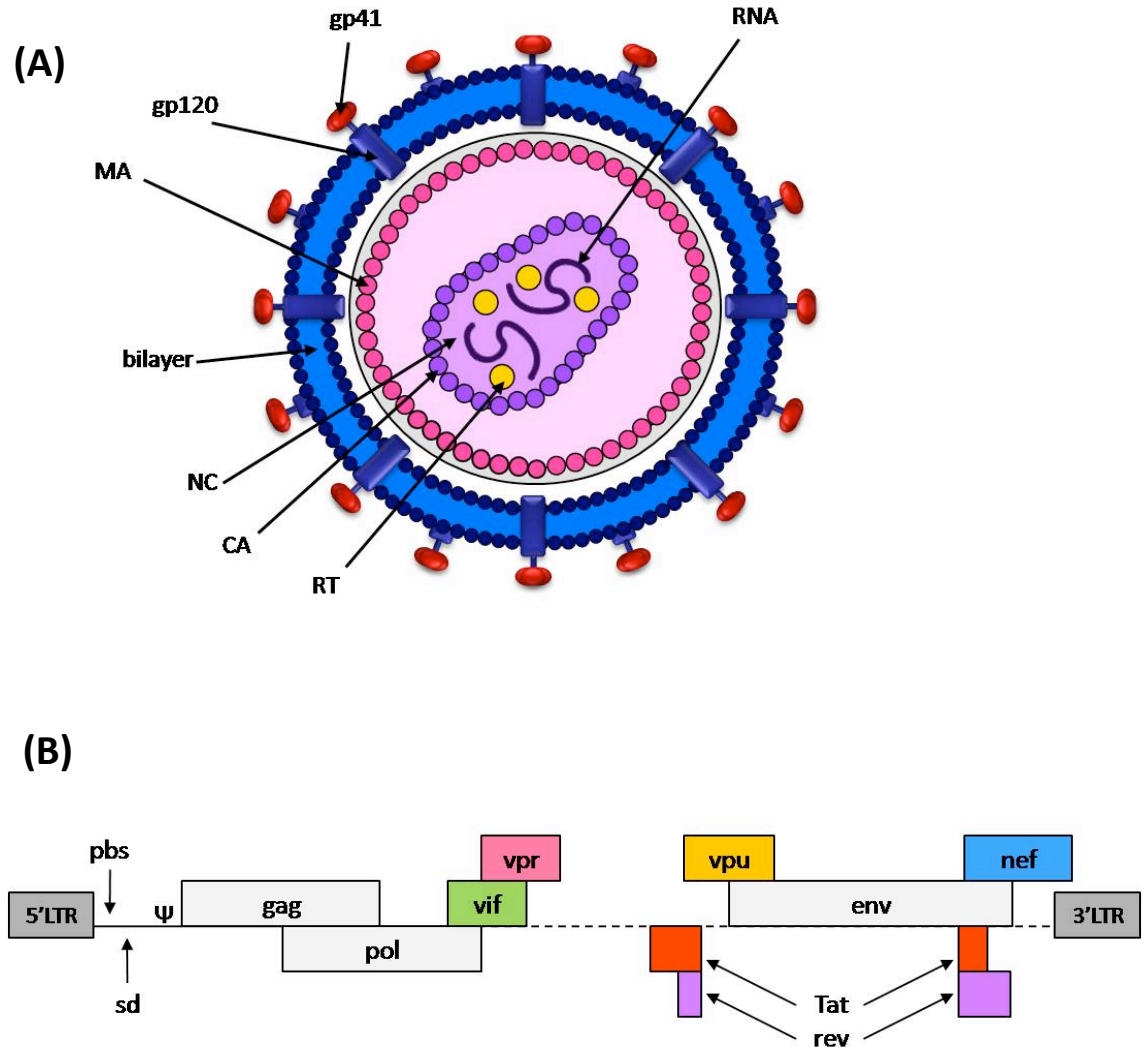

Figure 1.3. HIV-1 structure and genome

(A) The HIV-1 virion consists of a nucleocapsid containing 2 identical 9.2 kb single stranded RNA molecules surrounded by a lipid bilayer incorporating envelope glycoprotein spikes. MA- matrix, CA- capsid, NC- nucleocapsid, RT- reverse transcriptase.

(B) Viral DNA is flanked by long terminal repeats (LTRs). Gag/pol encodes structural proteins and viral enzymes. Env encodes envelope glycoproteins. Genes with regulatory functions: tat, rev,nef, vpu, vpr, vif. Pbs- primer binding site, sd- major splice donor site, Ψ- packaging signal, RRE- rev responsive element.

1.8. HIV-1 LIFE CYCLE

The life cycle of the retroviruses is divided into two phases. The early phase involves virus entry into the cell, reverse transcription of viral RNA, insertion of viral DNA into the host genome and establishment of integrated provirus. During the late phase viral RNA and proteins are expressed, virion particles become assembled and released by budding through the plasma membrane (Goff 2001).

1.8.1. VIRUS ATTACHMENT AND ENTRY

The first step of retroviral life cycle is the adsorption of viral particles to the surface of their target cells which is thought to be mediated by molecules distinct from the viral receptor responsible for the entry process (Nisole and Saib 2004). Envelope glycoproteins of HIV-1, HIV-2 and Simian Immunodeficiency Virus (SIV) interact with the C- type mannose binding lectins DC-SIGN (Dendritic cell- specific intercellular adhesion molecule 3-grabbing non- integrin) and DC-SIGNR (DC-SIGN related) to bind to the surface of dendritic cells. In the case of HIV-1, it is the high mannose structures on gp120 that are recognized by DC-SIGN (Hong, Flummerfelt et al. 2002). DCs bind and capture viral particles at peripheral sites of infection through these interactions and then carry them to the lymph nodes promoting infection of CD4+ cells (Geijtenbeek, Kwon et al. 2000).

Retroviral entry is a complex multi-step mechanism starting with glycoprotein gp120 recognizing the primary receptor CD4. This results in conformational changes in CD4 and gp120 and recruitment of coreceptors CXCR4 and CCR5 (Berger, Murphy et al. 1999). At this point gp120 starts to interact with these coreceptors, which in turn leads to new conformational shifts in the envelope glycoproteins (Kwong, Wyatt et al. 1998). This results in dissociation of gp120 from gp41 (gp120 is present on the surface of viral particles as gp41/gp120 trimers) and to the transition of gp41 to its fusogenic conformation. Insertion of the gp41 fusion peptide into the target membrane leads to fusion of viral and cellular membranes, entry of virions into the cell and release of the viral core in the cytoplasm.

This is followed by uncoating – progressive disassembly of viral core - which results in generation of subviral particles: reverse-transcription complexes (RTCs) and pre-integration complexes (PICs).

Reverse transcription of the viral RNA into DNA begins within 2 hours of infection and takes place in the cytoplasm (Goff 2001). It is not known what triggers the reaction, but it is likely to be initiated by exposure of RTC to the high concentration of deoxyribonucleotides in the cytoplasm.

Host cells contain several mechanism of resistance to retroviral infection. For example, a human protein APOBEC3G has been shown to inhibit HIV replication at the step of reverse-transcription (Sheehy, Gaddis et al. 2003). However, HIV-1 Vif protein protects the virus from APOBEC3G-mediated inactivation by preventing its incorporation into virions. Also the cellular protein cyclophilin A (CypA) is believed to protect the viral capsid from restriction by human factor Ref1, thus leading to an increase in HIV-1 infectivity (Nisole and Saib 2004).

1.8.2. NULCEAR ENTRY AND INTEGRATION

The reverse-transcribed DNA associates with viral proteins and forms the preintegration complex (PIC) (Nisole and Saib 2004). Retroviruses use the cytoskeleton as a track for intracellular trafficking of the PIC. In the case of lentiviruses such as HIV-1, PICs are able to actively cross the nuclear membrane and infect quiescent or terminally differentiated cells in which the cell cycle is stopped in the G0 phase (e.g. macrophages and microglia) (Suzuki and Craigie 2007).

How retroviral PICs cross the nuclear envelope in non-dividing cells is still not fully understood, but for HIV-1 it is generally accepted to be an energy-dependent, active process. Several HIV-1 PIC proteins contain karyophilic signals that make them actively imported into the nucleus (Suzuki and Craigie 2007).

Four different viral components have been reported to contribute to the nuclear import of HIV-1. Those are integrase, matrix protein, Vpr and the viral DNA. The exact function of each remains to be fully understood (Nisole and Saib 2004).

Following nuclear entry integrase inserts viral DNA into the host genome. This process of integration is described in details in chapter 3.

1.8.3. PROVIRUS TRANSCRIPTION AND TRANSLATION

The proviral DNA integrated into host DNA serves as the template for viral transcription. The 5'-long terminal repeat (LTR) is used as the promoter to direct synthesis of the full-length viral RNA. Retroviral transcription is mediated by RNA polymerase II and cellular basal and promoter specific factors. In addition, HIV-1 encodes its own transcriptional activator (Pollard and Malim 1998).

Efficient activation of the LTR promoter is largely driven by Tat which has been suggested to be involved in remodelling nucleosomes to relieve transcriptional blockage imposed by chromatin. Tat interacts with a specific 59-residue stem-loop structure, TAR, on the RNA leader sequence and is thought to cause a dramatic increase in transcriptional levels upon binding to TAR (Wu 2004).

At first, only multiply spliced RNAs are generated, which results in expression of Tat, Nef and Rev. Gag/pol requires export of full-length viral genomic RNA into the cytoplasm. Full-length transcripts not only serve as templates for translation (Gag and Gag-Pol), but also function as precursor RNAs (pre-mRNAs) for the production of diverse subgenomic mRNAs. When enough Rev is generated, it binds the Rev response element (RRE) present in all incompletely spliced viral mRNAs and induces their nuclear export as Rev contains a nuclear export signal.

1.8.4. ASSEMBLY AND BUDDING OF PROGENY VIRUS

Retroviral RNAs transported to the cytoplasm are translated to produce viral proteins. The products of the gag and gag-pol genes initially assemble into immature nucleocapsids containing two copies of full-length viral RNA (Pollard and Malim 1998). The nucleocapsid portion of Gag contains a domain that recognizes the packaging signal on RNA and ensures incorporation of genomic RNA into the virions (Gottliner 2001).

Figure 1.4.

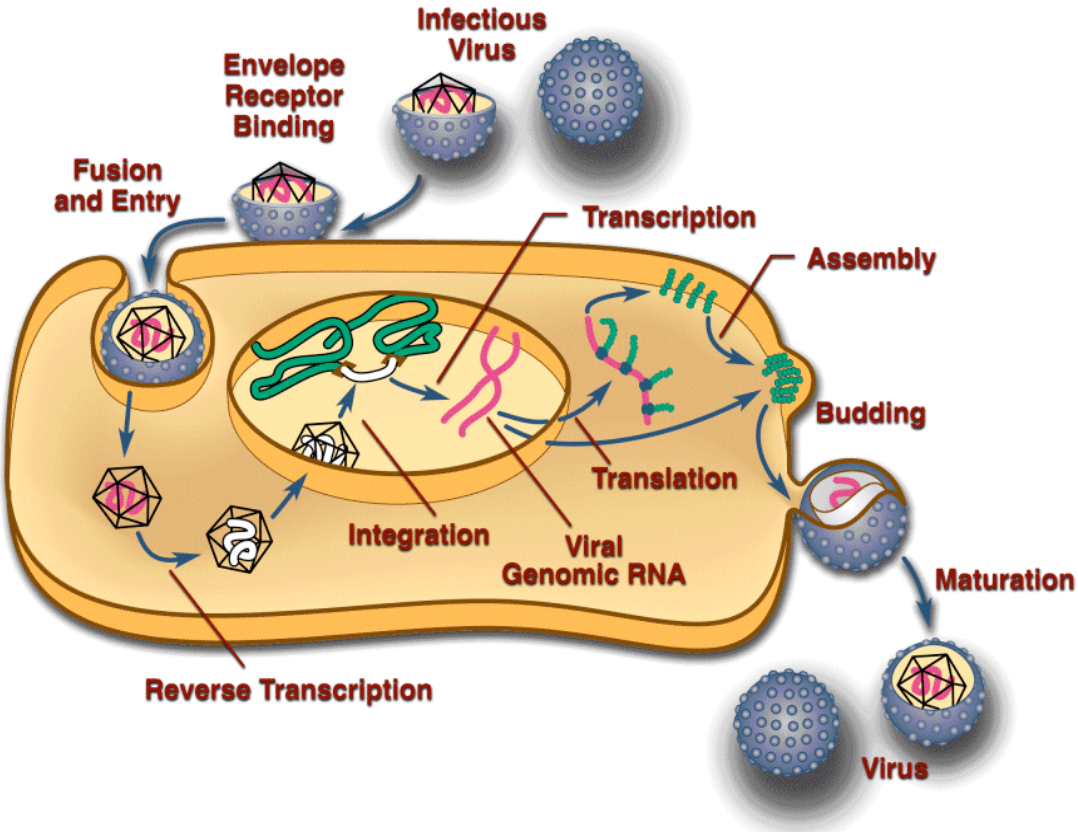

Figure 1.4. HIV-1 replication cycle. *Main steps of HIV-1 replication cycle: fusion and entry, reverse transcription, integration, transcription and translation, budding and maturation.*

Reproduced from http://www.ncbi.nlm.nih.gov/retroviruses/

The immature nucleocapsids bud through the plasma membrane. At this point they become encapsulated by a layer of membrane containing viral Env glycoproteins. Those are introduced into the plasma membrane as trimers of gp120 and gp41 (following env translation into gp160, its trafficking to the ER, and cleavage into gp120 and gp41). The plasma membrane becomes the viral envelope and is rich in cellular proteins like HLA class II (Pollard and Malim 1998).

The budding virions are non-infectious. The HIV viral enzyme protease (PR) then cleaves the core proteins (Gag and Gag-pol polyproteins) into their final forms. This process is termed maturation (Sierra, Kupfer et al. 2005).

1.9. LENTIVIRAL VECTOR DESIGN

Lentivirus based vectors have been developed by modifying virus genome in a way that removes its pathogenicity and capacity to replicate, yet it retains its ability to integrate into the host genome and mediate stable transgene expression.

Lentivector design is based on three principles. First of all, there is a separation between trans- (genes required for assembly of virus particles, like enzymatic, structural, accessory and envelope proteins) and cis- (crucial for packaging, reverse transcription and integration of transcripts from packaging plasmid) acting elements. These elements are provided on different vectors used for transient transfection. Removal of cis-acting sequences from the packaging vector prevents generation of replication-competent recombinants as this would require multiple rearrangements and recombination events (Naldini, Blomer et al. 1996).

In the second step, the safety of LVs is reinforced by removal of vif, vpr, vpu, nef and tat genes. Vif, vpr, vpu and nef are accessory genes encoding proteins essential for viral growth and pathogenesis *in vivo*. These can be deleted without affecting viral replication *in vitro.* Tat is a regulatory gene essential for viral replication. However, the trans-acting function of Tat is dispensable provided that a part of the upstream LTR in the transfer vector is replaced by constitutively active promoter sequences (Zufferey, Nagy et al. 1997; Dull, Zufferey et al. 1998; Zufferey, Dull et al. 1998).

Last, but not least, the safety of lentivectors has been further strengthened by generation of self-inactivating lentivirus vectors (SINs). In this approach a deletion in the U3 region of the 3' long terminal repeat (LTR) has been introduced. During reverse transcription this deletion is transferred to the 5' LTR. This eliminates the transcriptional activity of LTRs and production of full-length vector RNA in transduced cells, which minimizes the risk of generating RCRs (Zufferey, Dull et al. 1998).

Production of lentivectors is achieved through transient transfection of three plasmids into 293T cells: transfer, packaging and envelope vectors.

1.9.1. PACKAGING VECTOR

The initial packaging construct contained enzymatic, structural and accessory proteins. It consists of the human cytomegalovirus (hCMV) immediate early promoter driving expression of all viral proteins required in trans, but is defective for the production of the viral envelope and the accessory protein Vpu. The packaging signal (ψ) and adjacent sequences were removed from the 5' untranslated region. The 3' LTR is substituted by a heterologous polyadenylation signal (Naldini, Blomer et al. 1996). This was the first generation vector. In the second generation vectors the vif, vpr, vpu, nef accessory genes have been deleted (Zufferey, Nagy et al. 1997). Vectors were further modified by deletion of tat and a new design of the packaging component split in two separate non-overlapping expression constructs, one for the *gag* and *pol* genes and the other for the *rev* gene (Dull, Zufferey et al. 1998). This third-generation packaging system is similar in the performance to the first and second generation vectors in terms of yield and transducing efficiency, yet it brings about a significant increase in the predicted biosafety of the vector.

1.9.2. EXPRESSION VECTOR

The transfer vector plasmid contains the gene of interest, and the *cis-acting* sequences needed for packaging, reverse transcription and integration. They contain two elements designed to improve transgene expression and transduction efficiency:

the central polypurine tract (cPPT) and a WPRE element. The central polypurine tract from HIV *Pol* has been shown to enhance second strand synthesis (Follenzi, Ailles et al. 2000). The WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) enhances viral titer and transgene expression (Zufferey, Dull et al. 1998).

The gene of interest included in the expression vector is driven by a heterologus promoter. The expression constructs in this study use the SFFV promoter as it is superior to the CMV promoter in driving expression in haematopoietic repopulating cells.

1.9.3. ENVELOPE VECTOR

Lentiviral vectors are most commonly pseudotyped with the glycoprotein (G) of vesicular stomatitis virus (VSV) which allows them to infect a broad range of tissues and efficiently transduce postmitotic cells such as the retina, respiratory epithelium, muscle, brain, and liver. Other advantages of pseudotyping with VSV-G include high titers and increased stability of the vector particle during purification. However, it has been reported that VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. The extent of inactivation of VSV-G pseudotyped vectors across human sera was variable yet always substantial.

Several glycoproteins alternative to VSV-G have been tested. Those include glycoproteins from the following families: Rhabdoviridiae, Arenaviridae, Togaviridae, Filoviridae, Retroviridae, Coronaviridae, Flaviviridae and Baculoviridae (Cronin, Zhang et al. 2005). Each of those envelopes is cell type specific.

It has been of special interest to restrict gene transfer to relevant cell type depending on the application. Approaches towards this goal include for example the use of tissue-specific promoters (promoter targeting). However, it would be ideal to restrict gene transfer at the step of cell entry. For vaccination purpose, it would be most advantageous to restrict infection to APCs and DCs in particular. This can be achieved by modification of existing envelopes. For example Yang et al (2008) introduced mutations into the envelope of Sindbis virus which blocked the affinity for its natural receptor heparan sulfate, while still allowing it to bind to DC-SIGN expressed

on some DC subsets (Yang, Yang et al. 2008). Another way of targeting DCs has been achieved by conjugating viral envelope to single-chain antibodies (scFv) specific for C-type lectins such as DEC-205 and DC-SIGN (Bonifaz, Bonnyay et al. 2002)

Figure 1.5.

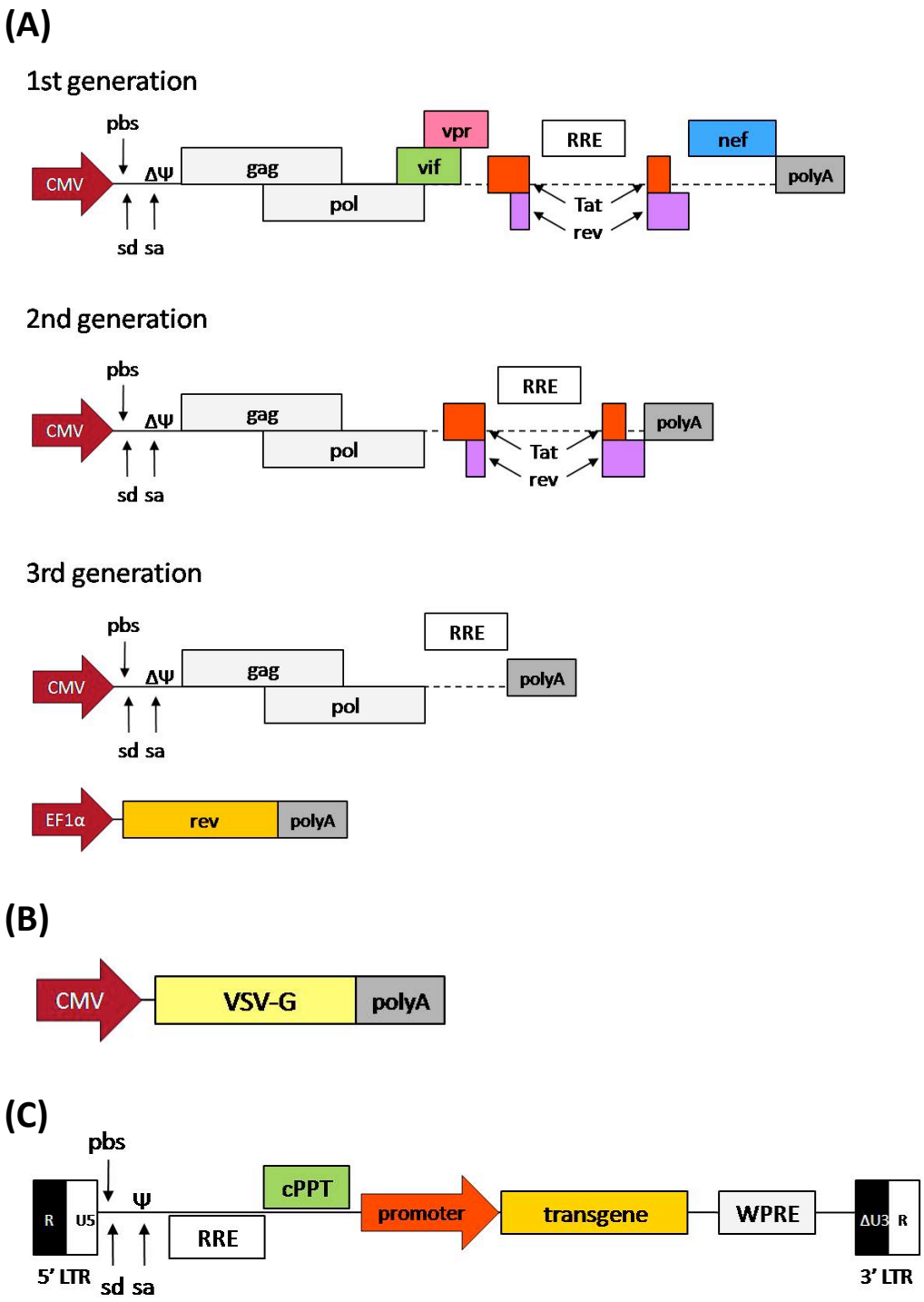

Figure 1.5. Vectors used for lentivector production

(A)Three generations of packaging vectors. The hCMV immediate early promoter drives the expression of all viral proteins required in trans, the packaging signal (ψ) and adjacent sequences were removed from the 5' untranslated region. The 3' LTR is substituted by a heterologous polyadenylation signal.

First generation vectors are defective for the production of the viral envelope and the accessory protein Vpu. Second generation vectors have additionally deleted accessory genes: vif, vpr, vpu and nef. Third generation vectors are further modified by a deletion of tat and are split in two separate non-overlapping expression constructs, one for the gag and pol genes and the other for the rev gene. (B) Envelope vector- vectors used in this study are pseudotyped with glycoprotein (G) of vesicular stomatitis virus (VSV). (C) Expression vector – transgenes are under control of SFFV and UBI promoters.

RRE-Rev responsive element, cPPT-central polypurine tract, WPRE-the woodchuck *hepatitis virus post-transcriptional regulatory element , LTR-long terminal repeats.*

1.10. LENTIVECTOR IMMUNOTHERAPY

Lentivectors gained interest as tools for vaccination as they are able to transduce dendritic cells. Both murine bone marrow derived DCs and human DCs derived from monocytes or CD34+ haemotopoietic precursors can be transduced with VSV-G pseudotyped lentivectors at high transduction rates of 70-90%. This efficiency is superior to the one obtained with adenoviruses and Moloney murine leukemia virus-based retroviral vectors (Mo-MuLV). Moreover, modified DCs maintain strong allostimulatory capacity, remain responsive to activation signals such as CD40 and LPS and secrete bioactive IL12 (Esslinger, Romero et al. 2002). As shown for the ovalbumin (OVA) antigen,transduced DCs efficiently process and present both MHC class I and class II restricted epitopes from the expressed antigen (He and Falo 2006).

Lentivectors have been used to transduce dendritic cells *in vitro*. *In vivo*, those cells efficiently induce potent and long-lasting T cell responses as measured by *in vivo* killing assay and IFN-γ ELISpot (Zarei, Abraham et al. 2004). In tumour experiments, injection of LV-transduced DCs was more efficient than Ag-pulsed DCs while in infectious disease model of LCMV this approach confers a protective antiviral immunity *in vivo* (Metharom, Ellem et al. 2001).

Also direct LV injections proved to be successful in inducing immune responses. Lentivector administration into a footpad of mice targets and transduces DCs which later appear in the lymph node (Esslinger, Chapatte et al. 2003). In addition it induces very strong systemic antigen-specific CTL responses in mice (Esslinger, Chapatte et al. 2003; Lopes, Dewannieux et al. 2008). Finally, it has been demonstrated that direct lentivector injection stimulates both CD4+ and CD8+ responses in mice (Rowe, Lopes et al. 2006). Tables 1.3. and 1.4. present an overview of studies using lentivectors as cancer vaccines.

Table 1.3. Overview of studies using lentivectors as cancer vaccines, part 1

Antigen	Route of administration	Boost?	End result	Reference
Cw3, Melan-A	Footpad, base of tail	---	High level of Ag-specific CD8+ T cells, effective in vivo CTL assay	esslinger
NY-ESO-1	i.v. tail vein	VV-NY-ESO-1, day 8	Effective in vivo CTL assay, improved response upon boosting	palmowski
Melan-A	s.c. at the base of tail	---	Memory T cells detectable several months later, high specific in vivo killing	chapatte
OVA	i.v. tail vein	VV-OVA, week 3	Protection against tumoral challenge	rowe
Full length HIV-1 Rev/Env coding sequence, codon optimized HIV-1 gp120	Intramuscular	---	Efficient induction of cellular and humoral responses	buffa
OVA	Subcutaneous	LV-OVA, day 150	Effective in vivo CTL responses, reduced tumour growth	dullaers

Table 1.4. Overview of studies using lentivectors as cancer vaccines, part 2

Antigen	Route of administration	Boost?	End result	Reference
Codon-optimized HIV-1 gp120	i.m.	---	Significant cellular and humoral immune response	negri
NY-ESO-1	s.c. base of tail	---	Induction of antibodies, CD8+ and CD4+ T cell specific responses	Garcia-casado
NY-ESO-1	s.c. base of tail, i.v.	VV-NY-ESO-1, week 3	Induction of specific CD8+ and CD4+ T cell responses	Lopes 2008
Mutated Trp1	s.c. footpad	---	Induction of potent CD8+ T cell responses, elimination of small tumours	liu
Gag	s.c./f.p./i.m./i.p./ i.d.	LV-Gag, DNA prime/LV-Gag boost	Induction of durable HIV Gag specific responses, enhancement of responses by prime/boost regimen	dai
hTERT	s.c. at abdominal flank	pY572 in IFA, week 3	Better/more sustained CD8+ T cell responses compared to peptide vaccination, drastic improvement of responses with prime/boost strategy	adotevi

1.11. PhD aims

In the light of up to date research on lentivectors, I set out to achieve the following goals:

- Test and compare the efficacy of non-integrating lentivectors to the integrating ones in the context of vaccination and tumour therapy
- Enhance responses elicited by NILVs by inclusion of a DC activator: MKK6 or NF-Kb
- Alter interactions between DCs and T cells to enhance responses elicited by immunization with lentivectors by using LVs to deliver shRNA against PDL1

CHAPTER 2. MATERIALS AND METHODS

2.1.MOLECULAR BIOLOGY

2.1.1. MOLECULAR BUFFERS USED FOR SUBCLONING

Molecular buffers and reagents used for subcloning are summarized in table 2.1.

Table 2.1. Molecular buffers used for subcloning

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

2.1.2. PREPARATION OF COMPETENT BACTERIA AND TRANSFORMATIONS

XL1 blue *E.Coli* were streaked onto a LB-plate containing tetracycline (10µg/ml) and grown at 37°C. A single colony was picked and cultured overnight in a small volume of LB containing tetracycline (10µg/ml). 1 ml of overnight culture was transferred into 100ml LB without antibiotics and grown for about 2 ½ - 3 hours at 37°C. Cells were cooled on ice for 5 min and spun at 3000 rpm for 10 minutes at 4°C. Next, they were gently suspended in TFB-I buffer using 50 ml of buffer for 100 ml of culture and left on ice for 5 minutes. After this time, they were centrifuged at 3000 rpm for 10 min at 4°C and gently resuspended in ice-cold buffer TFB-II (4 ml per 100ml culture) and put on ice for 15 min. Finally, they were aliquoted and stored at -80°C.

For transformation cells were defrosted on ice for 20 minutes. When purified plasmid was used, around 100 ng was added to the cells. For transforming ligations, 5µl of ligation reaction (out of 10 µl) was used. The mixture was incubated on ice for 20 minutes, treated with heat-schock (37°C for 2 mins) and put back on ice for 60-90 seconds. Finally, bacteria were plated on an agar plate containing a selection antibiotic and left at 37°C until colonies were visible on a plate.

2.1.3. PLASMID PREPARATION

Single colonies were inoculated in LB medium and grown overnight at 37°C in 2ml - for minipreps, or 100 ml – for midipreps. Selection antibiotic (usually ampicillin, 50µg/ml) was added to the medium. QiaPrep Spin Miniprep kit (Qiagen) was used to prepare minipreps which were usually stored in EB buffer and used for further cloning.

Plasmid Midi Kit (Qiagen) was used for midipreps. DNA prepared using midiprep kit was stored at a concentration of 1µg/µl in TE buffer.

2.1.4. RESTRICTION ENZYMES DIGESTIONS

All enzymes and digestion buffers used in this thesis were purchased from Promega (Madison, WI) or New England Biolabs (Ipswich, MA). Reactions were usually

performed in 20µl total volume with 2 µl of recommended 10x buffer, 1 µl of each enzyme, 1-4 µg of DNA and water for 3 hours or overnight if required. Agarose gel electrophoresis was used to isolate cut fragments.

2.1.5. AGAROSE GEL ELECTROPHORESIS

1% agarose gel was used for extraction of most DNA fragments following restriction enzyme digest and PCR reactions. Gels were prepared by dissolving agarose (Invitrogen, Carlsbad, CA) in 1xTAE buffer. 0.5 µg /ml of ethidium bromide was added (Dutscher Scientific, Essex, UK) and gels were left to solidify. DNA samples were loaded with 6x loading buffer and run alongside 1 kb Plus DNA ladder (Invitrogen, Carlsbad, CA) for comparison of fragment sizes. Gels were visualized in UV light and desired fragments were cut out with a scalpel and purified using Gel Extraction Kit (Qiagen, Hilden, Germany).

2.1.6. PCR REACTIONS

PCR reactions were performed using either Taq Hot Start Polymerase (Qiagen) or KOD polymerase (Novagen). Reagents for both reactions are listed in tables 2.2. and 2.3. :

Table 2.2. Taq Polymerase reaction reagents

Component	Volume (µl)	Final concentration
dNTPs (2.5mM)	1.5µl	200µM of each
10x Buffet	5µl	1x
MgCl2 (25mM)	4µl	1.5mM
Forward primer (50ng/µl)	1µl	1ng/µl
Reverse primer (50ng/µl)	1µl	1ng/µl
DTT (1M)	0.05µl	1µM
DNA		
H2O	to 50µl	

Table 2.3. KOD polymerase reaction reagents

Component	Volume	Final Concentration
PCR grade water	To 50 µl	
Sense primer (10µM)	1.5 µl	0.3 µM
Anti-sense primer (10 µM)	1.5 µl	0.3 µM
Template DNA		
KOD hot start master mix (0.04U/ µl)	25 µl	0.02 U/ µl

Reactions were run in a Hybrid termal cycler using parameteres shown in tables 2.4. and 2.5.:

Table 2.4. Taq Polymerase reaction parameters

Step	Time	Temperature
1.Polymerase activation	10 minutes	95°C
2. Denaturation	30 seconds	94°C
3. Annealing	30 seconds	5°C below lowest primer Tm°C
4. Extension	1 min/ 1 kb of product	72°C
Final extension	10 minutes	72°C
Repeat steps 2-4	25 cycles	

Table 2.5. KOD polymerase reaction parameters

Step	Target size			
	<500 bp	500-1000 bp	1-3 kbp	>3000 bp
1. Activation	95°C 2 min	95°C 2 min	95°C 2 min	95°C 2 min
2. Denaturation	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec
3. Annealing	Lowest primer Tm°C for 10 sec			
4. Extension	70°C 10s/kbp	70°C15s/kbp	70°C 20s/kbp	70°C 25s/kbp
Repeat steps 2-4	25 cycles			

2.1.7. LIGATIONS

Ligations of fragments cut with compatible ends enzymes were performed in 10µl, using 1µl of T4 ligase (Promega) and a 10x ligation buffer. Ligations of PCR products generated by Taq polymerase to pGEMT plasmid were also performed in 10µl total volume, with 1µl of T4 ligase (Promega), 1µl of pGEMT vector and 5µl of 2x ligation buffer (Promega). PCR products generated with a proofreading polymerase were ligated to pJET vector (Fermentas) according to manufacturer's instructions.

2.1.8. DNA QUANTIFICATION

DNA was quantified using Hitachi U-1500 spectrophotometer. DNA concentration was calculated using the following formula:

$$\text{Concentration (µg/ml)} = A_{260nm} \text{ reading} \times \text{dilution factor} \times 50\text{µg/ml}$$

2.1.9. SEQUENCING

Sequences were verified by sequencing service provided by Imperial College London or University College London. Standard primers were provided directly by the service, while custom primers were sent along with the DNA samples.

2.2. LENTIVECTORS

2.2.1. LENTIVECTOR PREPARATION

Lentivectors were prepared by a transient transfection on 293T cells using 3 plasmids: expression vector, second generation HIV-1 derived packaging plasmid p8.91 or p8.74, and a plasmid encoding for VSV-G envelope – pMD.G. The cells were split a day before the transfection in a way that would yield around 80% confluency on the following day. Fugene 6 (Roche Diagnostics, Mannheim, Germany) was used as a transfection reagent. Transfection was performed with the following amount of plasmids/reagents per 15 cm plate:

Table 2.6. Transfection reaction components/15 cm plate

Plasmid/reagent	Quantity
Expression plasmid	3.75µg
p8.91/p8.74	2.5 µg
pMD.G	2.5 µg
H_2O	91.25µl
Optimem (Gibco)	500µl
Fugene 6 (Roche)	45µl

The mixture was incubated for 20 minutes at room temperature. In the meantime fresh medium was added to cells used for transfection. After 20 minutes the solution of fugene 6, Optimem and DNA was added dropwise to plates with cells. Supernatants were collected 24, 48 and 72 hours after transfection, passed through 45µm filter and concentrated around 200 times by centrifugation in Sorvall ultracentrifuge at 115, 000 g for 2 hours at 4 °C. HBSS was added to the spun virus, and the tubes were left on ice for at least 30 minutes before the virus was collected, aliquoted and frozen at -80°C in HBSS.

2.2.2. LENTIVECTOR TITRATION

To titer EGFP-expressing lentivectors, 1-2 x 10^6 cells were transduced with 1-2 µl of vector preparation. 48 hours later, cells were harvested and run on FACS Calibur to determine the percentage of transduced cells. Titer (in infectious units/ml) was calculated using the following equation:

Titer (iu/ml)= no. of cells * (% EGFP / 100) * (1000/ no. of µl used)

Quantity of reverse transcriptase (RT) was measured using Reverse Transcriptase Assay colorimetric kit (Roche) according to manufacturer´s instructions. Typically 1:50-1:200 dilution and a RT reaction incubation time of 3 hours were used. The result is given in ng RT/µl.

2.2.3. PREPARATION OF LENTIVIRAL CONSTRUCTS

Figure 2.1. shows a schematic representation of lentiviral transfer constructs used in this thesis. The pHRSIN-CSGW (Fig. 2.1.A.) was provided by A. Thrasher and is referred from now on as lvEGFP. The vector is a self-inactivating HIV derived vector with 400 bp deletion in the 3'LTR U3 region. It contains a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and a spleen focus-forming virus (SFFV) promoter driving expression of emerald green fluorescent protein (EGFP).

Vectors lvEGFP-OVA, lvMKK6-OVA, lvvFLIP-OVA (Fig. 2.1.B.) have been previously described (Escors, Lopes et al. 2008). Ovalbumin transgene in these constructs consists of the C-terminal portion of the invariant chain fused to amino acids 242-353 of chicken ovalbumin (OVA) and termed IiOVA. Class I H2-K^b restricted epitope SIINFEKL ($OVA_{257-264}$) and class II H2-IA/IE^b restricted ISQAVHAAHAEINEAGR ($OVA_{323-339}$) epitope are encoded within the IiOVA. Sites with mutations in attachment sites are indicated with arrows.

Vector lvYFP (Fig. 2.1.C.) was derived from lvEGFP, by replacing EGFP with YFP and deleting one of the NotI restriction sites from the backbone of the vector. LvYFP.shRNA PDL1 (Fig. 2.1.E.) contains one of the shRNA directed against mouse PDL1 (P2, P3, P4, or P5).

lvOVA-EGFP (Fig. 2.1.D.) has already been described (Rowe, Lopes et al. 2006). LvOVA.P5 (Fig. 2.1.F.) was constructed by including shRNA coding against PDL1 (referred in the text as P5) driven from the same promoter and IiOVA.

Figure 2.1.G. presents constructs used for *in vivo* bioluminescence imaging. They are derived from lvEGFP-OVA vector, where OVA was replaced with firefly luciferase (Fluc).

Packaging plasmids with D64V and DNW mutations have been previously described (Apolonia, Waddington et al. 2007).

Described plasmids were used to generate the following lentiviral preparations:

Table 2.7. Lentiviral preparations

Prep	Expression vector	Packaging plasmid	Integrating?
OVA	lvEGFP-OVA	p8.74	Yes
EGFP	lv-EGFP	p8.74	Yes
D64V	lvEGFP-OVA	p8.74 D64V	No
DNW	lvEGFP-OVA	p8.74 DNW	No
DNW/2Δatt	lvEGFP-OVA 2Δatt	p8.74 DNW	No
2Δatt	lvEGFP-OVA 2Δatt	p8.74	No
MKK6/DNW	lvMKK6-OVA	p8.74 DNW	No
Vflip/DNW	lvvFLIP-OVA	p8.74 DNW	No

2.3. CELLULAR ASSAYS IN VITRO

2.3.1. MAINTENANCE OF CELL LINES

293T cells were used for lentiviral preparation and determination of titer by measuring expression of fluorescent proteins. They are adherent cells derived from human embryonic kidney cells, with a large T antigen from simian virus 40. 293T cells were grown in Dulbecco´s Modified Eagle Medium DMEM (Gibco, Paisley, UK) supplemented with 10% foetal calf serum FCS (Serotec, Oxford, UK), 2mM L-glutamine (Gibco), 100U/ml penicilling and 100µg/ml streptomycin (Gibco). Cells were passaged every few days using trypsin/EDTA (Gibco) and grown at 10% CO_2.

EG7.OVA (OVA transfected EL4 cells) cell lines were grown in 5% CO_2 in Rosswell Park Memorial Institute RPMI (Gibco) supplemented with 10% foetal calf serum FCS (Serotec, Oxford, UK), 2mM L-glutamine (Gibco), 100U/ml penicilling and 100µg/ml streptomycin (Gibco). Since they are non-adherent cells, they were passaged without trypsin. Additionally, EG7.OVA cells were grown in the presence of G418 (Invitrogen) at a concentration of 0.4mg/ml to keep the OVA plasmid.

All cells lines were frozen in FCS with 10% DMSO at a density of 5×10^6 cells/ml.

2.3.2. PREPARATION OF BONE MARROW DERIVED DENDRITIC CELLS

Bone marrow derived dendritic cells were prepared by flushing out the bone marrow from tibia and femura with HBSS (Hank´s balanced salt solution, Gibco) – 2% FCS using an insulin syringe. Red blood cells were removed using Red Blood Cell Lysis Buffer (Sigma). After washing, cells were resuspended in IMDM medium containing 10% foetal calf serum FCS (Serotec, Oxford, UK), 2mM L-glutamine (Gibco), 100U/ml penicilling and 100µg/ml streptomycin (Gibco), 50ng/ml of growth-macrophage colony stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ) and 50µM 2-mercaptoethanol (Gibco). Cells were grown at a density of $5\text{-}7.5\times10^6$ cells/ml of medium. Transductions were performed on days 4-6 with an MOI of 20. To induce maturation, cells were treated with 100ng/ml LPS 24 hours before the analysis.

Figure 2.1.

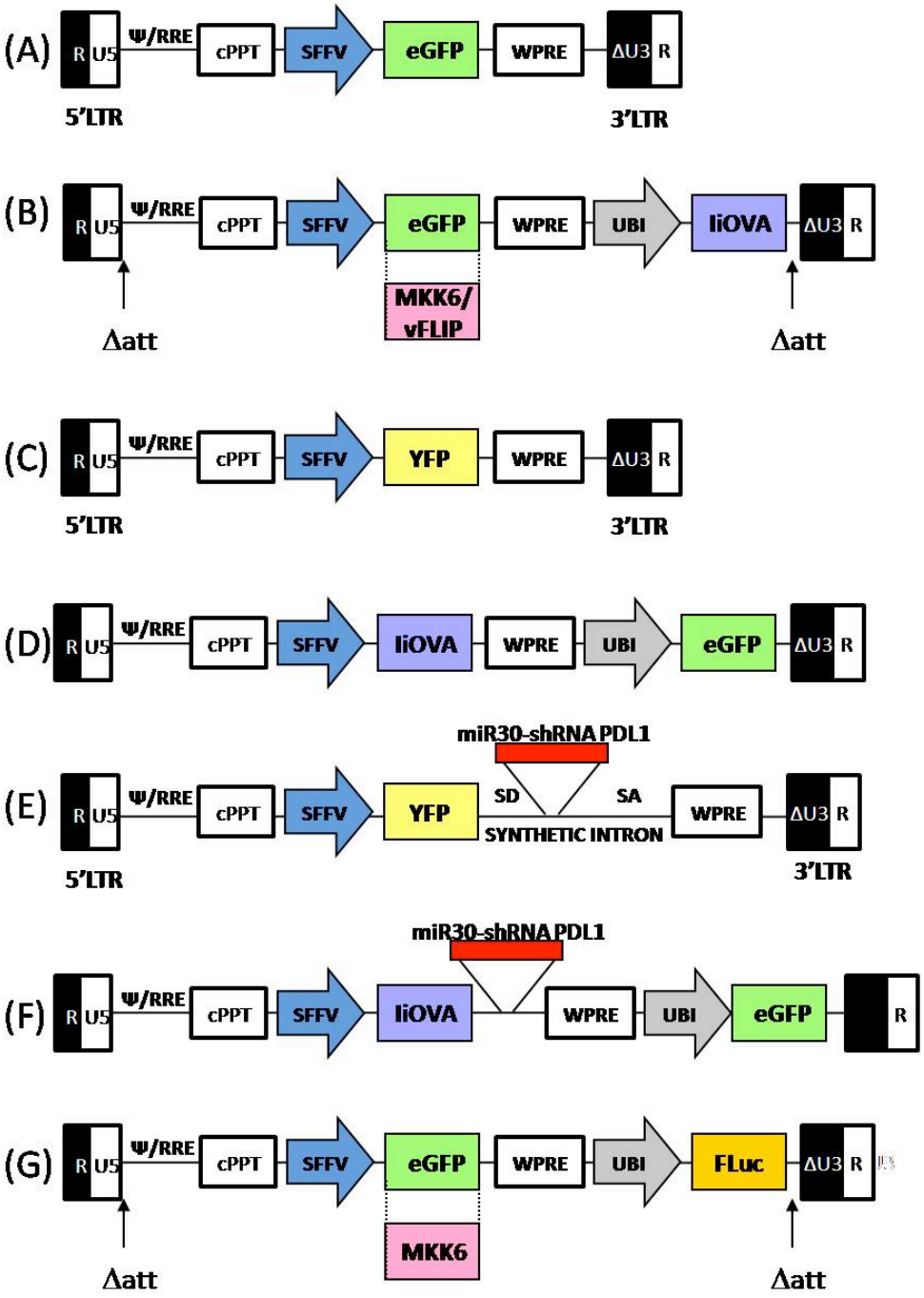

Figure 2.1. Lentivector constructs used in this thesis *(A)lvEGFP (pHRSIN-CSGW), (B)lvEGFP-OVA, lv-MKK6-OVA, lv-vFLIP-OVA, (C)lvYFP, (D)lvOVA, (E) lvYFPshRNA PDL1, (F) lvOVA.P5, (G)lvEGFP-Fluc, lvMKK6-Fluc*

2.3.3. FLOW CYTOMETRY SURFACE STAINING

Stainings were performed on ice in the dark using ice-cold buffers. Antibodies used for staining are listed in table 2.7. When staining dendritic cells, they were additionally blocked beforehand with anti-FcR and 10% mouse serum (Serotec) in HBSS for 20 minutes.

Stainings were performed in V-shaped 96 well plates with 1-2 × 10^6 cells/well. Antibodies were diluted to the correct concentration in staining buffer (HBSS, 2%FCS, 0.1% sodium azide). Samples were then incubated for 30 minutes and washed twice. If a biotin-conjugated antibody was used, samples were additionally labelled with streptavidin-conjugated fluorochromes for 20 minutes. FACS Calibur was used to perform fluorescence activated flow cytometry (FACS) and the results were analysed using Cell Quest (BD) or FlowJo 7.5 (Tree Star Inc., Ashland, OR) software.

2.3.4. INTRACELLULAR STAINING

Following surface staining, samples were fixed using Cytoperm/Cytofix solution (BD biosciences) for 20 mins, washed twice with 1xBD Perm/wash buffer. Antibodies diluted in 1x Perm/wash buffer were added and left for 30 mins. Cells were again washed two times in 1xbuffer and resuspended in staining buffer until FACS was performed.

Table 2.8. Antibodies used for FACS staining

Antibody	**Clone**	**Company**	**Dilution**
AnnexinV			5µl/sample
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
CD8-PE	53-6.7	eBioscience	1:200
CD11c-FITC	HL3	BD Pharmingen	1:400
CD11c-APC	HL3	BD Pharmingen	1:400
CD11c-FITC	HL3	BD Pharmingen	1:400
CD40-biotin	1C10	eBioscience	1:100
CD80-biotin	16-10A1	eBioscience	1:100
PDL2-biotin		eBioscience	1:100
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
Hamster IgG-biotin	G235-2356	BD Pharmingen	1:200
Streptavidin-FITC		DakoCytomation	1:500
Streptavidin-PE		eBioscience	1:500
Streptavidin-APC		eBioscience	1:500
Streptavidin-PeCy7		eBioscience	1:500

2.4. *IN VIVO* AND *EX VIVO* EXPERIMENTS

2.4.1. MICE

C57/BL6 mice were purchased from Charles River, maintained at UCL Biological Services facilities and used at 7-8 weeks of age. OT-1, OT-2 and Thy1.1 mice were bred at the same facilities. Local ethical approval was obtained for all animal experiments. Experimental guidelines from UCL and UK Home Office were followed.

2.4.2. IMMUNIZATIONS (DIRECT AND WITH DENDRITIC CELLS)

For direct lentivector (LV) immunization, mice were subcutaneously injected with lentivectors using doses indicated in the Results chapters. Mice serving as negative controls were injected with pHIVSIN-CSGW (EGFP-expressing virus). For mice vaccinated with dendritic cells, 1×10^6 bone marrow derived DCs were transduced with LV at an MOI of 20, and two days later cells were injected subcutaneously. All experiments were repeated at least three times.

2.4.3. HARVESTING SPLEENS AND LYMPH NODES

Spleens and lymph nodes were collected in HBSS and mashed through 70µm nylon mesh (BD Falcon). Cells were washed and spleens were subject to treatment with Red Blood Cell lysing buffer (Sigma-Aldrich) for 5 minutes at room temperature. Next, both spleens and lymph nodes were washed again three times in HBSS-2% FCS.

2.4.4. RNA ISOLATION

RNA was isolated using TRI reagent (Ambion) according to manufacturer's instruction. Briefly, isolated lymph nodes were homogenized in TRI reagent solution. Chloroform was added to the homogenate and samples were incubated at room temperature. After a spin, the aqueous phase was transferred to a fresh tube and mixed with isopropanol. Following RT incubation and centrifugation, precipitated RNA was washed with 75% ethanol and air-dried.

2.4.5. PENTAMER STAINING

Isolated lymph node cells (1-2 × 10^6 cells/sample) were stained first with MHC pentamer H2-Kb SIINFEKL (Proimmune, Oxford, UK) according to manufacturer's instructions for 10 minutes in room temperature. Next, they were washed and stained with APC-conjugated anti-CD8 antibody and finally resuspended in running buffer for FACS.

2.4.6. IFN-γ ENZYME-LINKED IMMUNOSORBENT SPOT (ELISPOT) ASSAY

ELISpot plates (Millipore, Billerica, MA) were covered with 15μg/ml of anti-IFNγ (BD Pharmingen, San Diego, CA) and left overnight at 4°C. The following day, the plate was washed with HBSS and blocked for 2 hours with RPMI medium containing 10% FCS, 2mM L-glutamine, 100U/mL penicillin and 100μg/ml streptomycin. Splenocytes prepared as described in 2.4.3. were resuspended in RPMI medium at different concentration (1×10^6, 5×10^5 and 2.5×10^5), allocated in an ELISpot plate and incubated for a minimum of 19 hours at 37°C. Medium alone was used as a background control, while other samples were were re-stimulated with OVA class I peptide SIINFEKL (Peprotech, Rocky Hill, NJ) at a final concentration of 50ng/ml. The following day the plate was washed with HBSS, the remaining cells were lysed for 5 minutes with water, plate was washed again and incubated for two hours with biotinylated anti-IFN-γ antibody (BD Pharmingen). IFN-γ spots were counted after using streptavidin-conjugated alkaline phosphatase (Caltag, Burlington, CA) at a 1:10000 dilution and Alkaline Phosphatase Conjugate Substrate Kit (BioRad, Hercules, CA). AID ELISpot counter was used to count the spots.

2.4.7. TRANSGENE DETECTION *IN VIVO*

24 and 48 hours after subcutaneous injection, TRI reagent (Ambion) was used to isolate total RNA from local draining lymph nodes. First-strand cDNA synthesis kit (NEB) and poly(A)-specific primers were used to generate first-strand cDNA. HotStart Taq polymerase (Qiagen) was used to PCR amplify OVA with specific primers(forward:CCTATCTTCTGGCCTGGGAGTG; reverse:TCACAGGGTGGCAGCATCCAC).

2.4.8. CFSE STAINING AND OT-1 CELLS TRANSFER

Splenocytes were isolated from OT1 transgenic mice, and re-suspended in HBSS with 0.1% bovine albumin at a concentration of 1-2 × 10^7. Cells were stained with 5μM carboxyfluorescin succinimydil ester (CFSE, Invitrogen, Carlsbad, CA) for 10 minutes at 37°C. The dye was quenched with ice-cold medium and cells were washed several times. After the last wash splenocytes were re-suspended and 10-20 × 10^6 cells were injected intravenously into vaccinated and control mice (C57/BL6 or Thy1.1 congenic mice). Five to seven days later splenocytes or lymph nodes were isolated and stained for Vα2.1. (Caltag) and Vβ5.1, 5.2(BD Pharmingen) chains of the OT-1 TCR receptor. Cells were run on FACS and proliferation was quantified after gating on the TCR chains.

2.4.9. TUMORAL EXPERIMENTS

For tumor protection experiments mice were injected subcutaneously on day 0 with 2×10^6 EG7.OVA cells in one of the flanks. Mice were immunized with lentivectors on days 3 and 10. Tumoral growth started to be visible on day 5 and from then on the tumour area was measured every 1 to 2 days until the mice had to be sacrificed due to the size of the tumour. The size of the tumour was calculated by multiplying width and length.

2.4.10. SIRNA KNOCKDOWN

Lentivector mediated shRNA-delivery system was constructed through introduction of a μRNA mIR30 sequence into synthetic chimeric intron IVS derived from the plasmid HygEGFP (Clontech) and has already been described (Escors, Lopes et al. 2008). PDL1 shRNAs were selected using the shRNA design tool at ¨http://hannolab.cshl.edu/GH_shRNA.html¨. Selected sequences were cloned into the mIR30 sequence within the synthetic intron and the whole cassette was inserted downstream of the SFFV promoted.

2.4.11. *IN VITRO* PROLIFERATION ASSAY

To assess the influence of PDL1 knockdown in dendritic cells on proliferation of T cells, 0.1×10^6 dendritic cells were transduced with vectors encoding OVA, OVA.P5 or EGFP as a control, CFSE-stained CD3+ cells from OT1 mice were added to the culture at a ratio of DC:T cells of 1:10. DC-T cells cultures were grown in RPMI containing 10% FCS, 2mM L-glutamine, 100U/mL penicillin and 100ug/ml streptomycin and supplemented with 10 units/ml of mouse IL-2. At specified time points cells were stained for Vα2.1 (Caltag) and Vβ5.1, 5.2 (BD Pharmingen) chains of the OT1 TCR receptor and analysed by FACS for CFSE dilution.

2.4.12. *IN VITRO* APOPTOSIS ASSAY

DC-T cell cultures were prepared as for *in vitro* proliferation assay but without staining with CFSE. At specified time points, cells were collected and labelled with Annexin V (eBioscience) according to manufacturer´s instructions. Namely, cells were washed with HBSS and resuspended 1x Binding Buffer. 5µl of Annexin V – APC was added to the cells and incubated at room temperature for 10 to 15 minutes. Just before cells were analysed on FACS, propidium iodide (PI) was added to cell solution at a concentration of 20µg/ml.

2.4.13. *IN VIVO* ANALYSIS OF TCR DOWNMODULATION

BMDCs were transduced with lvOVA or lvOVA.P5 lentivectors. LvEGFP was used as a negative control. Two days later they were injected subcutaneously into C57/BL6 mice. Seven days later splenocytes purified from OT-1 mice were transferred intravenously into vaccinated mice. Analysis was performed 1 week later. Spleens from vaccinated mice were purified and stained with Vα2.1 and Vβ5.1, 5.2. antibodies and analyzed by FACS.

2.4.14. T CELL (CD3+) PURIFICATION

CD3+ cells were purified using negative selection Mouse T Cell Enrichment Columns (R&D systems). Splenocytes were depleted of erythrocytes, washed several times and re-suspended in 1x column wash buffer. Cell suspension was applied to the top of a column washed with 1x column wash buffer, incubated at room temperature for 10 minutes and eluted (T cells) from the column with 1x column wash buffer. Cells in column wash buffer were centrifuged at 250g for 5 minutes and either stained with CFSE for proliferation experiments or directly added to dendritic cell cultures for apoptosis assay.

2.4.15. *IN VIVO* BIOLUMINESCENCE IMAGING

BMDCs were transduced with vectors expressing firefly luciferase (Fluc) and injected subcutaneously into $Trp2^{-/-}$ BL6 mice. Bioluminescence signal was quantified using IVIS imaging system upon intraperitoneal injection of luciferin-D.

2.4.16. STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 5.0. Means were compared using T-student or Mann-Whitney tests, as indicated in the experiments. Log-rank test was used to analyze differences in survival plots.

CHAPTER 3: INTEGRATION DEFICIENT LENTIVIRAL VECTORS FOR IMMUNIZATION AS A SAFER ALTERNATIVE FOR STANDARD VECTORS

3.1. INTRODUCTION

3.1.1. LENTIVIRAL INTEGRATION INTO THE GENOME CARRIES A RISK OF INDUCING INSERTIONAL MUTAGENESIS

Despite the fact that lentiviral vectors posess several advantages over other vectors such as no expression of viral proteins, large coding capacity, transduction of both dividing and non-dividing cells and stable integration into the human genome upon transduction, the latter poses a risk of inducing insertional mutagenesis. This problem has been highlighted by two separate clinical trials for SCID-X1 using γ-retroviral vectors (Hacein-Bey-Abina, Von Kalle et al. 2003; Hacein-Bey-Abina, Garrigue et al. 2008; Howe, Mansour et al. 2008).

In one of the trials 4 out of 9 patients treated to restore IL2 receptor γ developed T cell leukaemia after the therapy. Patients' blast cells contained activating vector insertions near the LMO2, BMI1 or CCND2 proto-oncogens as well as other genetic abnormalities like chromosomal translocation, gain-of-function mutations in NOTCH1 and deletion of tumour-suppressor CDKN2A (Hacein-Bey-Abina, Garrigue et al. 2008).

Also another independently performed gene therapy trial reported clonal T cell acute lymphoblastic leukaemia (T-ALL) caused by insertional mutagenesis and acquired somatic mutations in patients (Howe, Mansour et al. 2008).

The risk associated with using those vectors could be even higher if replication-competent recombinants were generated (RCRs) or upon vector mobilization after wild-type virus superinfection. These 2 mechanisms would lead to uncontrolled spread of the vector and a potential rise in the number of integration events. Lentivectors are engineered to have these risks reduced. Targeted elimination of promoter sequences

from 3'LTR U3 region makes the expression from a vector dependent on the presence of an internal promoter. These vectors are termed SIN (self-inactivating).

It is believed that lentivector mediated transfer is safer than the one achieved by γ-retroviral vectors (Montini, Cesana et al. 2006; Felice, Cattoglio et al. 2009; Montini, Cesana et al. 2009). However, a recent study reported clonal dominance after using a β-globin lentiviral vector (Kaiser 2009). Therefore, other alternatives, like using non-integration lentiviral vectors (NILVs) should be explored.

3.1.2. RETROVIRAL INTEGRATION IS CARRIED OUT BY INTEGRASE

The lentiviral preintegration complex (PIC) - consisting of integrase, central DNA flap, vpr and matrix – is responsible for crossing of the nuclear membrane in non-dividing cells. Once the PIC and the host chromosomal DNA associate, integrase catalyzes insertion of viral DNA into host sequences.

Integrase consists of 3 functional domains: the N-terminal domain, catalytic core domain and the C-terminal domain (figure 3.1.A). The N-terminal domain contains HHCC type putative zinc finger believed to be important for the catalytic function of integrase as mutations in any of the residues of the domain result in a complete or nearly complete abolishment of integration and reduction in the level of zinc binding. The catalytic core domain contains 3 highly conserved residues called the DD-35-E motif (in the case of HIV: D64, D116 and E152). Most mutations of these residues results in generation of an inactive enzyme. The C-terminal domain is the least conserved of the 3 domains and binds non-specifically to host chromosomal DNA. In several retroviruses this domain contains nuclear localization signals used for PIC entry into nucleus (Follenzi, Ailles et al. 2000).

Integration consists of two steps termed "3'-end processing" and " DNA strand transfer". The first step begins with nucleophilic attack of water molecules on 2 nucleotides localized in viral LTRs to the 3' side of highly conserved CA dinucleotide (figure 3b). Their removal exposes terminal 3'-hydroxyl groups. In the second step, processed LTRs and target DNA are brought together. This is followed by nucleophilic

attack of 3'-hydroxyl groups on the exposed 3'-end strands which results in insertion of viral DNA into target DNA, leaving 2 bases-long overhangs on the 5' ends (figure 3c). The process is completed by removal of overhangs, gap and nick repair (Craigie 2001).

3.1.3 RETROVIRAL EPISOMES

Accumulation of high levels of unintegrated viral DNA is a natural feature of retroviruses. Unintegrated DNA is present mainly as 1-LTR or 2-LTR episomal circles, remains stable in non-dividing cells and undergoes dilution with each division of the infected cell. Human macrophages are a natural non-dividing target of HIV-1. It has been demonstrated that 2-LTR circles persist in these cells for up to 21 days after infection (Gillim-Ross, Cara et al. 2005).

Non-integrated viral DNA can be produced in cells in at least 5 different ways: NHEJ non-homologous end joining, homologous recombination through strand invasion, homologous recombination through single-strand annealing, closure of intermediate products of reverse transcription and autointegration (Shoemaker, Goff et al. 1980). In terms of vector production such non-integrated forms can be achieved by generating mutants deficient in integration. These forms are superior to plasmid DNA for vaccination as they have a higher efficiency of transduction and do not contain any bacterial sequences.

One way to prevent integration in a lentivector is through introduction of mutations in the *gag/pol* which results in a mutant integrase. Integrase cannot be completely deleted as it is involved in a number of different processes besides integration, e.g.: virion morphogenesis, reverse transcription, levels of peptide-associated integrase, PIC nuclear translocation, viral DNA synthesis (Wanisch and Yanez-Munoz 2009). For the same reason 2 classes of mutations are possible to engineer: class I mutations – which are specific for integration and result in normal levels of viral DNA and class II mutations - which affect several other functions of integrase (pleiotropic). A number of mutations and their outcomes have been described. Table 3.1 provides the summary of identified class I mutations.

Figure 3.1.

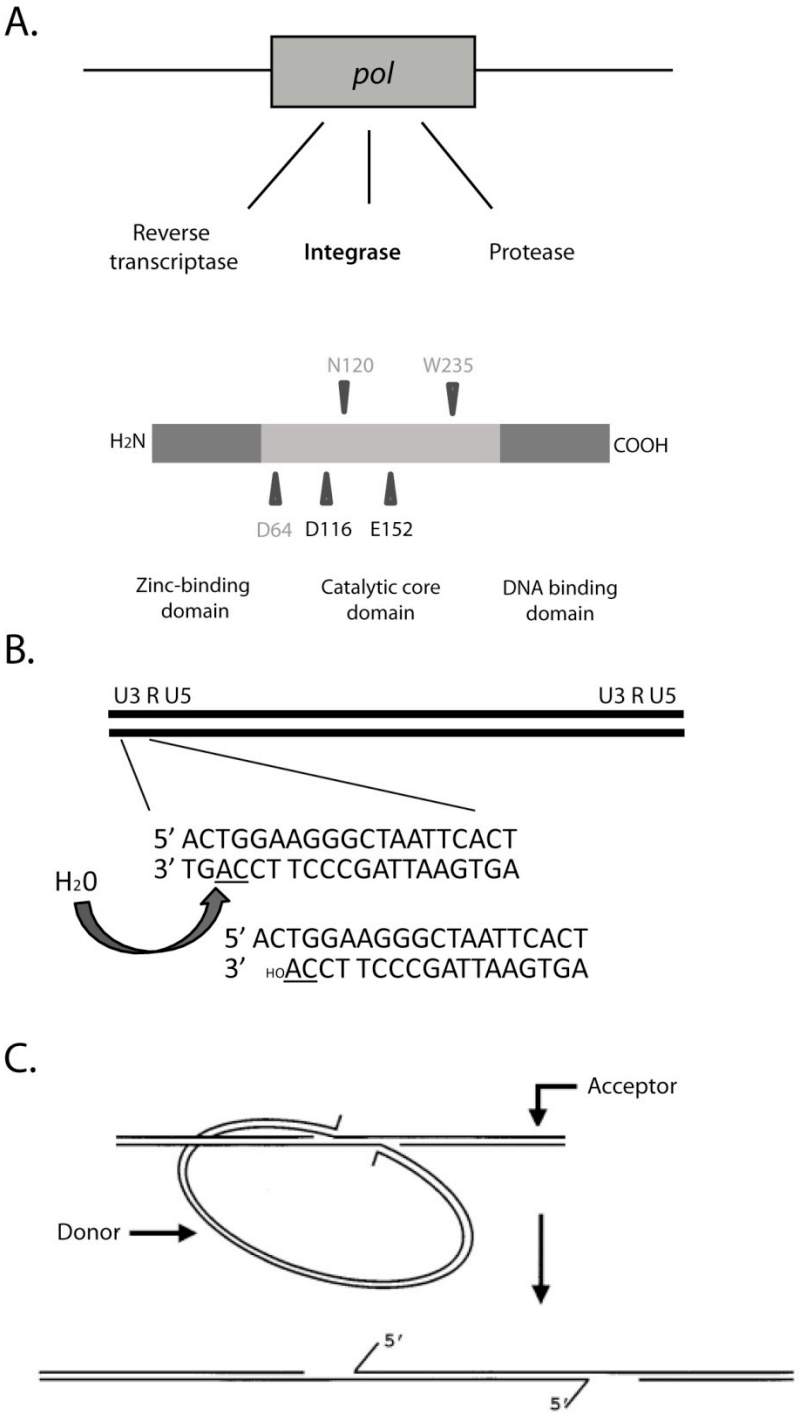
A.
pol
Reverse transcriptase
Integrase
Protease
N120
W235
H2N
COOH
D64
D116
E152
Zinc-binding domain
Catalytic core domain
DNA binding domain
B.
U3 R U5
U3 R U5
5' ACTGGAAGGGCTAATTCACT
3' TGACCT TCCCGATTAAGTGA
H20
5' ACTGGAAGGGCTAATTCACT
3' HOACCT TCCCGATTAAGTGA
C.
Acceptor
Donor
5'
5'

Figure 3.1. HIV-1 integration

(A) Schematic diagram of integrase. Integrase is encoded on the pol gene along with reverse transcriptase and protease. 3 domains of integrase are shown: N-terminal zinc binding domain, catalytic core domain and C-terminal DNA binding domain. Invariant residues termed DD-35-E motif are marked on the catalytic core domain. Residues marked in grey were used to generate mutant integrase. (B) Representation of the first step of integration: 3'-end processing. Viral DNA and 20 base pairs of the terminal HIV-1 U3 LTR are shown. In this step nucleophilic attack of water molecules on 2 nucleotides localized in viral LTRs to the 3' side of highly conserved CA dinucleotide causes loss of these nucleotides and exposure of terminal 3'-hydroxyl groups. (C) Representation of the second step of integration: DNA strand transfer. Processed LTRs and target DNA are brought together, followed by nucleophilic attack of 3'-hydroxyl groups on the exposed 3'-end strands. The result is insertion of viral DNA into target DNA, leaving 2 bases-long overhangs on the 5' ends. Removal of overhangs, gap and nick repair complete the reaction.

Fig.3.1. B and C reproduced from (Patrick Hindmarsh 1999)

Table 3.1. Class I mutations in retroviral integrase

Residue	Function
Q148	critical for interaction with the att sites (Esposito 1998; Gerton 1998) and strand transfer (Johnson 2006)
K156, K159	prevents 3' processing and strand transfer (Jenkins 1997)
W235E	blocks integrase binding to chromosomal DNA (Cannon and A.J. 1996; Leavitt 1996)
N120K	prevents binding to the target DNA (Lu 2005)
D64, D116, E152	specifically inhibit integration of viral DNA into the host genome (Leavitt 1993; Ansari-Lari 1995; Wiskerchen 1995; Leavitt 1996)
K264, K266, K273	Strand transfer requires acetylation of lysines in the C-terminal domain of integrase and mutating these amino acids to arginines (K264, K266, and K273) inhibits strand transfer and therefore integration (Cereseto 2005)

Inhibition of integration can also be achieved through mutating attachment sites (att) at the viral genome termini – *cis*-acting elements essential for proviral integration. Attachment sites in retroviruses contain a highly conserved CA dinucleotide. 7-13 base pairs around this motif are required for efficient integration (Masuda, Kuroda et al. 1998). Alterations in the CA motif have a significantly negative impact on several functions of integrase: 3'- end processing, strand transfer and disintegration. Mutations introduced at both LTRs block HIV integration (Masuda et al., 1995, Brown et al., 1999). They could also serve another function. As active IN is required for HIV-1 based replication, mutations in the *gag/pol* rendering lentivectors integration-deficient won't support replication. However, the risk of vector mobilization still exists. Attachment site mutants would reduce the risk of integration of vector mobilized by wild type virus. For this reason in a clinical trial it would be advantageous to use vectors with combination of mutations in *gag/pol* and att sites (Apolonia, Waddington et al. 2007).

The first studies analyzing transgene expression from non-integrating vectors were carried out in non-SIN vectors. These showed a much lower transduction efficiency of various cell lines and gene expression *in vivo* when compared to integrating vectors (Naldini, Blomer et al. 1996; Haas, Case et al. 2000; Vargas, Gusella et al. 2004). However, in the case of SIN-HIV vectors, introduction of a longer deletion in the vector's U3 region was shown to improve expression levels from integration deficient vectors (Bayer, Kantor et al. 2008). Transduction from these vectors in dividing cells is efficient but transient. However, in non-dividing cells like growth arrested C2C12 (Apolonia, Waddington et al. 2007) and non-dividing neural primary cultures *in vitro* (Philippe, Sarkis et al. 2006) it is not only efficient but also stable. Also *in vivo* long term eGFP expression (up to 9 months) and therapeutic efficiency in 2 rodent models of retinal degradation were demonstrated after administration of integration-deficient lentivectors (Yanez-Munoz, Balaggan et al. 2006). This study was further confirmed by another group showing long-term eGFP expression in the brain for up to 4 weeks after injection (Philippe, Sarkis et al. 2006) and long-term eGFP expression in the muscle for at least 8 months (Apolonia, Waddington et al. 2007). Also stable transgene expression in the liver following injection with non-integrating vectors has been demonstrated for up to 6 months (Bayer, Kantor et al. 2008). Finally, specific immune responses against HIV-1 gp120 were demonstrated following vaccination with a NILV expressing a codon-optimized HIV-1 gp120 along with mGM-CSF (Negri, Michelini et al. 2007).

3.1.4. DC ACTIVATION STATUS INFLUENCES THE OUTCOME OF IMMUNIZATION

Dendritic cells are the most potent initiators of immune responses, responsible for both innate and adaptive immunity. Immature DCs circulate in the peripheral tissues sampling antigens. They are characterized by high phagocytic activity yet poor ability to present antigens. However, upon encountering pathogens dendritic cells undergo maturation, which results in upregulation of MHC molecules, upregulation of several co-stimulatory molecules like CD80, CD86, CD40 and ICAM-I, upregulation of chemokine receptors like CCR7 and upregulation of secreted cytokines like IL12 or IL10 crucial for generation of Th1 and Th2 responses effectively. Mature DCs guided by

chemokine receptors migrate to regional draining lymph nodes and present the processed antigen to T cells. Depending on the state of a dendritic cell, antigen presentation can result in tolerance – if the antigen is presented by an immature DC, or an immune response – if the antigen is presented by a mature DC.

Several studies on signaling pathways following pathogen recognition point to 2 pathways that are especially important for DC activation and secretion of IL12 – p38 MAPK and NFκB pathways (Escors, Lopes et al. 2008)

P38 MAP kinase is activated in response to cellular stress and inflammation. MKK3 deficient mice (MKK3 being a specific p38 MAPK activator required for full p38 activation) exhibited impaired type 1 cytokine responses highlighted by greatly reduced production of IFN-γ after immunization with peptide antigen and impaired *in vitro* differentiation of naive T cells (Lu, Yang et al. 1999). Strikingly, macrophages isolated from these mice stimulated with LPS and DCs activated through CD40-CD40L interactions produced almost no IL12. As IL12 induces IFN-γ production, this explains inhibition of Th1 CD4+ responses. This evidence suggests that p38 MAPK kinase is required by both APCs and CD4+ T cells for efficient cytokine production. The effect of specific inhibition of p38 MAPK on LPS- or TNF- triggered maturation of human DCs was also analyzed (Arrighi, Rebsamen et al. 2001). Phenotypic changes normally induced by these stimulants – upregulation of CD1a, CD40, CD80, CD86, CD83, HLA-DR - were greatly affected. Also, upregulation of CD40 was blocked at higher concentrations of p38 inhibitor. Overall, allostimulatory capacity of DCs as well as DC maturation induced by contact sensitizers were reduced. Thus, yet again it was concluded that p38 MAPK is critical for maturation of immature DCs. Finally, expressing a constitutive p38 activator (MKK6EE mutant) on a lentivector along with an antigen led to stimulated DC maturation and increase in response to immunization not only with a model antigen OVA, but also with a clinically relevant antigen NY-ESO. This resulted in increased survival in mouse lymphoma model (Escors, Lopes et al. 2008).

The role of NFκB pathway in effective antigen presentation has been thoroughly investigated. DC mediated antigen presentation is NFκB – dependant as adenoviral transfer of IκBα – an endogenous inhibitor of NFκB - results in

downregulation of HLA class II and co-stimulatory molecules: CD80, CD86 and CD40, as well as greatly reduced production of immunostimulatory cytokines IL12 and TNF-α. NFκB is also believed to influence the duration of T cell – DC contact as transfer of IκBα resulted in upregulation of adhesion molecules on the surface of a DC (Yoshimura, Bondeson et al. 2001).

Blocking NFκB results in induction of T cell anergy/tolerance, inhibition of production of Th1 cytokines (IL2 and IFN-γ) *in vitro* (Yoshimura, Bondeson et al. 2001) and inhibition of DC antigen presentation *in vivo* (Yoshimura, Bondeson et al. 2003). As expected, *in vitro* NFκB activation results in upregulation of a number of cytokines (TNF-α, IL-6¸ IL-12, IL-15, and IL-18), chemokines, MHC class I and II molecules, and costimulatory molecules CD80 and CD86 thus resulting in increase in DC antigen presentation (Andreakos, Williams et al. 2006). *In vivo* enhancement of immune responses against a vector-encoded antigen with a shift toward Th1 response, increased IgG2a levels, T cell proliferation and IFN-γ production was observed upon NFκB activation. vFLIP from Kaposi's sarcoma- associated herpesvirus (KSHV) was found to be a constitutive activator of NFκB and expressing it from a lentivector led to enhanced maturation of mouse bone marrow-derived DCs (BMDCs) *in vitro* and improved immune responses to OVA upon immunization with a lentivectors encoding OVA *in vivo* (Rowe, Lopes et al. 2008).

Thus, both p38 MAPK and NFκB play a significant role in DC maturation and targeting those pathways can improve vaccine efficacy.

3.1.5. THE EFFECT OF ANTIGEN PERSISTENCE ON THE OUTCOME OF VACCINATION

When an antigen-loaded dendritic cells encounters a cognate T cell, several parameters are used to decide on the outcome of this encounter. T cells can either become activated and later progress to being memory T cells, or on the contrary – they may become tolerogenic. The activation status of antigen presenting cells (APC), presence of cytokines, antigen distribution, the amount and finally duration of antigen presentation all have an influence on the fate of a T cell (Lanzavecchia and Sallusto 2000)**.** The most important limitations imposed on the time of antigen presentation is

the stability of peptide-MHC class I complexes and the life span of an APC. The average half life of peptide-MHC class I complexes is only 10 hours, while a naive T cell requires at least 20 hours of stimulation in order to become committed to proliferate. APC life span is determined by signals from pathogens and T cells (Hou and Van Parijs 2004). In the case of DCs, Toll-like receptors (TLRs) are stimulated by pathogen derived molecules that serve as ligands for TLRs, directing the cells towards an apoptotic pathway that can be blocked by Bcl-2 (Hou and Van Parijs 2004). Thus, Blc-2 acts a molecular timer for antigen-bearing DCs. TLR-independent signalling via CD14 was found to be necessary for induction of apoptosis in terminally differentiated DCs. T cells' influence on an APC life span comes from signals sent by cytokines, co-stimulatory molecules like CD40L and the interplay between FasL and TRANCE (Tumor necrosis factor – related activation - induced cytokine) (with Fas-FasL interactions being pro-apoptotic) (Chen, Xu et al. 2004) which induce a Bcl-XL dependant survival pathway (Hou and Van Parijs 2004). Also the presence of naive T cells has been shown to help promote DC survival (Chen and Wang 2010). It is very likely that activated T cells are capable of killing antigen-bearing DCs. It has been demonstrated that CD8+ T cells kill DCs during anti-tumour responses and that $CD62L^-$ $CCR7^-$ effector memory T cells kill antigen-loaded DCs in the lymph nodes. This killing of DCs by activated T cells can be mediated in Fas- or perforin-dependant manner (Chen and Wang 2010) as well as by release of cytotoxic granules (Yang, Millar et al. 2006).

The timing of the duration on antigen persistence is crucial for immune responses. If it is too short – it will result in poor responses, while a defect in DC apoptosis has been shown to lead to DC accumulation causing chronic lymphocyte activation and autoimmunity (Chen, Wang et al. 2006).

It has been implicated that prolonged antigen presence after vaccination leads to induction of potent immune responses (Kelleher, Puls et al. 2006; Tatsis, Fitzgerald et al. 2007). Continuously expressed small amounts of antigen were thought to maintain long-lived CD8+ and memory T cell responses. Persistent antigen presentation has been extensively studied in models of infectious diseases. In chronic lymphoytic choriomeningatis infection (LCMV), the persistently presented antigen

continually recruits new naïve antigen-specific CD8+T cells (Vezys, Masopust et al. 2006).

Likewise, during persistent murine cytomegalovirus (MCMV) infection, memory cells compartment is maintained by a continuous replacement of functional cells due to persistent antigen presentation (Snyder, Cho et al. 2008). Cockburn et al. showed that the complete development of optimal CTL responses against malaria liver stage parasites require prolonged antigen presentation. Reducing the time of primed CD8+ T cells exposure to antigen *in vivo* diminished the size of the developing memory population. Transfer of developed memory cells to naïve animals did not result in expansion of those cells, yet transfer to previously immunized animals did. Last but not least, the continually persisting antigen was able to activate naïve T cells which differentiated to functional effector cells (Cockburn, Chen et al. 2010).

Some studies show that persistent antigen presentation would rather lead to T cell death than immunity (Zajac, Blattman et al. 1998; Bucks, Norton et al. 2009; Mueller and Ahmed 2009). It was pointed out that chronic exposure to antigen after vaccination results in T cell phenotype typical for partial exhaustion. Nevertheless, even those cells kept robust protective immunity upon an *in vivo* challenge (Yang, Millar et al. 2006). Despite the fact that upon DNA vaccination persistent antigen expression results in higher number of specific CD8+ T cells, a short antigen burst induces significantly better expansion of those cells (Radcliffe, Roddick et al. 2007). This suggests that long-term antigen exposure can interfere with generation of secondary responses (Hovav, Panas et al. 2007; Radcliffe, Roddick et al. 2007).

Antigen persistence could also result in exhaustion. During chronic LCMV infections T cell responses become dysfunctional through losing the ability to produce IL-2, TNF-alpha and IFN-gamma, finally becoming exhausted. Muller et al found that sustained antigen presentation directly drives T cell exhaustion during a chronic viral infection (Mueller and Ahmed 2009).

Studies have also demonstrated that the maintenance or survival of CTL during a chronic viral infection requires antigen and extensive cell division, in contrast to normal memory cell maintenance, which is antigen independent (Mueller and Ahmed 2009).

The duration of antigen presentation can also affect helper T cells. Contrary to what is observed in CD8+ T cells, CD4+ T cells are dependent on the presence of antigen throughout their expansion phase, even in the presence of an inflammatory stimulus (Obst, van Santen et al. 2005).

Last but not least, prolonged antigen presentation can elicit full expansion and cytokine production in CD4 T helper cells even in the absence of DC activation (Obst, van Santen et al. 2007).

3.1.6. AIMS OF THE CHAPTER

The study presented in this chapter aims to define the efficacy of integration-deficient lentiviral vectors as tools to induce specific immune responses. Additionally, activators of p38 MAPK and NFκB pathways will be tested in the context of NILVs. Vectors will be compared to their integrating counterparts in terms of cellular responses, persistence of antigen presentation, DC trafficking and will be used in a tumoral model setting.

3.2. RESULTS

3.2.1. INJECTION OF INTEGRATION DEFICIENT LENTIVECTOR RESULTS IN EXPRESSION LEVEL DETECTABLE *IN VIVO*

To confirm that transgene presence *in vivo* from both integrating and integration-deficient lentivectors, we injected mice subcutaneously with 500ng RT of the following vectors: OVA, MKK6/DNW, vFLIP/DNW and an integrating lentivector expressing EGFP as a negative control. 24 and 48 hours later total RNA was extracted from regional draining lymph nodes of injected mice. Next, poly(T) oligos were used to reverse transcribe the encoded antigen and to generate cDNA. Finally, OVA-specific primers were used to amplify a region of the transgene from cDNA. As shown in figure 3.2., all vectors used for injection tested positive for the presence of ovalbumin in the regional lymph node. The negative control yielded no band. Plasmid DNA containing ovalbumin was used as a positive control. This experiment confirmed that transgene form both integrating and non-integrating lentivectors is present in the regional draining lymph nodes already 24 hours after subcutaneous injection.

3.2.2. VACCINATION WITH INTEGRATION DEFICIENT VECTORS GENERATE IMMUNE RESPONSES THAT ARE DOSE DEPENDANT

Even though injection of integration deficient vectors results in detectable transgene expression in various models (Philippe, Sarkis et al. 2006; Yanez-Munoz, Balaggan et al. 2006; Apolonia, Waddington et al. 2007) as well as in our model, it is possible that the expression level is too low to induce potent immune responses to an antigen encoded on the vector. Therefore, the next step was to vaccinate mice with varying doses of both integrating and non-integrating vectors and to compare IFN-γ secretion in an ELISpot assay.

In this experiment seven different vectors were used, each at 3 different doses: OVA, D64V, DNW, 2Δatt, DNW/2Δatt, MKK6/DNW, vFLIP/DNW. In addition, integrating OVA-expressing heat inactivated lentivector was used as a negative control. This was necessary as during vector concentration there is a possibility for co-

precipitation of protein. Moreover, vesicular stomatitis virus G protein (VSV-G) - pseudotyped vectors prepared by transient transfection are known to be contaminated with tubovesicular structures (TVS) carrying plasmid DNA used for transfection (Pichlmair, Diebold et al. 2007). Inactivating the vector at 95°C for 15 minutes will destroy RT activity.

Eleven days after subcutaneous immunization, mice were sacrificed and spleens prepared for ELISpot assay. After an overnight incubation with SIINFEKL – OVA class I peptide, plates were developed and spots were quantified giving a number of IFN – γ secreting cells per 10^6 splenocytes. Splenocytes incubated in medium without peptide were used as control. The number of IFN-γ spots in the control group was in each case close to zero and in the graph representing results (Fig.1.3) substracted from the number of spots in wells stimulated with peptide. Additionally, ELISpot assay on spleens from mice vaccinated with integrating lentivector expressing EGFP only resulted in no spots.

Figure 3.3. shows that vaccination with 7 ng RT of integrating vector produced strong responses in our assay. Vaccination with 10ng RT of the same vector does not change the outcome, from which one can conclude that at a dose of about 7 ng RT the immune response reaches a level of saturation and increasing the dose would most likely not results in an increase in the number of IFN – γ secreting cells.

Four non-integrating vectors in figure 3.3.: D64V, DNW, 2Δatt, DNW/2Δatt – made integration deficient by introduction of several mutations either in the integrase, attachment sites, or both – need to be used at much higher doses that their integrating counterpart to elicit similar response. Immunization with 50 ng RT of each of these vectors gives response similar to the one obtained by immunization with 4 ng RT of the integrating vector. Furthermore, one needs to use as much as 250 ng RT of these vectors to get a response as high as the one generated by 7 or 10 ng RT of the OVA vector.

Unexpectedly, immunization with MKK6/DNW and vFLIP/DNW did not result in a higher number of IFN–γ secreting cells than DNW vector (without dendritic cell activators). This came as a surprise as inclusion of p38 MAPK and NFκB pathways

activators was previously shown to enhance responses to immunization (Escors, Lopes et al. 2008)**.** Last but not least, immunization with the heat inactivated control generated no response indicating that all responses generated by other vectors were due to transgene expression from a lentivector and not a contamination.

Figure 3.2.

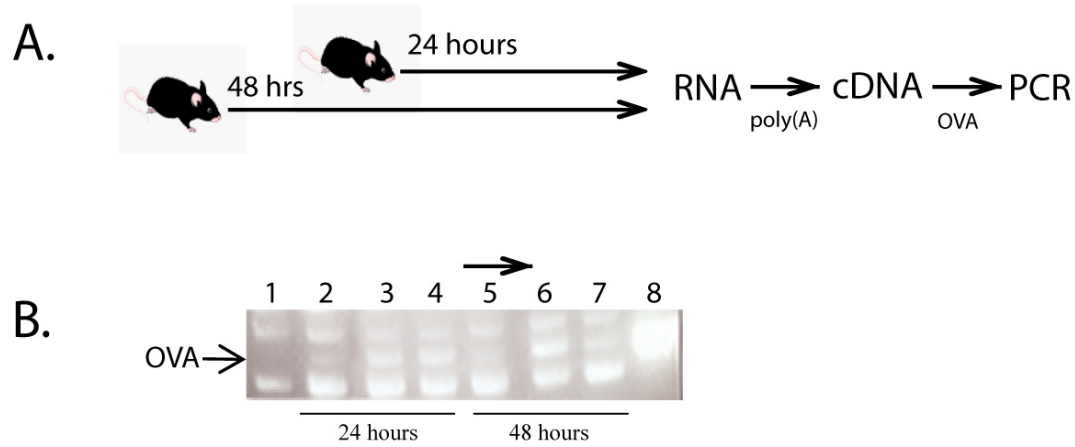

Figure 3.2. Transgene presence in vivo*. C57/BL6 mice were injected subcutaneously with 500 ng RT of the integrating vector expressing IiOVA (lvOVA), 2 nonintegrating vectors (lvMKK6/DNW and lv vFLIP/DNW and an integrating vector expressing EGFP (lvEGFP) as a negative control. 24 or 48 hours after injection local draining lymph nodes were collected and RNA was extracted and reverse-transcribed to cDNA with poly(T) oligos. Generated cDNA was used to specifically amplify 0.6 kb fragment of OVA. 1-lvEGFP (negative control), 2-lvOVA (integrating vector), 3-lvMKK6/DNW, 4-lv vFLIP/DNW, 5-lvOVA (integrating vector), 6-lvMKK6/DNW, 7-lv vFLIP/DNW, 8- plasmid expressing OVA (positive control).*

Figure 3.3.

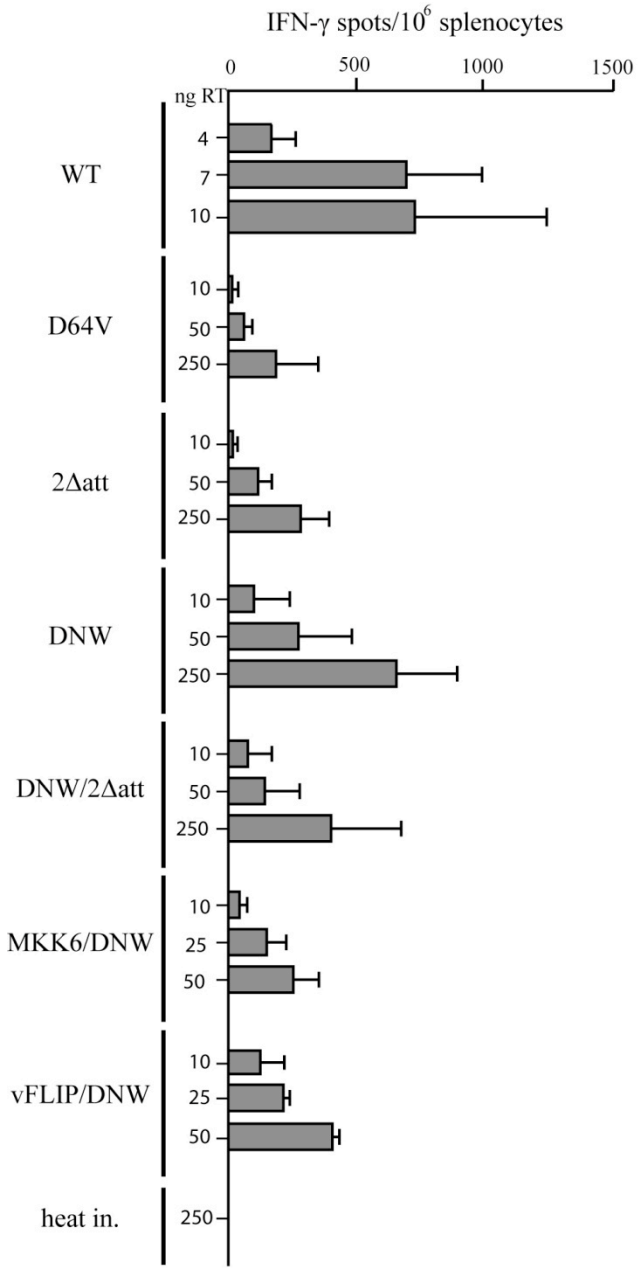

Figure 3.3. IFN-γ ELISpot assay at 11 days post-vaccination. *C57/BL6 mice were injected with 4-250ng RT (3 doses/each group) of the following lentivectors: OVA), D64V, DNW, 2Δatt, DNW/2Δatt, MKK6/DNW, vFLIP/DNW, HI (heat inactivated lvOVA). ELISpot assay was performed 11 days later. Processed spleens were stimulated overnight with 50ng/ml SIINFEKL. Splenocytes stimulated with no peptide were used as control. 3 mice and 3 repetitions were used for each group and dose. The result is shown as IFN-γ spots/10^6 splenocytes.*

3.2.3. IMMUNIZATION WITH NILVS LEADS TO AT LEAST 30 DAYS OF ANTIGEN PRESENTATION

It has been demonstrated that immunization with an integrating lentivector expressing OVA antigen results in prolonged antigen presentation resulting in effective T-cell memory (Arce, Rowe et al. 2009). Therefore, it was interesting to compare persistence of antigen presentation after subcutaneous immunization with integrating and integration-deficient vectors. Three different time points were selected: 30, 21 and 5 days. To check for how long we can detect antigen presentation upon immunization with selected vectors, mice were immunized with 100ng RT of OVA, DNW/2Δatt, MKK6/DNW and vFLIP/DNW vectors and after selected time points CFSE stained splenocytes from OT-1 transgenic mice were transferred into immunized mice. In this way, if the antigen was still presented, transferred cells should recognize the antigen and proliferate which can be visualized by FACS. Transferred cells were left to expand *in vivo* for five days and after this time spleens were extracted and stained for Vα2.1 and Vβ5.1, 5.2 chains of the OT-1 TCR receptor. Additionally, as a control mice were immunized with 100μg OVA protein in Complete Freund's Adjuvant (CFA).

Immunization with all of the used vectors resulted in striking expansion of transferred cells if the transfer was performed 5 days after immunization (figure 3.4.A.). However, also when transfer was done 21 and 30 days after immunization, there was a population of OT-1 cells that underwent division. The percentage of divided cells was quantified by dividing the number of proliferated cells (marked with a bar in figure 3.4.A.) by the total number of CFSE positive cells. This ratio was named proliferation index and it is presented for all the groups in figure 3.4.B**.** It is clear that at the 5 days time point at least 80% of cells proliferated in all the groups. After 21 days there is still 20% of cells that proliferated, and finally, after 30, about 10% of transferred cells proliferated.

This experiment leads to a conclusion that subcutaneous vaccination with integrating as well as integration – deficient vectors results in transduction of antigen presenting cells, transgene processing and finally presentation on an APC. It is not known whether after 30 days the presence and presentation of an antigen is a result of initial APC transduction (as in the case of dendritic cells this is beyond the life span of a

DC), the result of transducing a DC precursors (Arce, Rowe et al. 2009) or whether the antigen is presented by other APCs or non-professional antigen presenting cells.

Figure 3.4.

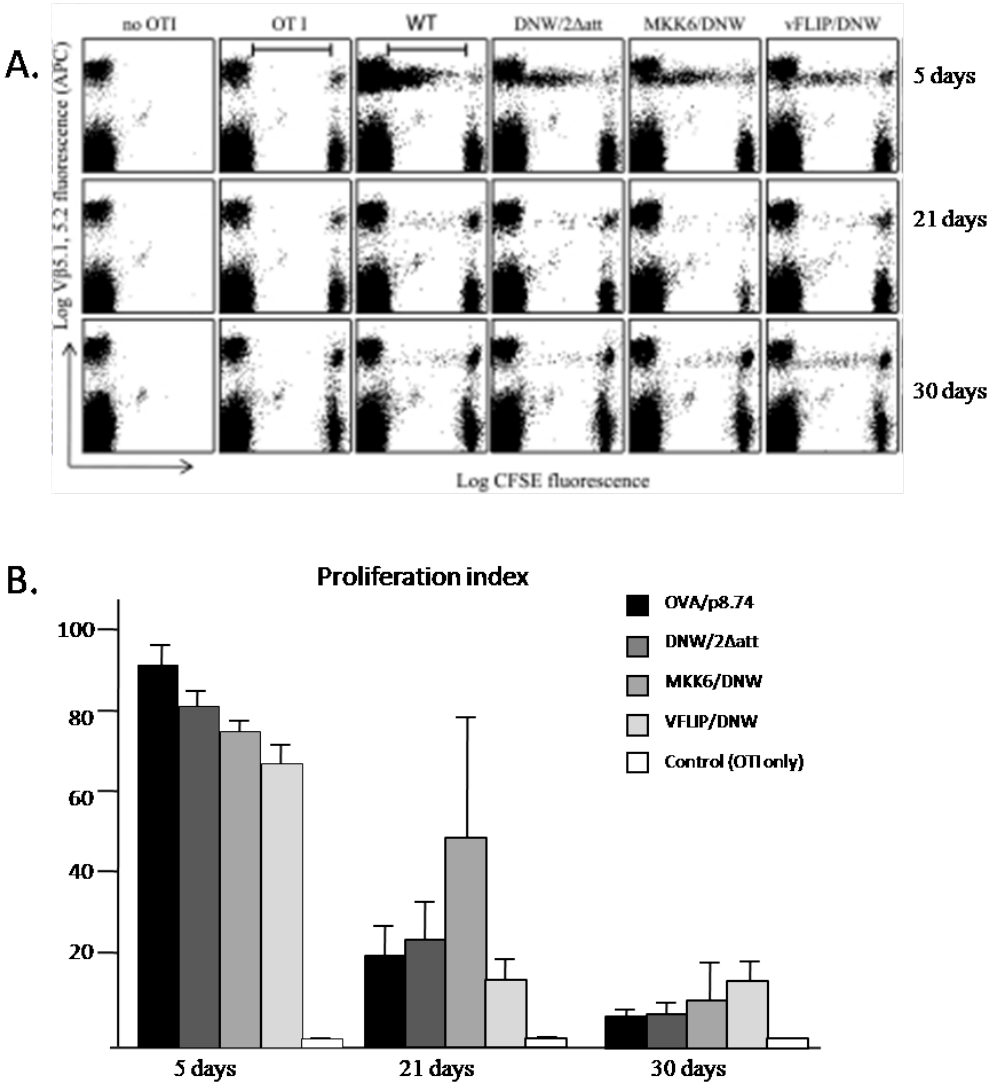

Figure 3.4. Duration of antigen presentation upon vaccination with integration-deficient lentivector. *C57/BL6 mice were injected with 150ng RT of each of the following vectors: OVA, DNW/2Δatt, MKK6/DNW, vFLIP/DNW. 5, 21 or 30 days after vaccination, adoptive transfer of CFSE-stained splenocytes from OT-1 transgenic mice was performed intravenously. Groups with no OT-1 cells transferred, OT-1 cells transferred without previous immunization, and immunization with 100µg OVA peptide in CFA followed by OT-1 transfer were used as controls. 5 days after transfer of OT-1 cells, spleens from immunized mice were collected, processed, stained and analyzed by FACS. (A) Cells are gated on Vα2.1. and Vβ5.1, 5.2 TCR chains. OT-1 proliferation is visualized as a shift on the FL1 axis (CFSE). A complete shift to the left indicates at least*

8 cell divisions. (B) Proliferation index was calculated using the following formula: (no. of proliferated cells)/)no. of total CFSE-stained cells), proliferated cells are indicated in (A) with a bar.

3.2.4. TUMORAL THERAPY

As vaccination with integration-deficient vectors was able to elicit CD8+ immune responses *in vivo*, they were tested in a tumoral therapy model with EG7 cell line (rapidly evolving into lymphoma when injected *in vivo)* transfected with OVA, and presenting OVA on the surface as the only antigen.

Mice were inoculated subcutaneously with 2×10^6 EG7.OVA cells, 3 days later primed with one of the vectors: control (integrating vector expressing EGFP), OVA (integrating), DNW/2Δatt, MKK6/DNW and vFLIP/DNW. Boost-vaccination was administered 7 days later. 150ng RT of each vector was used for both prime and boost vaccination. Inoculated tumours started to be visible after about 5 days from the time of inoculation and from this time on they were measured with a calliper. The size of the tumour was calculated as width × height. Mice were sacrificed when the size of the tumour reaches the size stated in Home Office regulations. Tumour scores are shown in figure 3.5.A. All mice in the control group had to be sacrificed by day 18. In the group vaccinated with integrating vector (OVA), tumours in 8 out of 10 mice started to regress after about 10 days. This is consistent with the expected time of induction of immune responses against OVA, as the mice were injected 3 days after tumour inoculation. As a result specific immune responses developed about 1 week after immunization. In 4 of the 8 mice with initial regression the tumours regrew and those mice finally had to be sacrificed. 4 mice were left alive and tumour-free after one 100 days. DNW/2Δatt had a similar response curve with initial regression. However, in this group only 1 mouse remained tumour-free at the end of the experiment. Strikingly, mice immunized with MKK6/DNW or vFLIP/DNW had the highest survival of all the groups. This was surprising, as it was previously demonstrated in this chapter that vaccination with those vectors did not result in high CD8+ responses in the ELISpot assay.

As shown in figure 3.5.B. , immunization with OVA, MKK6/DNW and vFLIP/DNW resulted in 40-60% of survival in those groups while immunization with DNW/2Δatt gave only 10% survival. Four or nine months later mice from all groups that survived were re-challenged with EG7.OVA cells. All of them remained tumour-free.

Figure 3.5.

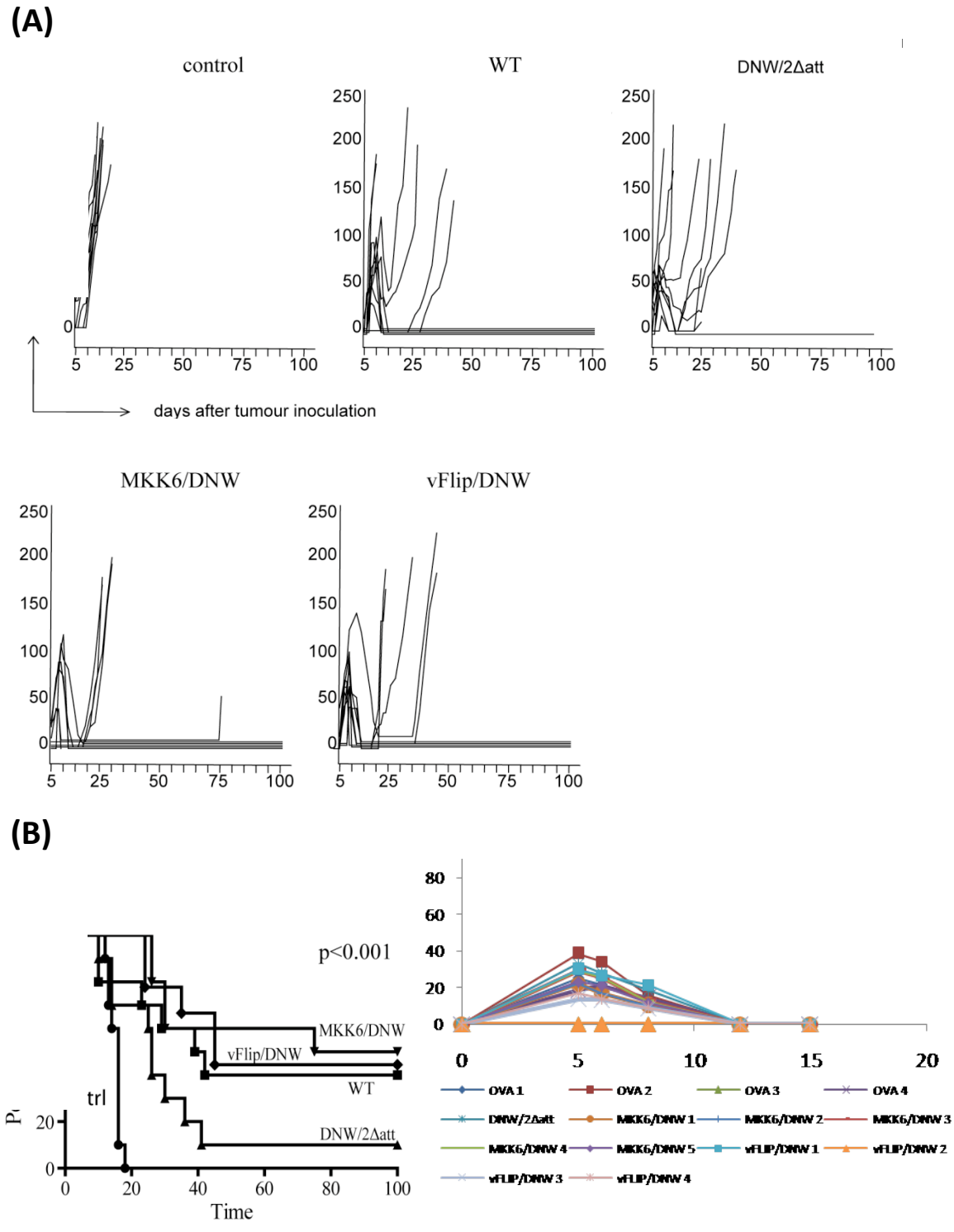

Figure 3.5. *Tumour therapy with non-integrating lentivectors*

(A) Tumour scores. 2×10^6 EG7.OVA cells were inoculated subcutaneously into C57/BL6 mice and mice were vaccinated 3 and 10 days later with 150ng RT of each vector used:

lvEGFP (integrating expressing EGFP – negative control), lvOVA, DNW/2Δatt, MKK6/DNA, vFLIP/DNW. Tumours started to appear visible about 5 days after implantation. From this point on, they were measured frequently and their size was calculated as width × height. Mice were sacrificed if the tumours exceeded 150mm^2. The size of tumours was measured for 100 days. After this time all tumour free-mice were left for several months before re-challenge with EG7.OVA cells. (A) Tumours size versus days after tumour inoculation (B) Survival curve and tumour-growth after re-challenge. For the survival curve the significance of differences in survival between different groups was calculated using Kaplan-Meyer algorithm. For the re-challenge, mouse were injected with 2×10^6 EG7.OVA cells 6 to 8 months post-vaccination.

3.2.5. IMMUNIZATION WITH LENTIVECTORS CO-EXPRESSING AN ANTIGEN AND DENDRITIC CELL ACTIVATORS RESULTS IN A FASTER RESPONSE AND GIVES TRANSDUCED DENDRITIC CELLS A HIGHER MIGRATORY CAPACITY

As immunization with lentivectors co-expressing DC activators did not result in higher responses in ELISpot assay, but gave significantly better survival in a tumoral model, it indicated that the response elicited by these vectors either had to occur earlier or last longer. Therefore, a time response ELISpot was performed for the following vectors: OVA, DNW/2Δatt, MKK6/DNW and vFLIP/DNW. The administered dose was adjusted to the one used in tumoral experiments, namely 150ng RT. ELISpots were performed at four different time points: 7, 14, 28 and 50 days in the same way as described earlier in this chapter. Strikingly, after 7 days from immunization, MKK6/DNW and vFLIP/DNW generate significantly higher responses than OVA and DNW/2Δatt vectors. Interestingly, 14 days after vaccination, DNW/2Δatt vector gave the highest number of IFN-γ secreting cells. After 28 days there are no significant differences between the groups, and after extra 22 days responses generated by DNW/2Δatt and MKK6/DNW went down (figure3.6.).

The "faster" immune responses after vaccination with lentivectors co-expressing dendritic cell activators was further explained by *in vivo* bioluminescence imaging. Bone-marrow derived dendritic cells were transduced with lvEGFP-FLuc or lvMKK6-FLuc (lentivectors expressing firefly luciferase with EGFP or MKK6 respectively). Transduced dendritic cells were injected subcutaneously into Trp2 $^{-/-}$ C57/BL6 mice and imaged after peritoneal injection of luciferin-D. Mouse without cell transfer but injected with D-luciferin was used as a control (located on the right side in Fig.3.7). Figure 3.7. shows that 1 day after injection all cells were localized at the site of injection. After 2 days cells started to migrate towards regional draining lymph nodes. This effect was more pronounced in the MKK6 group. Finally, 5 days after injection dendritic cells in the EGFP group in most mice are localized at the ipsilateral regional draining lymph nodes. Strikingly, in the MKK6 group, cells migrated not only to the ipsilateral regional lymph nodes but also to the contralateral ones.

Figure 3.6.

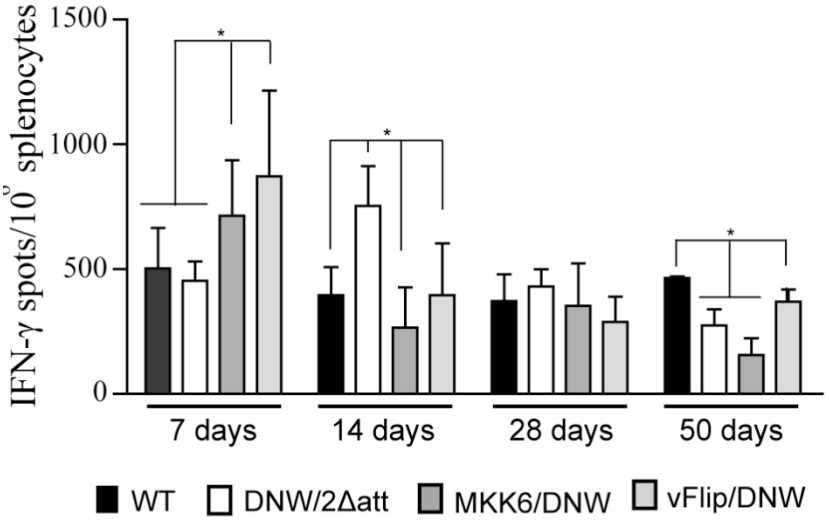

Figure 3.6. IFN-γ responses over time. *Mice were injected with 150ng RT of the following vectors: lvOVA, DNW/2Δatt, MKK6/DNA, vFLIP/DNW. 7, 14, 28 and 50 days later mice were sacrificed and spleens were processed for ELISpot assay. The result is shown as the number of IFN-γ secreting cells/ 10^6 splenocytes at specified time points. Splenocytes stimulated with medium containing no peptide were used as a control. The number of spots in the control wells was substracted from the number of spots in wells containing cells stimulated with SIINFEKL peptide. 3 mice and 3 repetitions were used for each group. * denotes p<0.05 (significantly relevant)*

Figure 3.7.

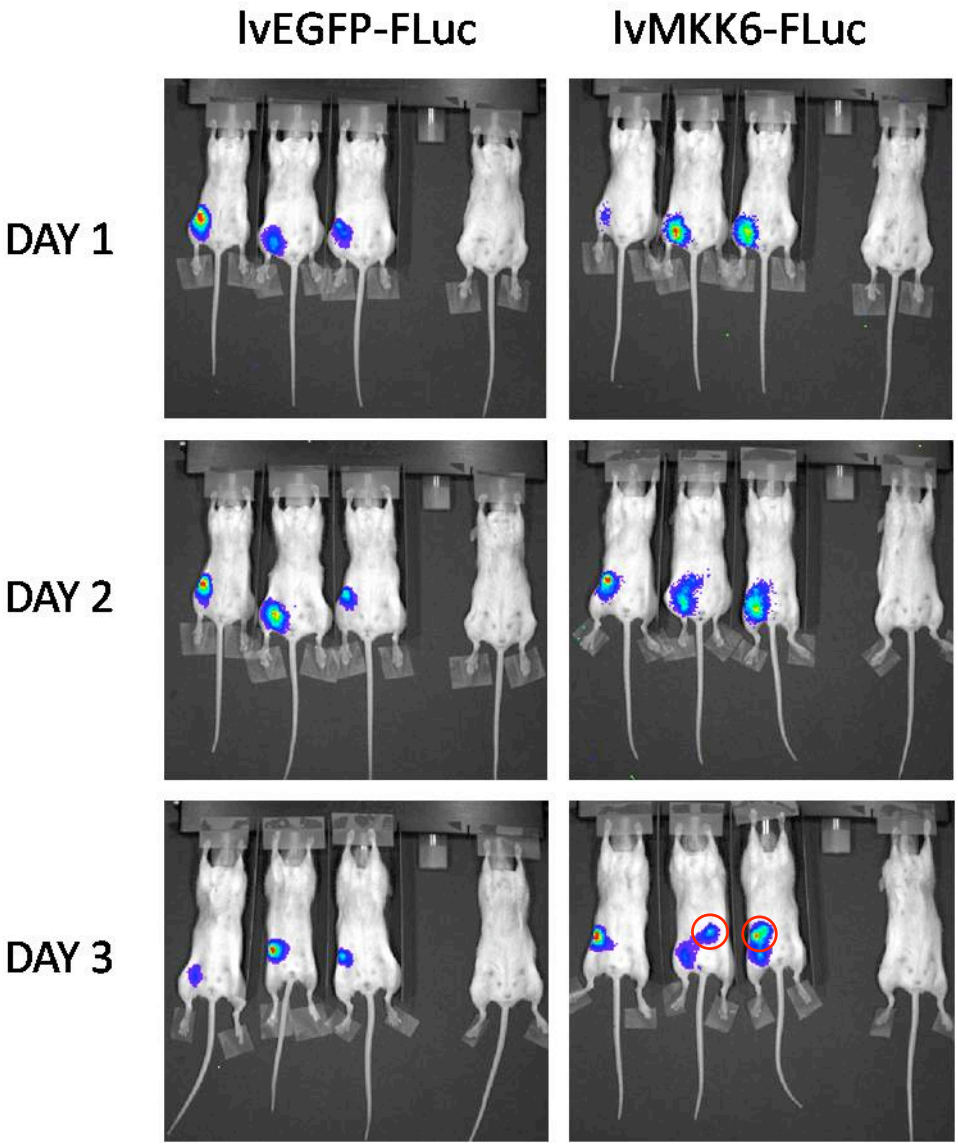

***Figure 3.7. In vivo bioluminescence tracking of lentivector-transduced BMDCs**. BMDCs were prepared from C57/BL6 mice, transduced with lv-EGFP-fLuc or lv-MKK6-fLuc and injected subcutaneously into Trp2$^{-/-}$ C57/BL6 mice. Image was acquired after intraperitoneal injection of luciferin-D. Three mice per group with one repetition were used. Mouse without cell transfer but injected with D-luciferin was used as a control (located on the right side).*

3.3. DISCUSSION

This study demonstrates that subcutaneous administration of integration deficient lentiviral vector expressing a model antigen results in sustained immunization over a period of several months (in tumoral experiments mice were re-challenged several months after first vaccination). Even though integration competent lentiviral vectors have previously been shown to induce strong specific and protective immune responses against tumours (Rowe, Lopes et al. 2006; Lopes, Dewannieux et al. 2008) and infectious pathogens, as integrating vectors their use comes with potential problems linked to random integration into the host genome and the risk of inducing insertional mutagenesis. This has become especially highlighted after 2 independent gene-therapy trials using γ-retroviral vectors (Hacein-Bey-Abina, Von Kalle et al. 2003; Howe, Mansour et al. 2008) and finally, lentivector based gene therapy trial designed to treat β-thalassemia (Kaiser 2009) . Development of integration deficient vectors overcomes this risk.

In this chapter NILVs are shown to express antigen in the regional draining lymph nodes after subcutaneous immunization suggesting that transduced cells migrate to the lymph nodes. These cells are probably skin-derived DCs. This is in agreement with previous studies using integration competent vectors showing transduction of skin-derived $CD11c^+$ DCs (He, Zhang et al. 2006; Rowe, Lopes et al. 2006) as well as LN-resident DCs (Lopes, Dewannieux et al. 2008).

IFN-γ ELISpot assay was used as a tool to evaluate CD8+ T cell responses. Of course other parameters like granzyme B secretion and cytokines secreted by CD4+ T cells could have been measured. However, it was only logical to think that, because of the multitude of IFN-γ effects on anti-tumour immunity, measuring this cytokine could be a good indicator of which constructs would generate best anti-tumour protection *in vivo*.

IFN-γ ELISpot results show that vaccination with NILVs is not as effective as vaccination with their integrating counterparts. In fact, to achieve the response obtained by vaccination with 7 or 10ng of integrating vectors, as much as 250 ng of NILV was required. This can be explained by the difference in expression levels

between integrating vectors and NILVs. Even though the transduction efficiency of both vectors was shown to be the same in non-dividing cells, the expression level from NILVs was always lower (Apolonia, Waddington et al. 2007; Coutant, Frenkiel et al. 2008). A previous report comparing anti-gp120 T cell and antibody responses showed that those elicited after immunization with non-integrating vector were somewhat lower than those elicited by using integrating vector (Negri, Michelini et al. 2010). Similarly, another paper published around the time this study was accepted for publication found that antibody responses after immunization with NILV expressing a secreted form of the envelope of West Nile virus were lower compared to those induced by immunization with integrating vector (Coutant, Frenkiel et al. 2008). As VSV-G pseudotyped lentivectors were shown to carry tubovesicular structures containing plasmid DNA used for vector preparation and co-precipitated proteins, heat-inactivated integrating lentivector was included in this study as a negative control. Sample treatment at 95°C for 15 minutes resulted in RT inactivation and proved that responses elicited in this study were due to RNA content in lentivector preparations.

This study tested vectors harbouring different mutations in the integrase (D64V, DNW), attachment sites (2Δatt) as well as a combination of both (DNW/2Δatt). The nature of introduced mutations did not have an impact on the outcome of vaccination. DNW/2Δatt vector was used for further experiments as it has the highest potential for clinical studies because of its safety profile.

Surprisingly, co-expressing DC activators MKK6EE and vFLIP did not augment IFN-γ ELISpot responses to immunization with non-integrating vector. This is contrary to what was reported before (Escors, Lopes et al. 2008). However, this conundrum is addressed and explained in the later part of the study.

Strikingly, subcutaneous immunization with both integrating and non-integrating vectors resulted in persistence of antigen presentation for at least 30 days with a similar proliferation index for both vectors. Even though the debate on the influence of the duration of antigen persistence is still ongoing, it was recently demonstrated that immunization with an integrating lentivector results in a prolonged antigen

presentation which leads to induction of effective CTL responses (Arce, Rowe et al. 2009). As shown here, also immunization with a non-integrating vector also leads to such a prolonged persistence of antigen. As 30 days is beyond the life-span of a DC, this result is in agreement with the study showing that immunization with a lentivector leads to transduction of DC precursors providing a reservoir of antigen (Kamath, Henri et al. 2002) (Arce, Rowe et al. 2009). This finding further supports the results of analysis of IFN-γ responses over the course of 50 days showing sustained and equal memory T cell responses at this time point elicited by both integrating and non-integrating vector despite the fact that initial responses (7 days) after using NILV were significantly lower.

Initial experiments showed that immunization with NILVs required a much higher RT ng dose to generate responses like the ones observed after immunization with integrating vectors. This applied even to NILVs expressing DC activators. However, when the latter were used in a tumoral model at a dose of 150ng/mouse (for both prime and boost), non-integrating vectors with MKK6EE or vFLIP showed improvement in the survival curve over integrating vectors expressing only the antigen.

As the initial experiments showed no improvement in responses upon co-expression of a DC activator, it was surprising to see that these vectors worked so much better in the tumoral model. This is explained by analysis of responses over time using an identical dose for each group adjusted to the one used in tumoral settings, namely 150ng RT/mouse. This experiment showed that inclusion of a DC activator contributes to generation of a faster immune response as indicated by results on day 7. At day 14 these responses are greatly reduced which might explain why in ELISpot performed 11 days after vaccination we saw no improvement when using lvMKK6-EGFP and lv vFLIP-EGFP.

This finding was additionally supported by the results of an *in vivo* bioluminescence tracking of dendritic cells. In this experiment, BMDCs were transduced with 2 different vectors: lvEGFP-FLuc or lvMKK6-FLuc. While BMDCs transduced with lvEGFP-fLuc moved only to ipsilateral regional lymph nodes, BMDCs transduced with lvMKK6EE-Fluc vector not only migrated to the regional lymph nodes

faster but also additionally moved to the contralateral lymph nodes. It is possible that this extra stimulation provided by MKK6EE that made the BMDCs migrate faster is also responsible for inducing a quicker response in earlier experiments. However, more experiments are needed to confirm if MKK6EE can indeed provide dendritic cells with a higher migratory capacity.

Overall, this study shows that integration deficient lentivectors are able to generate long lasting T cell responses, and thus provides a safer alternative to integrating vectors. However, it also shows that NILVs are not as efficient as their integrating counterparts in generating CTL responses. Therefore, other means of improving responses, like DC modification, should be explored, an example of which is inclusion of DC activators like applied in this study. Such non-integrating lentivectors co-expressing molecules altering maturation phenotype of an APC proved to be superior in a tumoural therapy model. Finally, not only were they efficient in treating growing tumours, but also induced long lasting memory T cells that protected immunized mice against rechallenge with the tumour for at least a year.

This chapter f ocused on analysis of CTL responses. However, one should not forget that *in vivo* protection is also mediated by other cell subsets, such as T helper cells, NK cells, macrophages.

CHAPTER 4. LENTIVIRAL MEDIATED PDL1 KNOCKDOWN TO ENHANCE RESPONSES TO IMMUNIZATION

4.1. INTRODUCTION

4.1.1. PD1-PDL1/PDL PATHWAY

There is an urgent need for better cancer vaccines as despite a growing number of clinical trials, tumour eradication remains rare. One of the factors contributing to this problem is the tumour microenvironment which blocks T cell responses by manipulation of T cell and APC signalling. PD1-PDL pathway is a part of this inhibitory milieu. Therefore, its manipulation could lead to better responses upon vaccination and prolonged survival in patients.

PD1-PDL belongs to the B7:CD28 family of pathways. It is thought to be critical for termination of immune responses as it is involved in regulation of balance between T cell activation, tolerance and immunopathology (Keir, Butte et al. 2008).

PD1 (programmed death 1/ CD279 receptor) is encoded by the *Pdcd1* gene. It is a 299 amino acid type I transmembrane protein made up of one immunoglobulin (Ig) superfamily domain, a 20 amino acids long stalk, a transmembrane domain, and an intracellular domain containing ITIM (immunoreceptor tyrosine based inhibitory motif) and ITSM (immunoreceptor tyrosine based switch motif) motifs. It can be expressed on T cells, B cells, natural killer cells, activated monocytes and dendritic cells. It is not expressed on resting T cells, but is induced upon T lymphocyte activation and its cell surface expression is detected within 24 hours after stimulation (Nishimura, Agata et al. 1996; Chemnitz, Parry et al. 2004).

PDL1 and PDL2 are ligands for PD1. PDL1 is a type I transmembrane protein encoded by *Cd274* gene consisting of 290 amino acids and 7 exons of which the first one is non-coding and contains 5'UTR. The second exon contains signal sequence, while third and fourth encode IgV-like and IgC like domains respectively. The binding interface is localized on the IgV-like domain. The transmembrane domain is encoded on the 5^{th} exon. Last, but not least 6^{th} exon codes for 30 amino acids of no known function, while the 7^{th} exon contains intracellular domain residues and 3'UTR. PDL2

has a similar structure but these two ligands differ in their affinities, with PDL2 having a 3-fold higher affinity for PD1 and PDL1 having an extra receptor – CD80, and expression pattern. PDL1 is constitutively expressed on mouse T cells, B cells, DCs, macrophages, mesenchymal stem cells, bone marrow-derived mast cells (Yamazaki, Akiba et al. 2005) and is upregulated on a number of cell types after activation with both type I and type II interferons (Eppihimer, Gunn et al. 2002; Schreiner, Mitsdoerffer et al. 2004). PDL2 has a much more restricted expression. It is inducibly expressed on DCs, macrophages, peritoneal B1 B cells, memory B cells, and cultured bone marrow (BM)-derived mast cells (Francisco, Sage et al. 2010). Figure 4.1. summarizes expression of PD-L1, PD-L2 and their binding partners on APCs and T cells.

Figure 4.1.

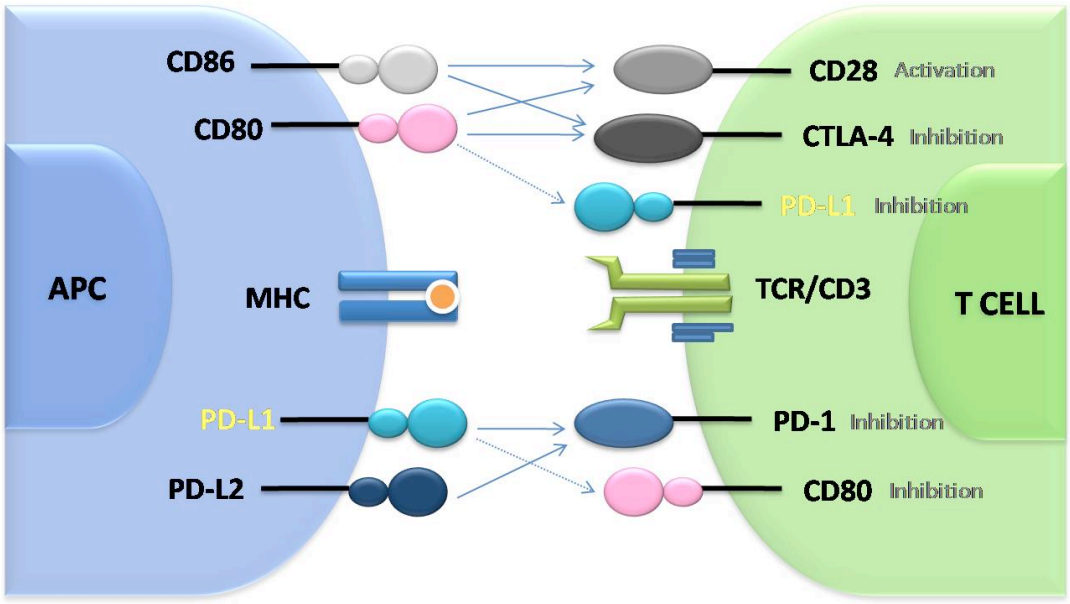

Figure 4.1. B7:CD28 family interactions. *CD80/CD86: CD28 interactions provide activation while B7:PDL/CTLA4 interactions are inhibitory. PDL1 is localized on both T cells and APCs. PDL1:CD80 interactions occur between APC:T cell and T cell:APC which can deliver bidirectional inhibitory signal. PD1 is localized only on T cells.*

Adapted from (Keir, Butte et al. 2008) with a permission from the publisher

4.1.2. PDL1 AND T CELL RESPONSES

PDL1 ligates PD1 and thus influences T cell responses by modifying TCR signalling (Keir, Butte et al. 2008). PD1 ligation acts in 2 different ways to regulate cell differentiation and survival: it can directly block early activation events (that are positively regulated by CD28) or it might block IL2 secretion leading to cell death (Carter, Fouser et al. 2002). There is also some evidence that ligation of PD1 inhibits induction of a cell survival factor Bcl-xL (Chemnitz, Parry et al. 2004). However, modification of immune responses through PDL1 is not limited to engaging PD1. Both PDL1 and PDL2 are able to reverse-deliver signals to cells on which they are localized. In fact, culture of bone marrow derived DCs with soluble PD1-Ig fusion makes DCs acquire suppressive phenotype characterized by inhibition of DC activation and increased IL10 production independent of IDO (Kuipers, Muskens et al. 2006). Figure 3.1. gives a schematic representation of PDL1 mediated suppression of immune responses.

4.1.3. PD1-PDL1 IN T CELL TOLERANCE AND T CELL EXHAUSTION

The PD-1:PD-L pathway controls peripheral T-cell tolerance in several ways. First of all, it can inhibit the initial phase of the immune response: activation and expansion of naïve self-reactive T cells as well as their differentiation into effector T cells (Francisco, Sage et al. 2010). It has been demonstrated that deficiency of PD1 on specific T cells results in an increase of CD8+ T cell responses to antigen-bearing resting DCs (Ansari, Salama et al. 2003; Probst, McCoy et al. 2005; Keir, Liang et al. 2006). Also reactivation, expansion and functions of effector T cells can be negatively influenced by PD1-PDL1 interactions (Ansari, Salama et al. 2003; Keir, Liang et al. 2006). Moreover, as PD-L1 and PD-L2 are expressed on tolerogenic DCs, both ligands may control the decision between T-cell activation and tolerance (Probst, McCoy et al. 2005).

Also PDL1 expressed on non-hematopotetic cells plays a crucial role in maintaining peripheral tolerance as it can inhibit self-reactive responses in target organs (Rodig, Ryan et al. 2003). It can control function of pathogenic effector cells and in this way limit autoimmune tissue destruction (Keir, Liang et al. 2006; Keir, Freeman et al. 2007). Furthermore, PDL1 on endothelial cells has been shown to restrict extravasation of T cells into target organs (Grabie, Gotsman et al. 2007).

Both PD1 and PDL1 are expressed on Tregs and regulate the interplay between Treg and T effector cells. Not only does PDL-PD1 ligation inhibit proliferation, survival and other functions of effector cells like cytokine secretion, but it also has an impact on induction of iTregs. In fact, PDL1 is essential for iTreg induction (Francisco, Salinas et al. 2009). PDL1 deficient APC are not able to fully convert naïve CD4+ T cells into iTregs. In addition to that, PDL1-Ig significantly increases *de novo* generation of CD4+FoxP3+Tregs from naïve CD4+T cells in the presence of TGF-β and CD3 stimulation (Francisco, Salinas et al. 2009).

In general, PDL1 has 4 different functions on Tregs: 1) induction of iTregs and conditioning DCs towards tolerogenic phenotype, 2) sustaining the function of nTregs, 3) suppression of auto-reactive T cells, 4) promotion of contra-conversion of T effector cells to iTregs. Thus, the PD-1: PD-L pathway stays in control of interactions between Tregs, T-effector cells, and APCs . Moreover, PDL1 - PD1 ligation has been demonstrated to affect the stability of DC-T cell contacts by inhibiting TCR-induced stop signals (Fife, Pauken et al. 2009). Since both PD1 and PDL1 are constitutively expressed on Tregs, they may regulate formation of stable and productive immunological contacts, which could constitute a possible mechanism of suppression by Tregs.

PD1 expressed on DCs has a negative impact on their function. Inducible Tregs expressing PDL1 can directly engage PD1 on a DC, modulate DC function and suppress immune responses (Yao, Wang et al. 2009). In this way PDL1 expressed on Tregs has a direct influence on the tolerogenicity of DCs. Dendritic cells condition with iTreg downregulate CD80 and CD86, which implies that Tregs might be acting through suppressing stimulatory capacity of DCs (DiPaolo, Brinster et al. 2007).

Effective CD8+ T cells possess several functional properties, such as cytokine production, cytotoxic potential, ability to proliferate, low apoptosis rate. These functions are often impaired during chronic infections. This state, called exhauston and defined as the persistence of virus-specific CD8 T cells that lacked effector functions was first described *during chronic LCMV infection (Zajac, Blattman et al. 1998)*. Exhausted T cells have been since observed during several infections in both primates (SIV infection) and humans (*HIV, hepatitis B, hepatitis C, human T lymphotropic virus-1 (Freeman, Wherry et al. 2006)).*

Several steps of exhaustion have been described, starting from mild characterized by low IL-2 and TNF-alpha production and poor cytotoxicity. This is followed by moderate exhaustion (modestly defective IFN-γ, cytotoxicity, and little IL-2 or TNF-α), severe exhaustion (lack of IFN-γ, TNF-α, IL-2, and cytotoxicity) and finally physical deletion of T cells. The PD-1–PD-L pathway has been identified as a key regulator of exhaustion. The correlation between the levels of PD1 and PDL1 has been noted tduring chronic viral infections: *an increased expression of PD-1 by virus-specific T cells, and PD-L1 by APCs caused more severe exhaustion* (Freeman, Wherry et al. 2006).

PD-1 is highly expressed by CD8+ T cells during chronic LCMV infection. Furthermore, the PD-1–PD-L is thought to have a major role in regulating T cell exhaustion during this infection as blocking the pathway *in vivo* enhances virus-specific CD8 responses- both the number of cells and their functions are increased (Barber, Wherry et al. 2006).

4.1.4. PD1-PDL1 SIGNALLING

PD1-PDL1 signalling affects many aspects of T cell function. First of all, it is thought to have an effect on T lymphocyte proliferation and even more, on the production of cytokines like IFN-γ, IL-2 and TNF-α. Induction of a cell survival factor – Bcl-xL – is blocked upon PD1 ligation (Chemnitz, Parry et al. 2004). This might explain why PD1 makes T cells more susceptible to apoptosis. Several transcription factors like GATA-3, Tbet and Eomes, are inhibited by the pathway (Nurieva, Thomas et al. 2006).

In general, PD1 ligation alters membrane-proximal signalling events in T cells. Ligation of TCR and PD1 causes tyrosine phosphorylation of ITIM and ITSM motifs of PD1. This recruits SH2-domain containing tyrosin phosphatase 1 (SHP-1) and SHP-2 which bind to ITIM and ITSM on PD1 and subsequently block activation of PI3K (phosphatidylinositol kinase) and downstream signalling events including activation of Akt (Fig. 4.2.) (Francisco, Sage et al. 2010). Since PI3K and Akt are involved in glucose uptake, PD1 ligation blocks the effect of costimulation on glucose uptake in T lymphocytes and ablates CD28 mediated increase in metabolism (Parry, Chemnitz et al. 2005). Furthermore, PD1 inhibits phosphorylation of CD3ζ, ZAP70 and PKCθ (Parry, Chemnitz et al. 2005) which are involved in activation of TCR-mediated T cell function. There is evidence that PD1 ITIM/ITSM motifs associate with Lck and Csk (Sheppard, Fitz et al. 2004) which might suggest that Lck and/or Csk mediate phosphorylation of PD1. Last, but not least, PD1 ligation inhibits Erk signalling pathway. This can be overcome by STAT5-activating cytokines, namely IL-2, IL-7 and IL-15 (Bennett, Luxenberg et al. 2003).

Figure 4.2.

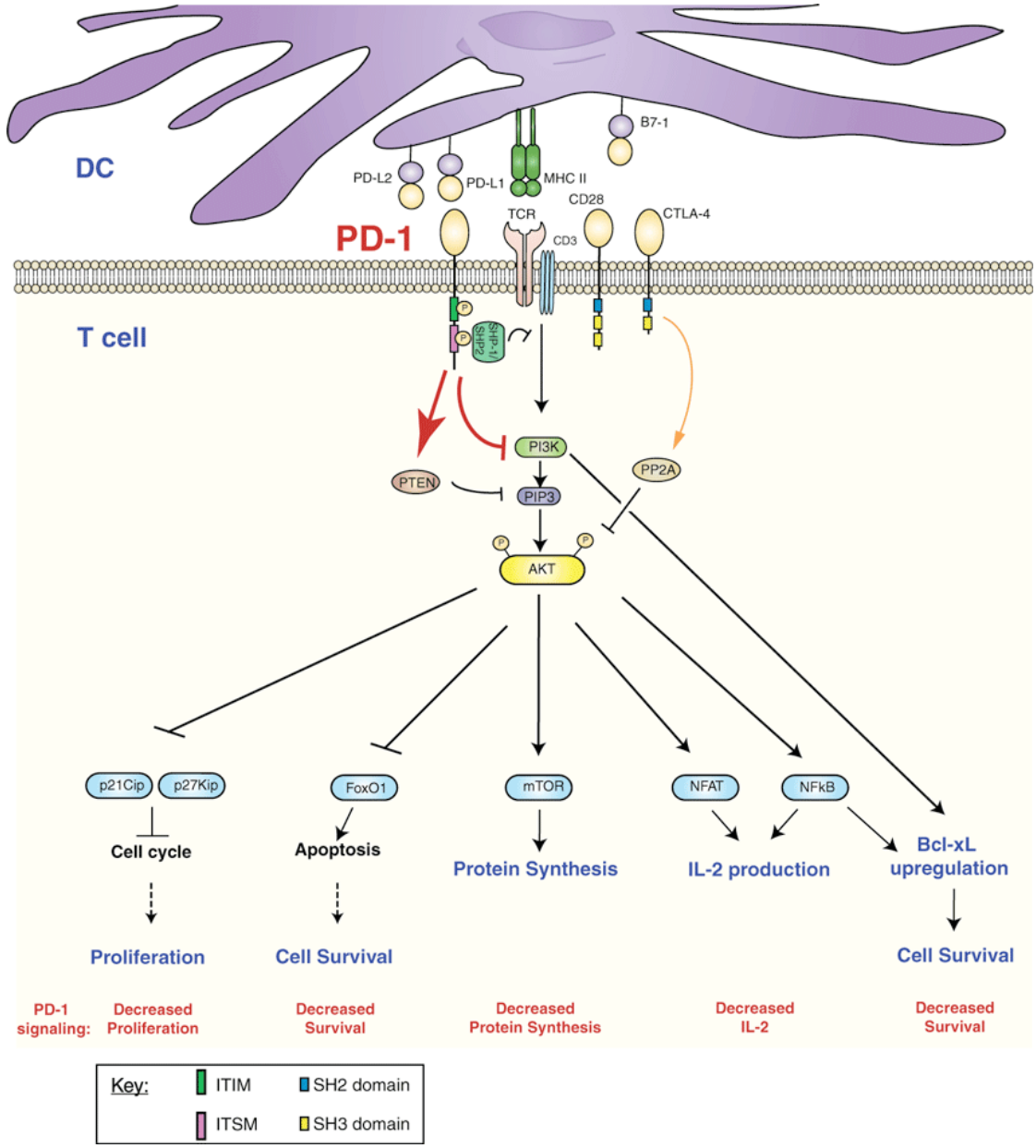

Fig. 4.2. PD-1 signalling. *Upon ligation of TCR and PD-1, the ITIM and ITSM motifs of PD1 are phosphorylated on the tyrosine residues. This induces binding of the ITSM by SHP-1 or SHP-2 which results in dephosphorylation of proximal signalling molecules. Expression of PTEN is augmented. The result is attenuation of PI3K and Akt pathways activation.*

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4.1.5. PD1-PDL1 PATHWAY IN TUMOR IMMUNITY

Even though a number of tumours express antigens that can be recognized by host T cells, it is very rare that they are eliminated by the immune system as the tumoural microenvironment causes immune suppression. PDL1 has been found to be a part of this environment. The ligand was first discovered in an expressed sequence tag clone of human ovary tumor (Dong, Zhu et al. 1999). This suggested that tumoural cells express PDL1. In fact, many groups confirmed PDL1 expression on a number of solid tumours including breast, ovarian, lung, colon, bladder, melanoma, liver, salivary, stomach, gliomas, thyroid, thymic epithelial, head and neck and demonstrated a link between the level of PDL1 expression and unfavourable prognosis in a number of cancers (Dong, Strome et al. 2002; Strome, Dong et al. 2003; Konishi, Yamazaki et al. 2004; Thompson, Gillett et al. 2004; Wu, Zhu et al. 2006; Hamanishi, Mandai et al. 2007; Inman, Sebo et al. 2007; Nakanishi, Wada et al. 2007; Nomi, Sho et al. 2007). In addition to that, most of PDL1 negative tumour lines start to express PDL1 in plasma membrane and cytoplasm after IFN-γ treatment (Dong, Strome et al. 2002) .

Tumours expressing PDL1 are more likely to advance and invade into deeper tissue structure (Konishi, Yamazaki et al. 2004; Thompson, Gillett et al. 2004; Wu, Zhu et al. 2006; Hamanishi, Mandai et al. 2007; Inman, Sebo et al. 2007; Nakanishi, Wada et al. 2007; Nomi, Sho et al. 2007). Despite this fact, PDL1 does not have a direct effect on tumorigenicity as transfection of PDL1 into PDL1 negative P815 tumor cells (mouse lymphoblast-like mastocytoma cell line) did not alter the tumorigenicity of P815 cells in both syngeneic DBA/2 mice and immunodeficient RAG-2−/− mice (Dong and Chen 2003). The PD1-PDL1 pathway has been found to influence both T cells and DCs within the tumoral microenvironment (Dong, Strome et al. 2002; Curiel, Wei et al. 2003; Hirano, Kaneko et al. 2005; Sharma, Baban et al. 2007).

PDL1 on tumours causes inhibition of T cell activation and blocks lysis of tumour cells (Dong, Strome et al. 2002; Hirano, Kaneko et al. 2005). Apoptosis caused by tumour associated PDL1 has been suggested as a potential mechanism of tumoural immune evasion (Dong, Strome et al. 2002). Interestingly, PDL1 on tumoral cells has not only been shown to increase apoptosis of T cells via a receptor on T cells, but also

through deletion of activated T cells *in vivo* (Dong, Strome et al. 2002). Apoptosis could be partially prevented by inclusion of neutralizing mAb to PDL1 (Dong, Strome et al. 2002). These results support the role for tumour-associated PDL1 in the programmed cell death of effector T cells as a major mechanism for the resistance of tumour cells.

PDL1 was also found to be upregulated on the surface of tumour associated blood monocyte-derived myeloid dendritic cells (MDCs) isolated from tissues or draining lymph nodes of ovarian carcinoma patients (Curiel, Wei et al. 2003). This causes a suppressive function of tumour – infiltrating dendritic cells and downregulates T cell immunity, thus causing inhibition of immune responses. MDC-mediated T cell activation could be partially restored by blockade of PDL1, followed by downregulation of IL-10, upregulation of IL-2 and IFN-γ.

A subset of plasmacytoid DCs in the tumour draining lymph nodes (TDLNs) is high in IDO - a potent suppressor for T cell activation. IDO^+ DCs directly activate resting $CD4^+CD25^+FoxP3^+$ Tregs (suppressing target T cell proliferation) which in turn upregulates PDL1 and PDL2 on target DCs. Antibodies against PDL1-PD1 pathway block suppression by mediated by Tregs. Thus, the majority of constant Treg activity in TDLNs is mediated via IDO-induced PD1-PDL1 dependant mechanism (Sharma, Baban et al. 2007).

As tumour-associated PDL1 prevents T cell activation and tumour lysis, blockade of the PD1-PDL1 pathway might restore T cell function. Indeed, treatment with PDL1 augments responses in mice, as measured by cytokine production and cytotoxicity (Iwai, Ishida et al. 2002; Hirano, Kaneko et al. 2005; Iwai, Terawaki et al. 2005). Blockade of PD1 inhibits spread of poorly immunogenic tumours. This effect is mediated by enhanced recruitment of effector T cells (Iwai, Terawaki et al. 2005). Also adoptive therapy by tumour – reactive T cells was shown to be improved upon PDL1 blockade in PDL1 expressing squamous cell carcinoma (Strome, Dong et al. 2003). In mice with established tumours, PDL1 expression confers resistance to therapeutic anti-CD137 antibody. This effect is reversed by blockade of PDL1 or PD1 by specific monoclonal antibodies (Hirano, Kaneko et al. 2005).

Figure 4.3.

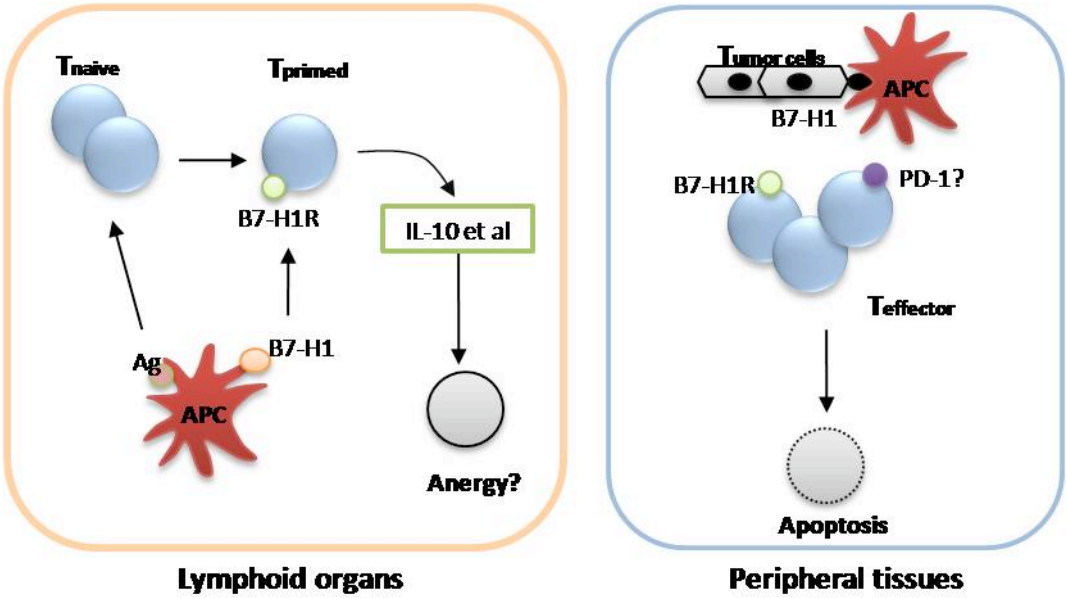


Fig. 4.3. Mechanisms of PD-L1 mediated inhibition of cell-mediated responses. *Encounter of antigen-presenting APC and a naive T cell specific for the antigen primes the T cells. PDL1 on the surface of APC interacts with this early primed T cell and makes it secrete IL10 which induces T cell tolerance and anergy. When a fully activated T cell encounters a tumoral cell or an APC in the periphery, PDL1 expressed on those cells delivers signals that terminate T cell response.B7-H1=PDL1, B7-H1R – receptor for PDL1*

Modified from (Dong and Chen 2003) with permission from the publisher.

4.2. AIMS OF THE CHAPTER

This chapter describes using lentivectors as a tool to deliver shRNA against PDL1 to dendritic cells to manipulate immune responses by alteration of PD1-PDL1 pathway. A potent shRNA is selected and its effects on dendritic cells and co-cultured T cells will be examined. Finally, such a lentivectors will be tested as a vaccine using cellular assays and its efficacy will be evaluated in a tumoral model.

4.3. RESULTS

4.3.1. IDENTIFICATION OF A POTENT PDL1 SHRNA

In order to identify shRNA that would target and efficiently downregulate PDL1, 4 different shRNAs were selected and cloned in the context of miR30 localized within a

synthetic intron into a lentivector containing SFFV-driven yellow fluorescent protein (YFP) (lv.YFP.miR30-shRNA PDL1). Selected shRNAs had the following sequences:

P2-ACCGAAATGATACACAATTCGA, P3–AGCCACTTCTGAGCATGAACTA,
P4-GCAGGCGTTTACTGCTGCATAA, P5-GGCGTTGAAGATACAAGCTCAA.

In order to determine which of these shRNAs was most efficient in knocking down PDL1, each of them (miR30-shRNA P2/P3/P4/P5) was tested *in vitro*. For this purpose bone marrow derived DCs were prepared from C57/BL6 mice and cultured in the presence of GM-CSF and β-ME. They were transduced with each of the lentivectors containing miR30-shRNA PDL1 at an MOI of 20 and left in culture for 4 to 5 days. Next, BMDCs were stimulated with 100ng/ml of LPS and analyzed 24 hours later. DCs transduced with lvYFP without LPS stimulation were used as a negative control, and BMDCs transduced with lvYFP and stimulated with LPS constituted a positive control for LPS activation and upregulation of PDL1. Even though PDL1 mRNA is constitutively expressed, mRNA levels do not correspond to the protein expression level, and PDL1 is upregulated on the surface of a T cell after its activation. For the analysis live cells were gated on YFP and PDL1 expression was compared between the samples. As presented in figure 4.4, transductions with all of the designed shRNAs resulted in a partial knockdown of PDL1 in DCs. ShRNA P5 repetitively generated the highest downregulation of PDL1 on the surface of DCs. To quantify the extent of PDL1 knockdown, the MFI of (shPDL1 + LPS) was divided by (lvYFP+LPS)MFI. The result is presented as a percentage of a positive control expression level. ShRNA P2 was the least efficient of the used sequences giving over 60% of control sample MFI. Transduction with a lentivector expressing shRNA P5 resulted in as little as 30% control MFI level. Since it was the highest PDL1 suppression achieved with generated PDL1 shRNAs, P5 was selected for further experiments.

Figure 4.4.

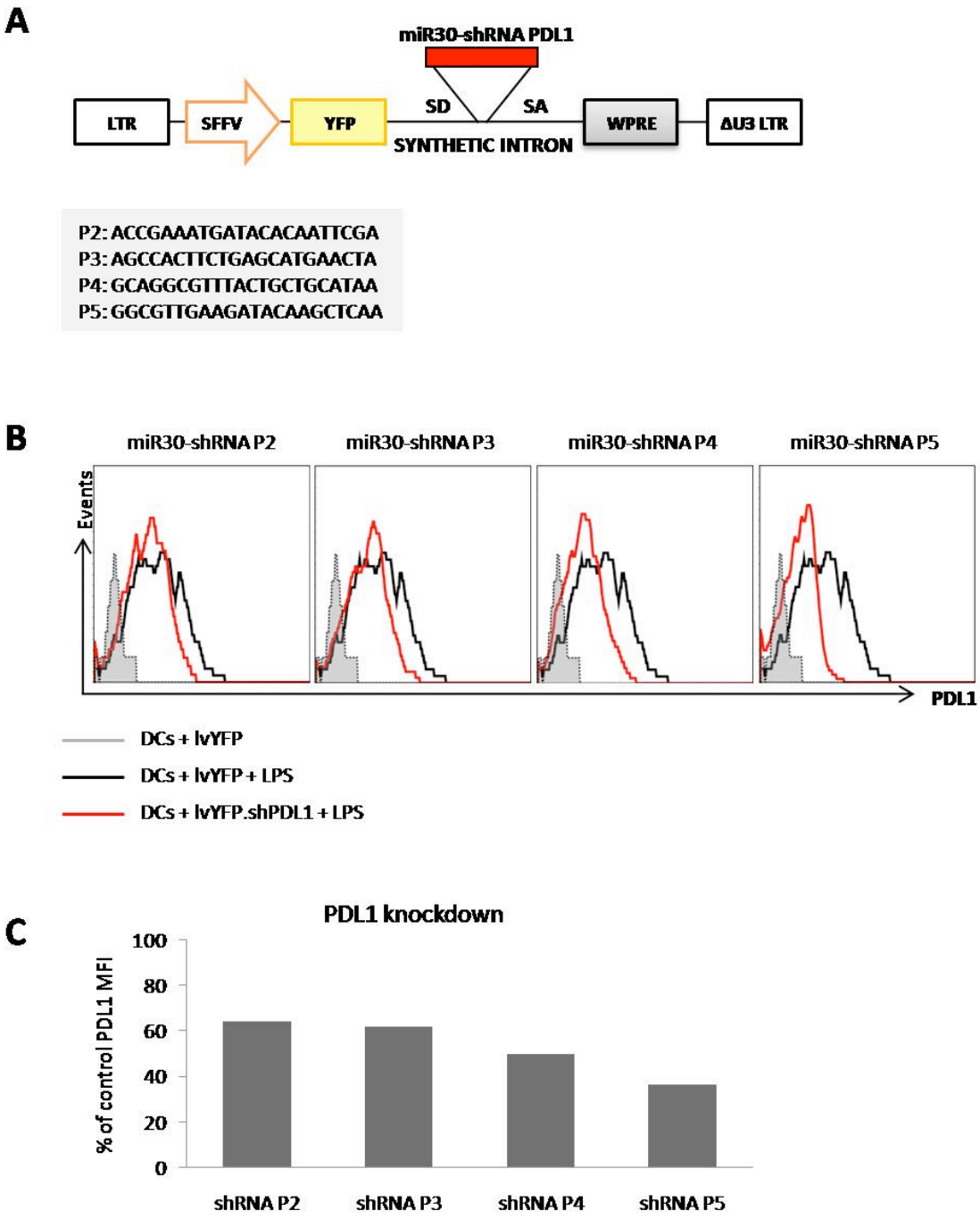


Figure 4.4. shRNA mediated PDL1 knockdown *(A)Schematic drawing of lv.YFP.shRNA PDL1. YFP is expressed from SFFV promoter. shRNA PDL1 is cloned in the context of miR30, localized within a synthetic intron. SD-splice donor, SA-splice acceptor. Below generated shRNA PDL1 sequences labeled P2, P3, P4, P5. (B)PDL1 knockdown by*

selected shRNAs. Lv.YFP.P2/P3/P4/P5 were tested for PDL1 suppresing capacity. BMDCs were transduced with each of the vectors and left in culture for 4-5 days. 24 hours before FACS analysis (surface staining for PDL1) they were stimulated with LPS to upregulate cell surface PDL1 expression. Grey shaded bars – BMDCs transduced with lvYFP (no shRNA) and not stimulated with LPS (negative control), black bar – BMDCs transduced with lvYFP and stimulated with LPS (positive control), red bar – BMDCs transduced with one of the lvYFP.shRNA PDL1 vectors and stimulated with LPS. (C)Level of PDL1 suppression by individual anti PDL1 shRNAs. Bars were calculated by dividing PDL1 MFIs obtained after transduction with each of the shRNA expressing vectors by PDL1 MFI of the samples transduced lvYFP and stimulated with LPS (positive control).

4.3.2. EFFECT OF PDL1 KNOCKDOWN ON DENDRITIC CELL

To determine the effect of PDL1 knockdown on the maturation status of dendritic cells, lv.YFP.P5 vector was used to transduce BMDCs. Lv.YFP vector was used as a control. Four days after transduction some of the lv.YFP samples were stimulated with LPS (positive control). Twenty-four hours later all samples were stained and analyzed by FACS and the result induced by lv.YFP.P5 was compared to the one induced by lv.YFP and lv.YFP stimulated with LPS. DCs were stained for PDL2, CD80, CD40 and ICAM-I. Isotype control was used for each of the stainings. Cells gated on YFP (FL1) were analyzed for the presence of surface markers (Fig. 4.5).

PDL1 knockdown caused upregulation of PDL2, which was even higher than the one induced by LPS. CD80 was also upregulated to an extent comparable to LPS treatment. CD40 was not induced, while ICAM-I was much higher than the negative control and LPS treatment.

Overall, this indicated that PDL1 knockdown drives BMDCs towards a matured phenotype. However, as CD40 is not upregulated, the full maturated-DC phenotype is not achieved.

4.3.3. PDL1 KNOCKDOWN EFFECT ON T CELL APOPTOSIS

As PD1-PDL1 pathway has been shown to be involved in inducing T lymphocyte apoptosis in the tumoural environment, PDL1 knockdown was evaluated for the ability to reduce apoptosis in a DC – T cell co-culture. For this purpose BMDCs were transduced with lv.OVA or lv.OVA.P5. Two days later splenocytes from OT-1 transgenic C57/BL6 mice were purified using a CD3+ negative selection kit and co-cultured with DCs in the ratio of T cells: DCs of 10:1 for 2, 4 or 7 days. At each time point cells were collected and stained with Annexin V to check for early stages of apoptosis and propidium iodide (PI) to stain nonviable cells. For each of the selected days, AnnexinV and PI log fluorescence is plotted for both lv.OVA and lv.OVA.P5 in the same histogram (Fig. 4.6.B). This allows for direct comparison of apoptosis between the two groups. Each PI and Annexin V plot was split into three depending on the intensity of AnnexinV

and PI staining into AnnexinV^{high}, AnnexinV^{med}, AnnexinV^{low} and PI^{high}, PI^{med}, PI^{low} respectively (Fig. 4.6.B). Bar graphs comparing percentages of cells from the lvOVA and lvOVA.P5 groups in each of this 6 sub-groups were plotted as well as a graph showing the percentage of PI and AnnexinV positive cells (Fig. 4.6.C).

At all 3 time points: 2, 4 and 7 days, there was a trend for more cells from the lvOVA group to be present in the AnnexinV ^{high} and PI ^{high} populations compared to cells in lvOVA.P5 group (Fig. 4.6.C). The significance of this experiments is not confirmed as more repetitions have to be performed. The result was contrary in the AnnexinV ^{low} and PI ^{low} population. This indicates that PDL1 knockdown from dendritic cells in a culture with T cells leads to a decrease in T lymphocyte death. This observation is confirmed by the percentage of AnnexinV/PI positive cells being higher in the lvOVA group (Fig. 4.6.C).

Figure 4.5.

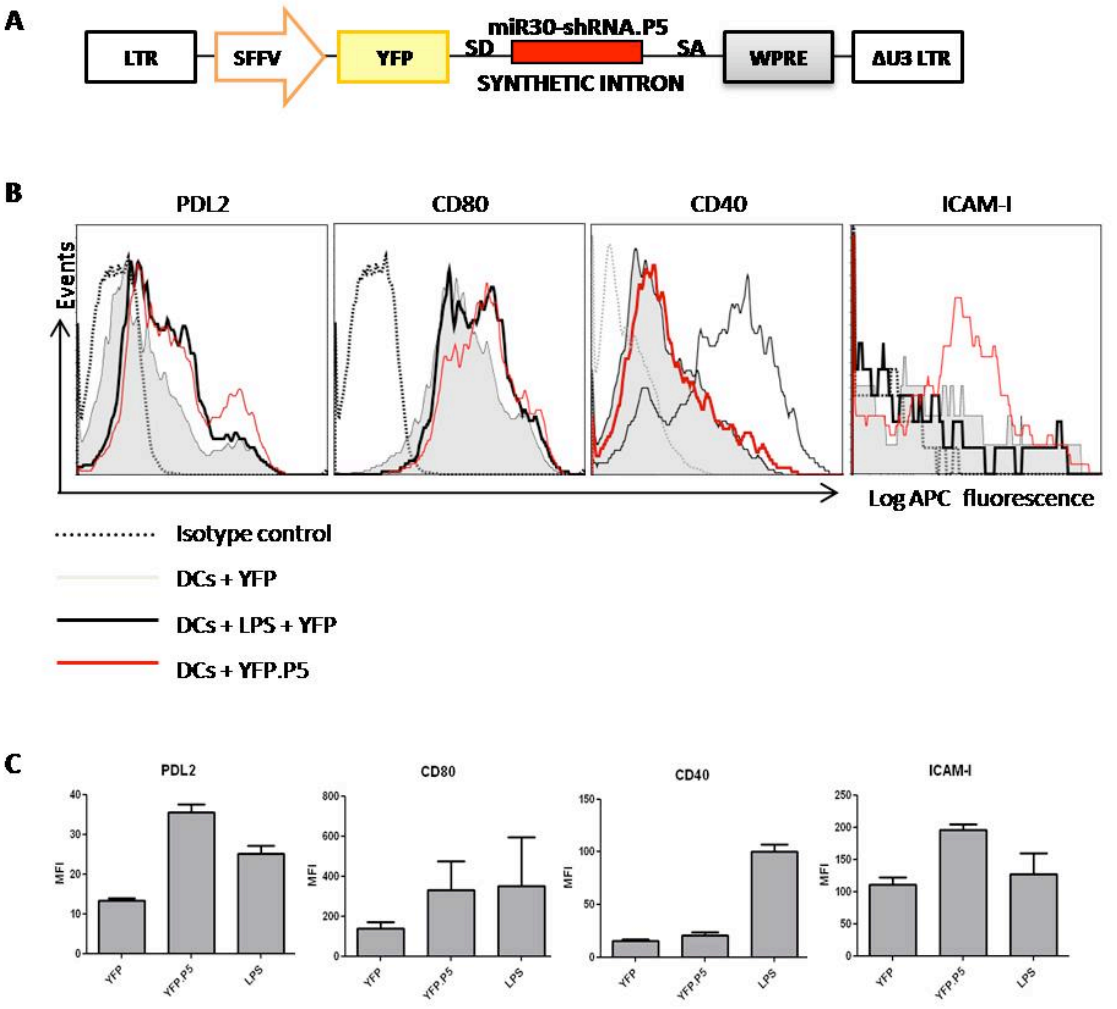
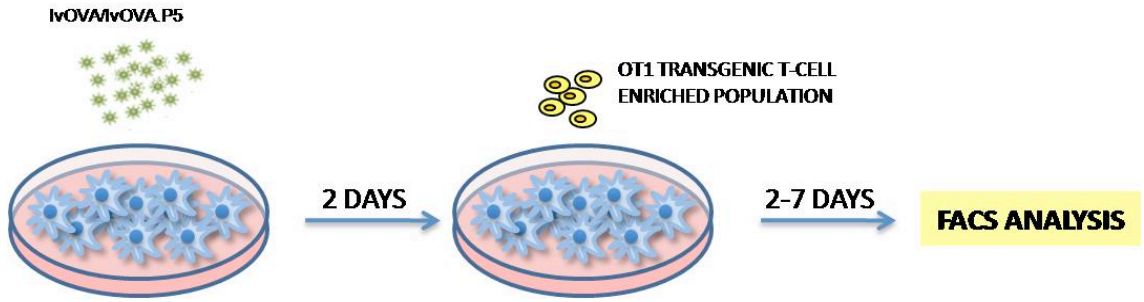


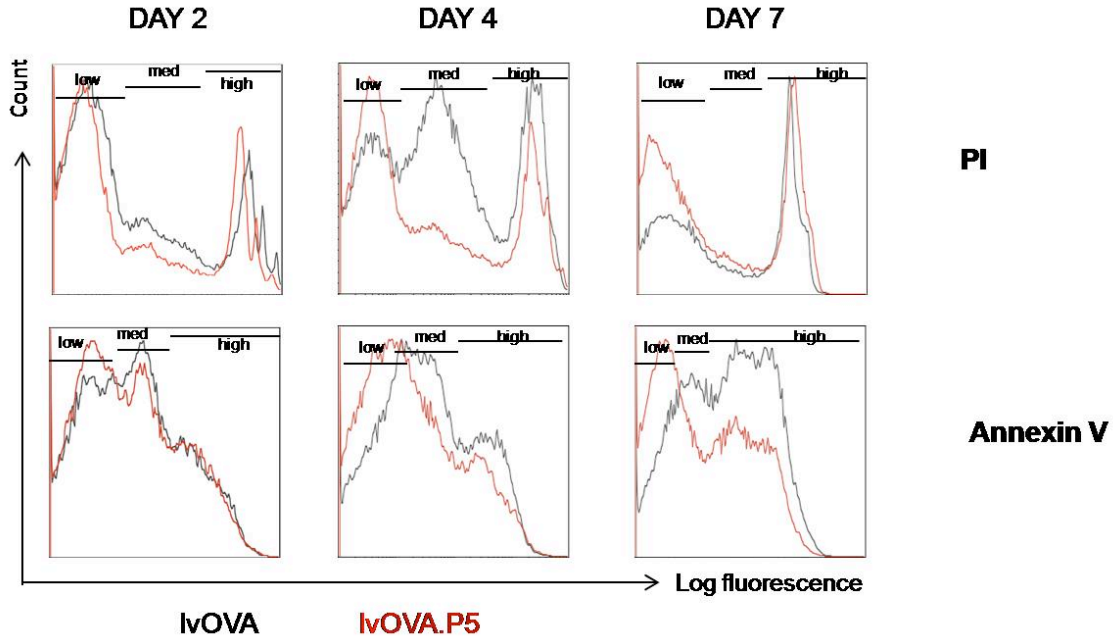
Figure 4.5. Effect of PDL1 knockdown on DC maturation *(A)Schematic drawing of lentivector used in this experiment. SFFV promoter drives YFP and previously selected shRNA P5. (B)Effect of PDL1 knockdown on dendritic cells. BMDCs were transduced with lvYFP.P5 or lvYFP as a control and let in a culture for 4 days. Dotted bar – isotype control, grey shaded bar – DCs transduced with lvYFP vector (negative control), black bar – DCs transduced with lvYFP and stimulated with LPS 24 hours before analysis (positive control), red bar – DCs transduced with lvYFP.P5. Results are presented also in the form of bar graphs.*

Figure 4.6.

(A)


lvOVA/lvOVA.P5
OT1 TRANSGENIC T-CELL
ENRICHED POPULATION
2 DAYS
2-7 DAYS
FACS ANALYSIS

(B)


DAY 2
DAY 4
DAY 7
Count
low
med
high
PI
Annexin V
Log fluorescence
lvOVA
lvOVA.P5

(C)

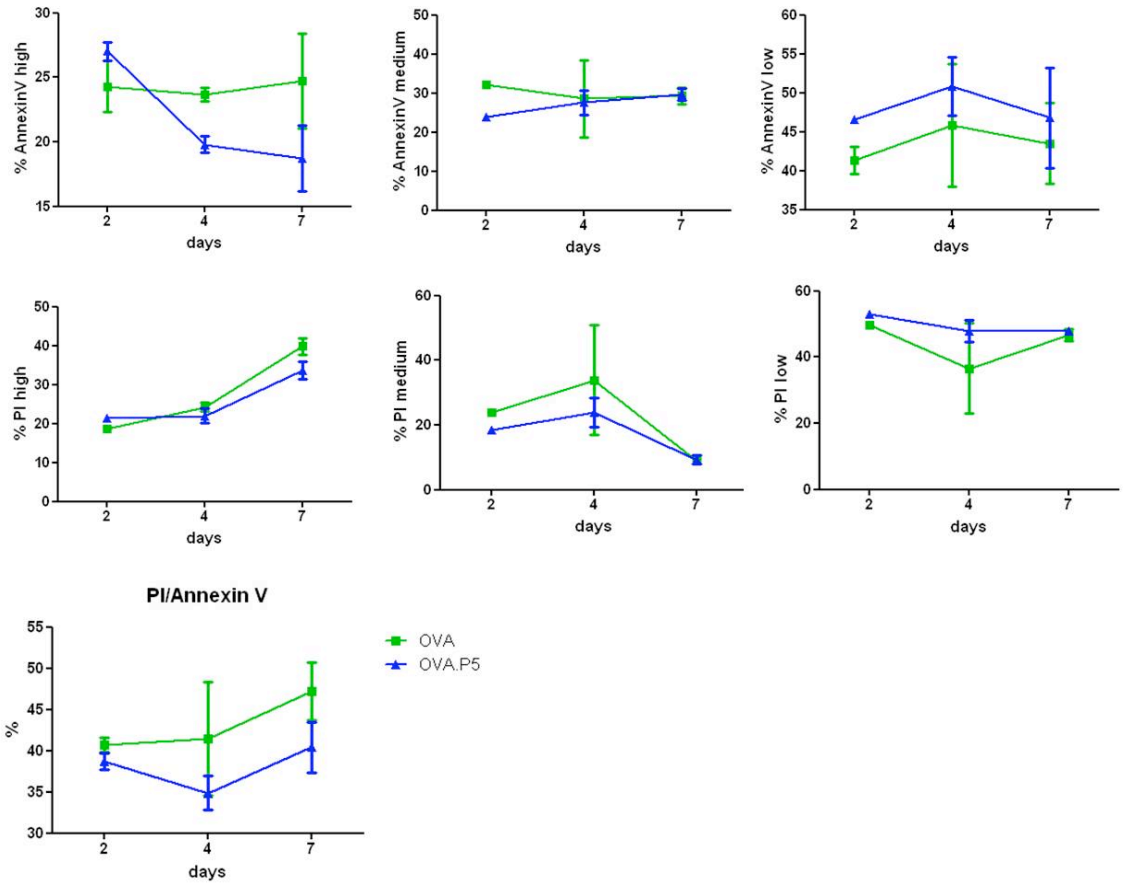


Figure 4.6. Effect of PDL1 knockdown in APCs on T cell apoptosis *(A)Schematic drawing of the experiment schedule. BMDCs were transduced with lvOVA or lvOVA.P5. Two days later CD3^{+} negatively selected splenocytes from OT1 transgenic mice were added to DCs and left in the culture for 2-7 days. At 2, 4 and 7 days time points cells were analyzed for apoptosis.(B)Upper panel - histograms presenting propidium iodide (PI) staining, lower panel – Annexin V staining. Left to right: days 2, 4 and 7. Black line denotes lvOVA, red line – lvOVA.P5. (C)Bar graphs presenting the percentages of cells over time in the following populations:* AnnexinV^{high}, AnnexinV^{med}, AnnexinV^{low} and PI^{high}, PI^{med}, PI^{low}, AnnexinV^{high}/ PI^{high}. Data is drawn from two separate experiments.

4.3.4. THE EFFECT OF PDL1 KNOCKDOWN ON ANTIGEN-INDUCED TCR DOWNREGULTION IN T-CELLS

Antigenic stimulation of T cells is known to cause internalization and degradation of the TCR/CD3 complexes from the surface of a T cell (Valitutti, Muller et al. 1997). Since PD1-PDL1 ligation induces an inhibitory signal, I checked whether PDL1 knockdown has an influence on the TCR degradation. BMDCs were transduced with either lvOVA or lvOVA.P5. Two days later OT1 CD3+ enriched transgenic T cells were put into culture with previously transduced dendritic cells. On days 2, 4 and 7 cultures were analyzed by staining T cells with specific TCR chains antibodies: Vα2.1 and Vβ5.1, 5.2 (Fig.4.7.A, B).

Changes in TCR complex downregulation were clearly visible between the 2 groups containing OVA loaded APCs already on day 2 (Fig. 4.7.B,C). Gating the control group (no OVA presented by DC) for Vα2.1 and Vβ5.1, 5.2 chains revealed a distinctive population of $V\alpha2.1^{high}V\beta5.1, 5.2^{high}$ cells. This is the population of OT1 transgenic cells that are specific for OVA and will expand if encountered by an activated APC presenting OVA. In the lvOVA group the Vβ chain is clearly downregulated. Since T cells stimulated with lvOVA.P5-transduced APCs were also exposed to antigenic stimulation, the same TCR downmodulation was expected. However, as seen in figure 4.7.C. (histogram plot), the Vβ chain was not as downmodulated as in the lvOVA group. The Vα chain was not downregulated in any of the groups at this time point.

This effect was further analyzed on days 4 and 7 (Fig. 4.8.A). While both Vα and Vβ chains are strongly downmodulated in the lvOVA group, in the lvOVA.P5 group their levels are closer to the ones in the control group. To explore this further, T lymphocytes were divided into $V\alpha^+$, $V\alpha^-$ $V\beta^+$ and $V\beta^-$ populations and the percentage of cells in $V\alpha^+$ and $V\beta^+$ was plotted and compared over time between the lvOVA and lvOVA.P5 groups (Fig. 4.8.B). It was found to be always higher in the lvOVA.P5 group and the difference between the lvOVA and lvOVA.P5 tends to reach its peak at day 7. For example, on day 7 only around 30% of cells in the lvOVA group are $V\alpha^+$ compared to around 60% in the lvOVA.P5 group. Similarly, about 25% of cells in the lvOVA group are $V\beta^+$, while in the lvOVA.P5 group it is around 50%.

4.3.5. THE EFFECT OF PDL1 KNOCKDOWN ON T CELL PROLIFERATION

As knocking down PDL1 in dendritic cells decreases apoptosis in co-culture with cognate T cells, it was interesting to see whether it also impacts the rate of proliferation of those lymphocytes. To investigate this, BMDCs were transduced with either lvOVA or lvOVA.P5. Two days later CFSE-stained OT1 CD3+ enriched transgenic T cells were put into culture with previously transduced dendritic cells. On days 2, 4 and 7 cultures were analyzed by staining T cells with specific TCR chains antibodies: Vα2.1 and Vβ5.1, 5.2 and analyzing CFSE dilution in cells positive for these 2 chains (Fig. 4.10.A).

Dilution of CFSE was assessed according to the expression level of the TCR chains. Four populations were gated: $V\alpha^{high}$, $V\alpha^{low}$ $V\beta^{high}$ and $V\beta^{low}$. Results after 2 days of co-culture showed very little proliferation in all groups but $V\alpha^{low}$. At day 4, analysis of $V\alpha^{high}$ and $V\beta^{high}$ populations revealed at least 5 to 6 divisions in both OVA and OVA.P5 groups. CFSE in $V\alpha^{low}$ and $V\beta^{low}$ populations was also diluted, more for the OVA.P5 group. However, those cells did not present a clear pattern of proliferation with visible separate populations like those seen in the $V\alpha^{high}$ and $V\beta^{high}$ groups. Still, there was more proliferation in the OVA.P5 group.

Similar results were found upon analyzing 7 days old co-cultures. At this time point, T cells were split into 6 groups: $V\alpha^{high}$, $V\alpha^{mid}$, $V\alpha^{low}$, $V\beta^{high}$, $V\beta^{mid}$ and $V\beta^{low}$ (Fig. 4.9.C). $V\alpha^{high}$ and $V\beta^{high}$ groups have a characteristic distinct divided populations showing at least 7 divisions in the lvOVA groups and at least 8 divisions in the lvOVA.P5 group. CFSE in the lvOVA.P5 group is more diluted, indicating that PDL1 knockdown improves T cell proliferation. Also the 4 remaining groups show improved proliferation in the lvOVA.P5 group. However, the pattern of division is not clear and no distinct populations can be distinguished.

To analyze the results further, I looked at the % of $CFSE^{high}$ cells (=undivided cells) and at the MFIs of divided cells. At day 2 there are no differences between the percentage of undivided cells in both groups. At day 4, the percentage is slightly lower

for the lvOVA.P5 group in the Vβ ^{high} population. However, at day 7, there are clear differences between the two groups (Fig. 4.10.B).

At day 2, in both groups the MFI of CFSE in divided cells was the same. However, at dat 4, it started to diverge, and at day 7, the MFI in the lvOVA.P5 group was 4-6 times higher depending on the analyzed population ($V\beta^{high}$ and $V\beta^{low}$ respectively) (Fig. 4.10.C).

Figure 4.7.

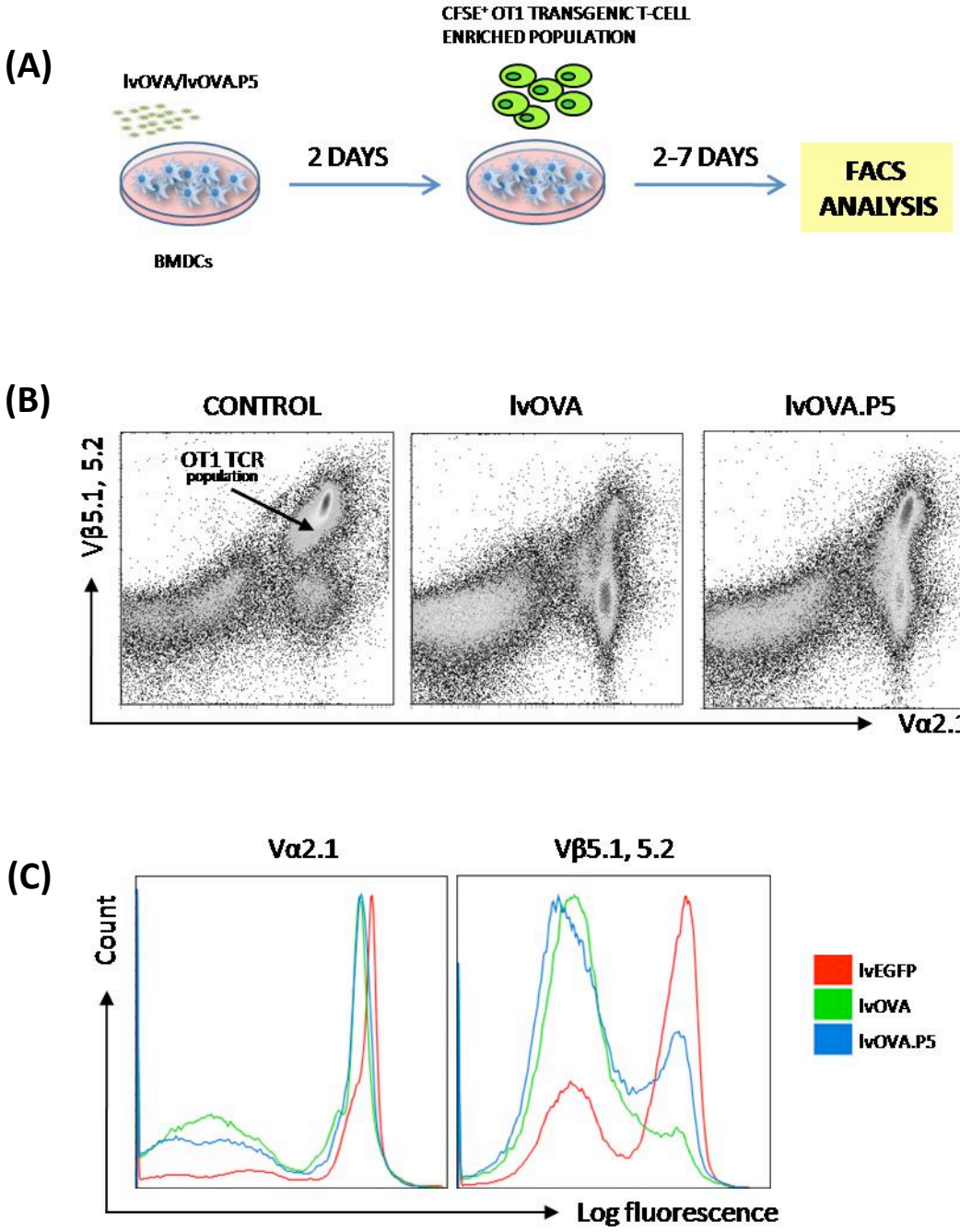


Figure 4.7. The effect of PDL1 knockdown in DCs on antigenic stimulation-induced TCR downregulation. *(A) Schematic representation of experimental design (B) An example of gating on both TCR chains (day 2) - dot plot, OT1 population is indicated with an arrow (C) An example of gating on single TCR chains – histogram. Before visualizing TCR chains, cells were gated on viable T lymphocyte population.*

Figure 4.8.

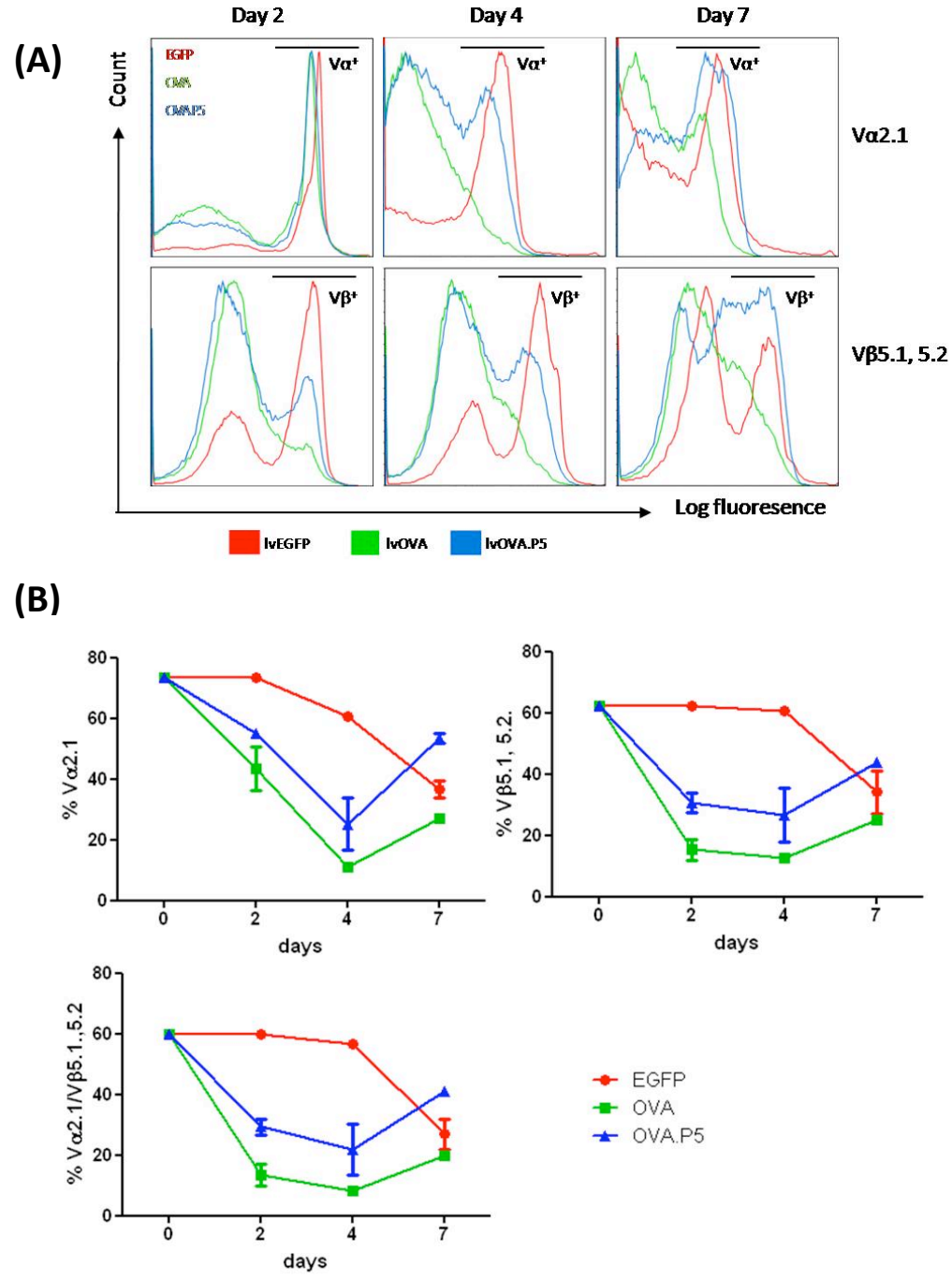
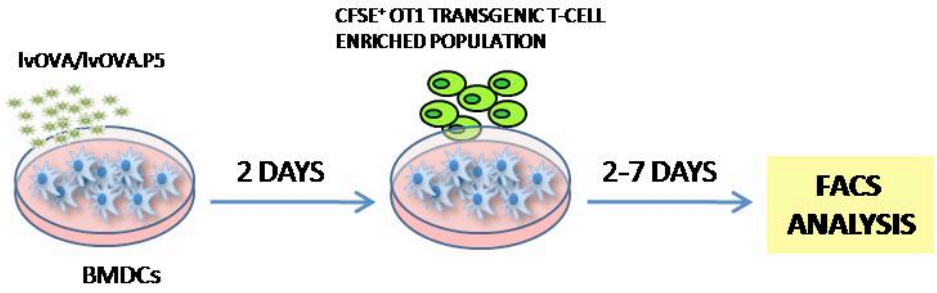


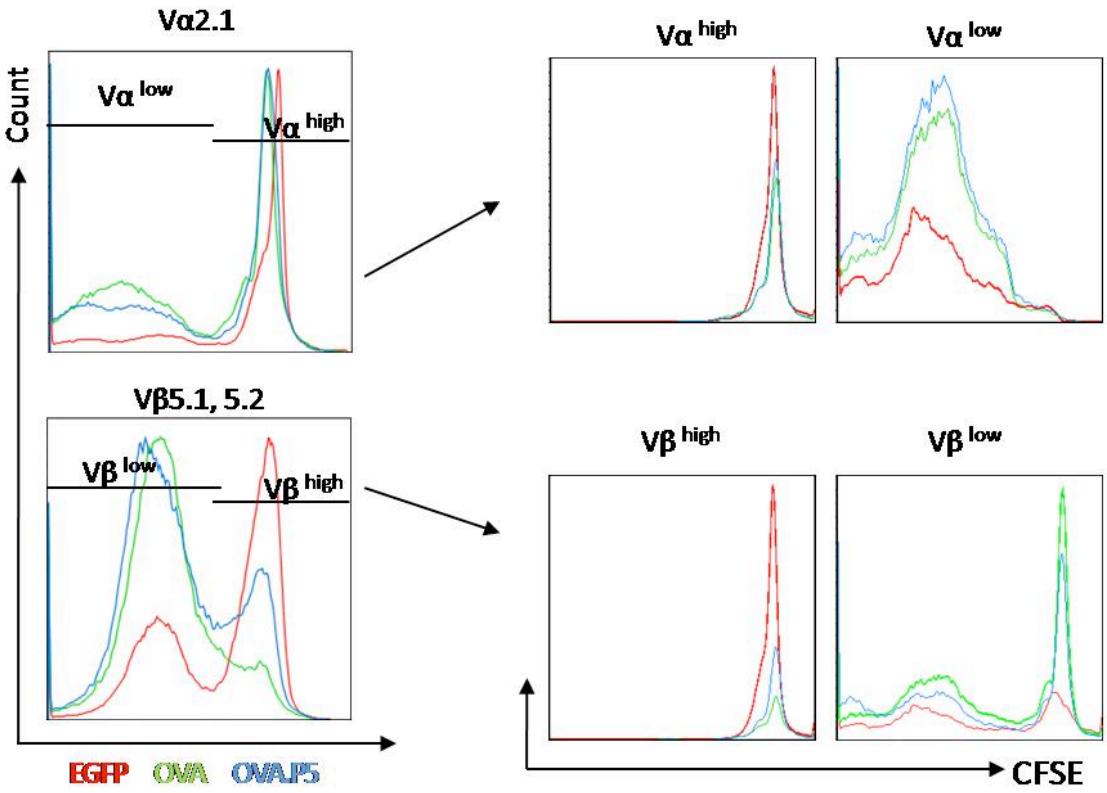
Figure 4.8. The effect of PDL1 knockdown in DCs on antigenic stimulation-induced TCR downregulation over time. *(A) Histograms of Vα2.1. and Vβ5.1,5.2 chains on days 2,4, and 7, populations regarded as positive for Vα2.1. and Vβ5.1,5.2 chains are marked with a bar (B) Bar graphs representing the percentage of cells from each group localized in populations positive for Vα2.1., Vβ5.1,5.2 and Vα2.1/Vβ5.1,5.*

Figure 4.9.

(A)


CFSE+ OT1 TRANSGENIC T-CELL
ENRICHED POPULATION
IvOVA/IvOVA.P5
2 DAYS
2-7 DAYS
FACS
ANALYSIS
BMDCs

(B)


Vα2.1
Count
Vα low
Vα high
Vβ5.1, 5.2
Vβ low
Vβ high
Vα high
Vα low
Vβ high
Vβ low
EGFP
OVA
OVA.P5
CFSE

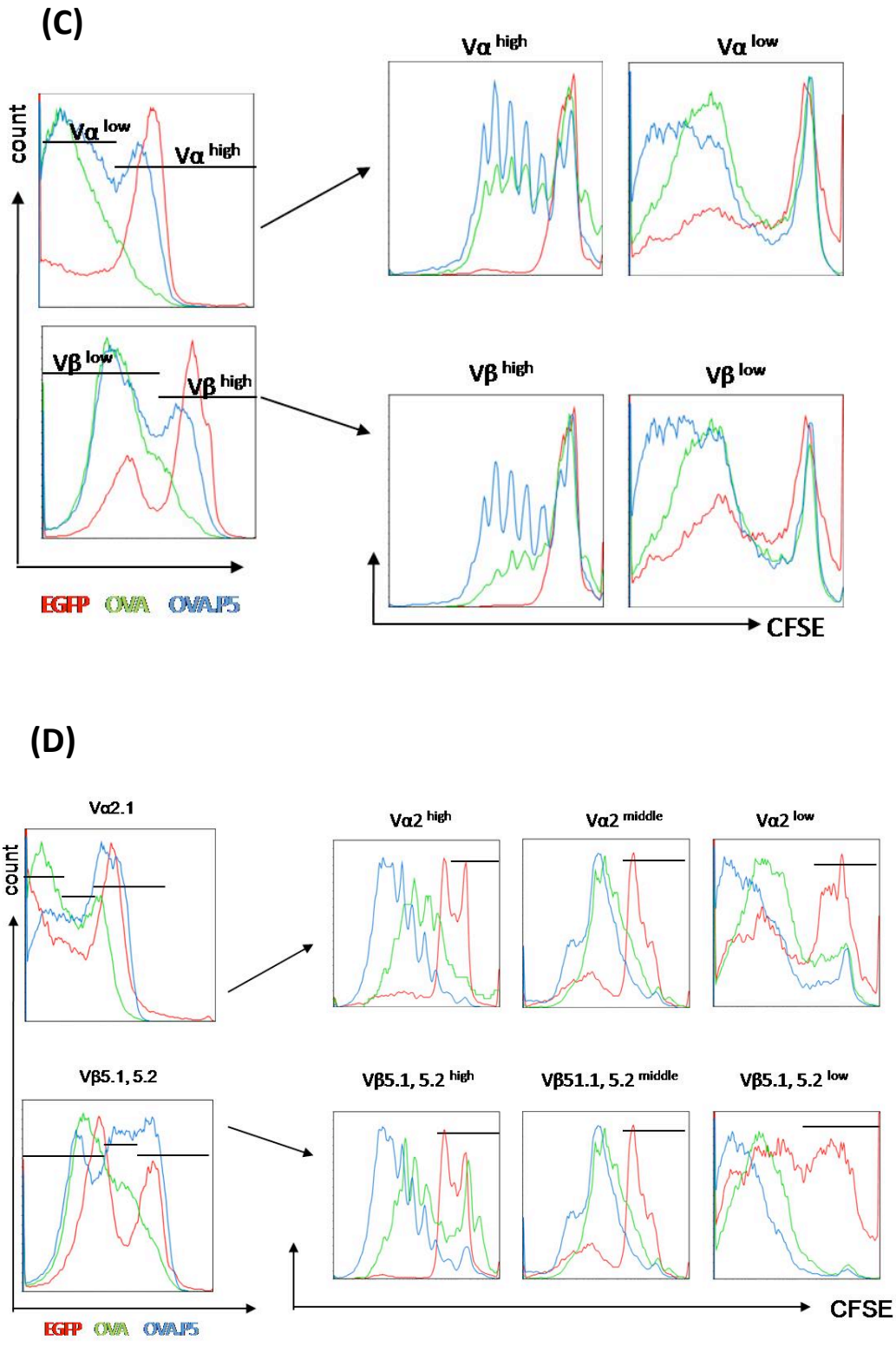


Figure 4.9. Effect of PDL1 knockdown in APCs on T cell proliferation *(A)Schematic drawing of experimental time-line. (B)Cell proliferation on day 2: Cells are gated on Vα2.1. and Vβ5.1, 5.2. TCR chains. Vα2.1.$^{high/low}$ Vβ5.1, 5.2.$^{hig/low}$ populations are analyzed for CFSE dilution. (C)Day 4: (D)Day 7. Cells in both lvOVA and lvOVA.P5 have considerably expanded compared to the control group.*

Figure 4.10.

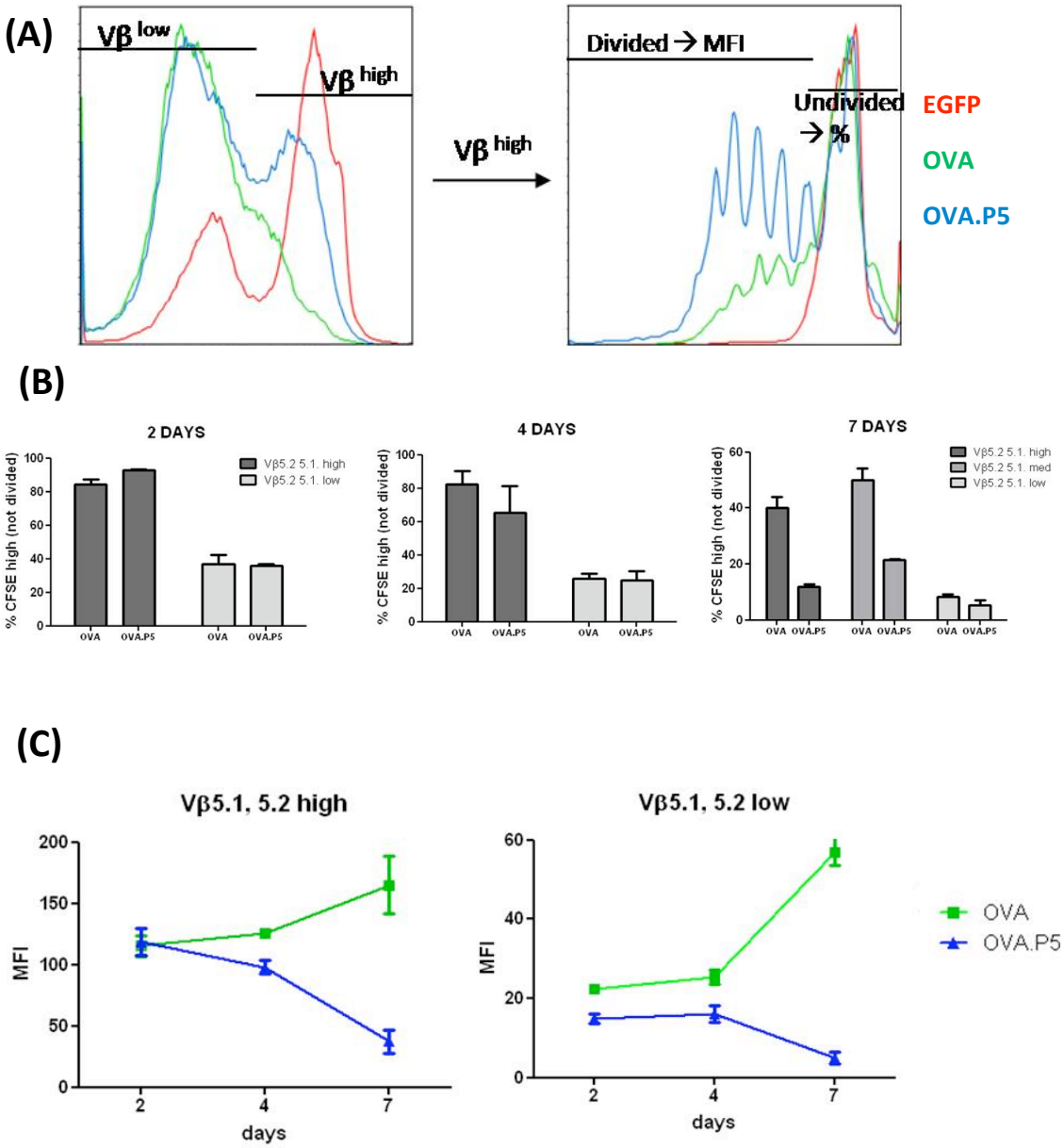


Figure 4.10. The effect of PDL1 knockdown on proliferation of co-cultured T cells. *Results are a representation of two independent experiments. (A) Schematic representation of analysis: cells were initially analyzed according to Vβ5.1,5.2 expression, CFSE plots were analyzed for the percentage of CFSE^{high} undivided cells and MFIs of divided cells (B) Bar graphs representing the percentage of undivided cells (C) graphs representing MFIs of divided cells.*

Higher end MFI can be explained by different FACS setting on the days of specific experiments.

4.3.6. PDL1 KNOCKDOWN EFFECT ON ANTIGENIC STIMULATION-INDUCED TCR DOWNREGULATION *IN VIVO*

In order to investigate whether PDL1 knockdown blocks TCR downregulation also *in vivo*, BMDCs were transduced with lvOVA and lvOVA.P5. Two days later transduced BMDCs were transferred subcutaneously into C57/BL6 mice. 7 days later CFSE-stained OT1 splenocytes were injected intravenously into the same mice. One week later spleens were collected and stained for Vα2.1 and Vβ5.1, 5.2. TCR chains (Fig. 4.11.A).

As expected, splenocytes in the control group had high expression of both Vα and Vβ chains as OT1 cells transferred into these mice did not encounter an APC expressing a specific antigen. In the lvOVA group, both Vα2.1 and Vβ5.1, 5.2. TCR chains were downregulated (Fig. 4.11.B) with MFIs equaling about 50% of the control group MFIs. In the lvOVA.P5 group, both chains were downregulated when compared to the control group. However, the downregulation in this group was lower than in the group without PDL1 knockdown.

4.3.7. THE EFFECT OF PDL1 KNOCKDOWN ON VACCINATION

As a potent anti-PDL1 shRNA was selected and showed to induce changes in dendritic cells, it was next tested in a vaccination model using ovalbumin as a control antigen. P5 shRNA was re-cloned into a vector expressing OVA (Fig. 4.12.A.) and termed lvOVA.P5. IFN-γ responses were evaluated with an ELISpot assay using 3 different time points: 7, 14 and 21 days. At day 0 mice were injected with 10^7 infectious units of lvOVA or lvOVA.P5. At indicated time points mice were processed and spleens were stimulated overnight with 50ng/ml SIINFEKL. Spleens from vaccinated mice were also stained with a pentamer and an anti-CD8 antibody to check for generation of TCR-specific T cells.

Even though both vectors generated good responses against ovalbumin, there were no significant differences in the magnitude of IFN-γ ELISpot responses between them at any of the time points (Fig. 4.12.B). However, pentamer staining showed an increase in the number of specific T cells over the control group for both lvOVA and

lvOVA.P5 with a trend for lvOVA.P5 to be higher (Fig. 4.12.C). To measure the fold increase over the control group, the percentage of generated specific T cells was divided by the percentage of pentamer positive T cells in the control group (Fig. 4.12.D.). For the lvOVA, the range was from 1.15 on day 7, through 1.4 on day 14, to nearly 2.2 on day 21. For lvOVA.P5 these values were higher: 1.6 on day 7, 1.85 on day 14 and 2.3 on day 21.

4.3.8. PDL1 KNOCKDOWN EFFECT ON TUMORAL THERAPY

The lv.OVA and lv.OVA.P5 vectors were also used for tumoural therapy. On day 0 mice were inoculated subcutaneously with 2×10^6 EG7.OVA cells and vaccinated with lentivectors on days 3 and 10.

Mice in the control group (lv.EGFP) had to be sacrificed on day 13 as the size of the tumours exceeded Home Office guidelines (Fig. 4.13.A). In the group vaccinated with lv.OVA, around day 10 tumours started to regress. This is consistent with the expected timeline of the generated anti-OVA response. Even though tumours completely disappeared in all of the mice in the group, they started to reappear around day 17 which was earlier explained to be a result of antigen loss in the cell line (Escors, Lopes et al. 2008). At day 40, 2 out of 5 mice were tumour-free. Similarly, in the group immunized with lv.OVA.P5, an initial tumour regression was observed consistent with the timeline of the responses. However, after initial regression phase some of the tumours regrew and finally, on day 40, 2 out of 5 mice were alive and tumour free. Vaccination with both lv.OVA and lv.OVA.P5 was significantly better in tumour treatment then vaccination with a control vector. However, there were no significant differences between those 2 groups (Fig. 4.13.B).

Figure 4.11.

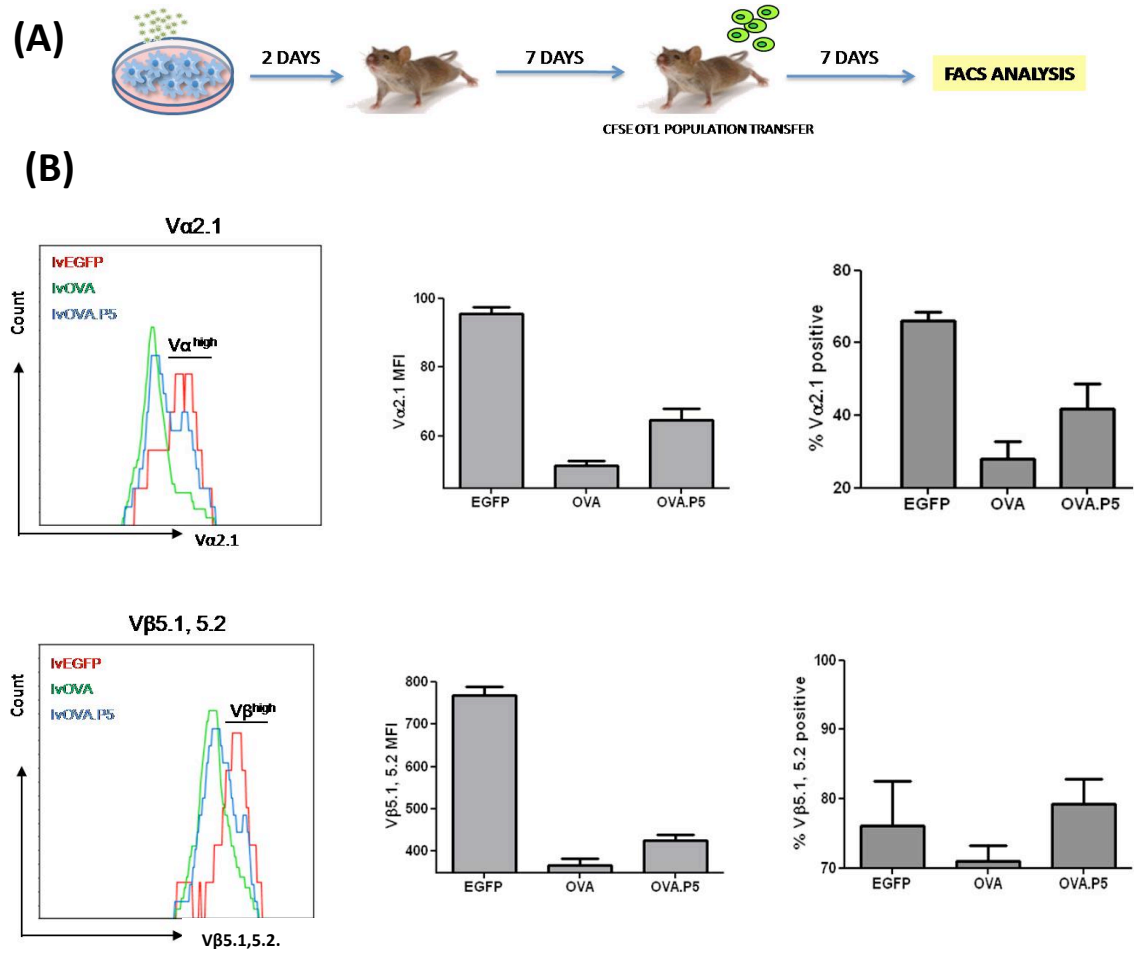


Figure 4.11. Effect of PDL1 knockdown on antigenic stimulation induced TCR downregulation in vivo. *(A)Schematic drawing of experimental time-line. BMDCs were transduced with lvOVA or lvOVA.P5 and 2 days later injected subcutaneously into C57/BL6 mice. Seven days later CFSE-stained splenocytes from OT1 transgenic mice were transferred intravenously into vaccinated mice and left to proliferate for another 7 days. After this time splenocytes from injected mice were collected and analyzed. (B)Vα2.1 and Vβ5.1, 5.2. staining of collected spleens. Cells were gated on live T lymphocytes population, followed by gating on CFSE positive cells before they were analyzed for Vα2.1 and Vβ5.1, 5.2.. Red bar – EGFP (negative control) ,green bar – lvOVA, blue bar – lvOVA.P5. Bar graphs show the percentage of Vα2.1^{high} and Vβ5.1, 5.2^{high}cells in Vα2.1 and Vβ5.1, 5.2 positive populations. Graphs show MFIs of Vα2.1 and Vβ5.1, 5.2 (total population).*

Figure 4.12.

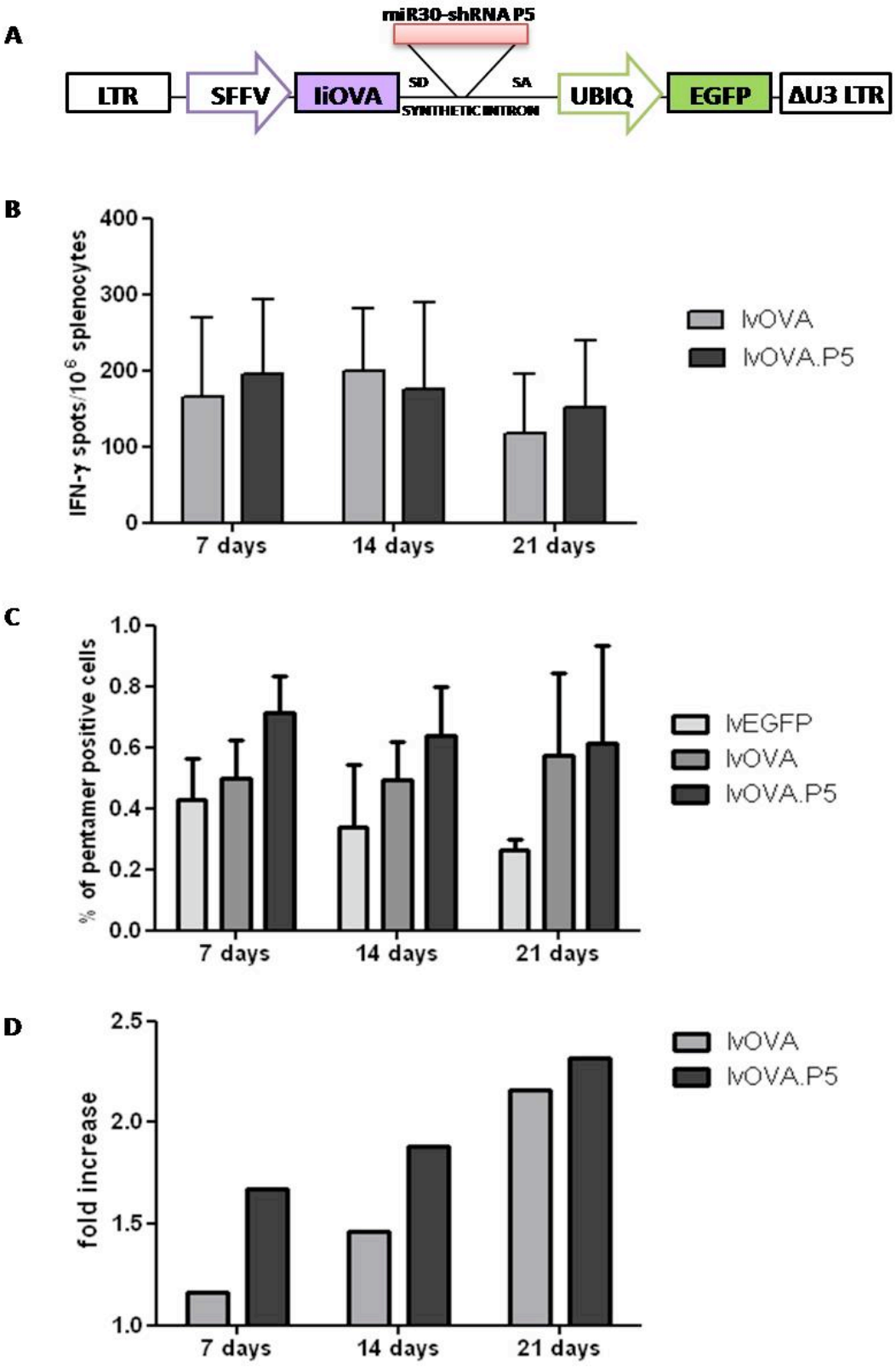

A
miR30-shRNA P5
LTR
SFFV
liOVA
SD
SA
SYNTHETIC INTRON
UBIQ
EGFP
ΔU3 LTR
B
IFN-γ spots/10^6 splenocytes
400
300
200
100
0
7 days
14 days
21 days
lvOVA
lvOVA.P5
C
% of pentamer positive cells
1.0
0.8
0.6
0.4
0.2
0.0
7 days
14 days
21 days
lvEGFP
lvOVA
lvOVA.P5
D
fold increase
2.5
2.0
1.5
1.0
7 days
14 days
21 days
lvOVA
lvOVA.P5

Figure 4.12. PDL1 knockdown effect on vaccination with a model antigen. *Data are representative of three independent experiments, each in triplicates. (A)Schematic drawing of vector used for vaccination and tumour experiments – lvOVA.P5. SFFV promoter drives IiOVA and shRNA P5 expression. (B)IFN-γ ELISpot. Results for days 7, 14 and 21. Y-axis: IFN-γ spots/10^6 splenocytes. Medium-grey bars: lvOVA, dark-grey bars: lvOVA.P5. (C)Percentage of SIINFEKL pentamer positive cells. Results shown for days 7,14 and 21. Light grey bars – lvEGFP (negative control), medium-grey bars: lvOVA, dark-grey bars: lvOVA.P5. (D)Increase in pentamer staining over control. To calculate the value of bars, the following equation was used: (%pentamer$^+$ cells in lvOVA or lvOVA.P5) / (%pentamer$^+$ cells in lvEGFP)*

Figure 4.13.

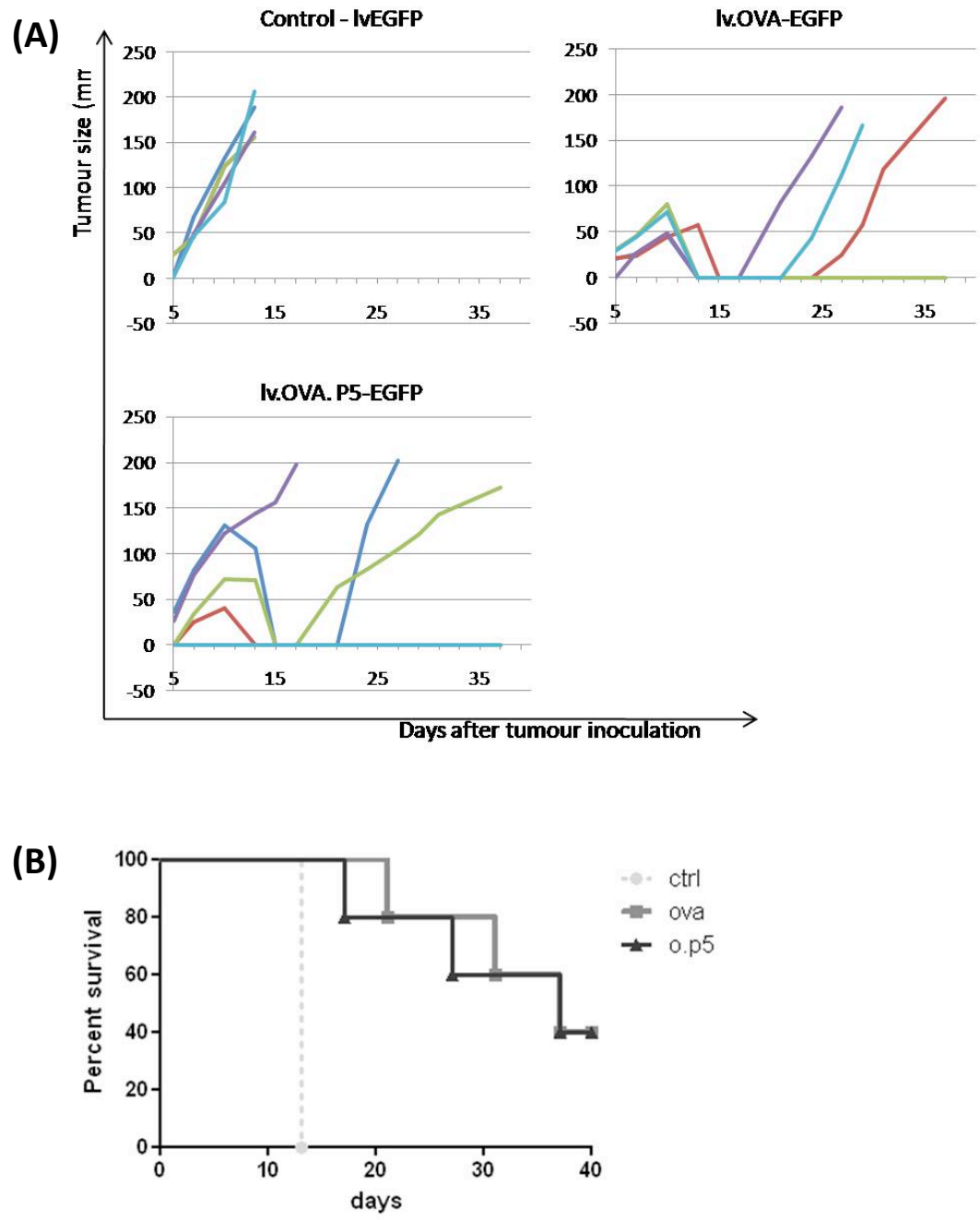


Figure 4.13. Effect of PDL1 knockdown in APCs on tumour treatment *(A)Tumour scores. Mice were inoculated subcutaneously with 2×10^6 EG7.OVA cells and vaccinated subcutaneously on days 3 and 10. Tumour scores were measured from day 5with a caliper and were calculated by multiplying height and width of the tumour. Tumour size is depicted on the Y-axis. X-axis shows days from tumour inoculation. (B)Kaplan-Meyer survival curve. No significant differences were found between lvOVA and lvOVA.P5 groups.*

4.4. SUMMARY

PDL1 is highly expressed on some tumours and its expression corresponds to poor prognosis in patients. Additionally, also tumour associated APCs tend to be both high in PDL1 and tolerogenic. All of these might limit expansion and functionality of T cells and induce apoptosis, inhibition and exhaustion of tumour draining lymphocytes. In recent years several studies blocking PD1 PDL1 pathway have been conducted and showed improvement in anti tumour responses. However, this kind of treatment requires long term administration of blocking antibodies. DC vaccination seems to be a promising strategy for treatment of tumours, but so far it has not been completely successful. This study combines DC therapy and blocking of PD1/PDL1 pathway by delivering short hairpin RNA against PDL1 to dendritic cells using lentiviral vectors as a gene transfer platform.

Four different sequences: P2, P3, P4 and P5 were tested for knockdown of PDL1 in BMDCs treated with LPS to induce cell surface expression of the co-inhibitory molecule. Based on this, P5 was selected for further experiments and cloned into a vector expressing a model antigen OVA. PDL1 knockdown is shown to induce changes in maturation status of dendritic cells. First of all, PDL2 is upregulated. Some studies show that PDL2 might act as a co-stimulatory molecule. However, there is a possibility that a reciprocal mechanism exists between PDL1 and PDL2. Therefore, it would be worth investigating whether PDL2 knockdown also induces changes in the expression level of PDL1. Furthermore, since there is still some confusion as to the role of PDL2 in stimulation and inhibition, perhaps knocking down PDL1 and PDL2 at the same time could contribute to a stronger effect in all experiments that follow.

Apart from upregulation of PDL2, PDL1 knockdown also induced changes in the levels of CD80 and ICAM-I. As the expression level of both of these molecules corresponds to the maturation level of a DC, this result suggests that PDL1 knockdown drives DCs towards a more mature phenotype. However, as the CD40 level was not changed, full maturation of those cells has not been achieved. Nevertheless, it is interesting to see that PDL1 knockdown induces changes already at the level of dendritic cells and not only alterations in T cell function. Interestingly, as high PDL1 expression on tumor associated APCs was shown to condition them towards being

tolerogenic, this study shows that knocking down PDL1 from dendritic cells drives DCs towards maturation.

Vaccination with a vector expressing ovalbumin and shRNA P5 was tested for T cell IFN-γ responses by ELISpot assay. Nevertheless, no significant differences were found between lvOVA and lvOVA.P5. However, pentamer staining of spleens used for ELISpot showed an increase in the level of pentamer positive T lymphocytes as compared to lvOVA vaccination. However, it is possible that this increase does not reflect the functionality of those T cells. LvOVA.P5 was further used in a tumor therapy experiment. In this study, mice were immunized after inoculation of tumours. No significant differences were found between survival of mice in lvOVA and lvOVA.P5 groups.

Despite the fact that PDL1 knockdown did not improve CD8+ IFN-γ responses, it decreased apoptosis and improved proliferation of cognate T lymphocytes in *in vitro* studies. In the apoptosis assay CD3+ OT1 cells were co-cultured with lvOVA/lvOVA.P5 transduced T cells and stained for propidium iodide and Annexin V at selected time points. At each of them, that is 2, 4 and 7 days, lvOVA.P5 group had a lower MFI of both PI and AnnexinV staining and a higher number of $PI^{-}AnnexinV^{-}$ cells. This implies that PDL1 knockdown in DC results in longer survival of T cells interacting with $PDL1^{-}$ DCs.

Likewise, transduction with lvOVA.P5 resulted in enhanced T cell proliferation at all time points. Interestingly, this study shows that inhibition of PD1 PDL1 pathway prevents downregulation of TCR chains from the surface of T cells. The same was observed for T cells that were transferred into mice vaccinated with lvOVA.P5. It is known that antigenic stimulation causes downregulation and degradation of TCR/CD3 complex (Salvatore Valitutti 1997) However, the precise mechanism of this phenomenon is not yet elucidated. Even though PD1 PDL1 pathway is believed to downmodulate TCR signaling to the T cell, to our knowledge this is the first study linking PD1/PDL1 signalling to regulation of degradation of TCR/CD3 complexes. However, further work is required to prove this.

In summary, we have identified potent shRNA inhibiting PDL1 and used lentivector as a tool to deliver it to dendritic cells. Even though PDL1 knockdown enhanced T cell survival and proliferation *in vitro,* this was not enough to generate higher CD8+ IFN-γ responses and improve survival in tumoural experiment. One could speculate that in those experiments injection of lentivector transduced DCs could give a better effect than direct immunization due to a low number of dendritic cells transduced by direct immunization. An even better system would consist of dendritic cells isolated from PDL1 knockout mice. Those could be transduced with lvOVA to express OVA antigen.

Several experiments could be conducted to complete the study. First of all, assays checking the change in cytokine production in T cells cultured with dendritic cells having PDL1 knocked-down could be run. It would be especially interesting to observe the levels of IFN-γ, IL-2 and TNF-α. Also, the change in cytokine secretion by dendritic cells could be measured. Moreover, a comparison of lentivector mediated PDL1 knockdown and antibody-mediated blockade of PDL1 should be investigated. The effect of PDL2 knockdown and a combination of PDL1/PDL2 knockdown should be checked.

This thesis does not investigate the CD4+ T cell responses which can be regarded as a major limitation. It would be crucial to check the influence of PDL1 knockdown in dendritic cells on the function of CD4+ T cells as it is thought that PD1/PDL1 pathway has a bigger influence on these cells than on CD8+ T cells. Dendritic cells from PDL1 knockout mice stimulated stronger CD4(+) T cell responses compared to dendritic cells from wild-type mice as measured by the number of cells as well as cytokines produced by the cells. Moreover, studies using EAE model showed that PD-L1 expression on T cells and host tissues limits responses of self-reactive CD4+ T cells *in vivo*. This stresses the critical role of PDL1 in T cell tolerance (Latchman, Liang et al. 2004). Futhermore, PDL1 is essential for induction of iTregs as PD-L1$^{-/-}$ antigen-presenting cells are not fully able to convert naive CD4 T cells to iT reg cells (Francisco, Salinas et al. 2009). Therefore, it would not only be very interesting to check CD4+ T cell responses in the model used in this thesis (by for performing IL-4 ELISpot) but it would also be crucial to test PDL1 knockdown in APCs in a more natural tumour model that involves peripheral tolerance to the tumour –associated antigen as the OVA-based model used in this thesis is artificial and thus very immunogenic.

Chapter 5. FINAL DISCUSSION AND SUMMARY

This thesis has explored the possibility of using integration-deficient lentiviral vectors for vaccination, as well as using lentivectors to deliver shRNA against the inhibitory molecule PDL1 to enhance immune responses to the antigen encoded on the same vector.

Since there is a constant need for better vaccines, this thesis was meant to develop the two ideas described here. I will now go on to summarize the results of both parts and present parallel developments in the fields.

5.1. SUMMARY AND CONCLUSIONS

The first part of the thesis dealing with development of non-integrating lentivectors (NILVs) for immunization (chapter 3) was based on the previous research on using NILVs for gene delivery (Philippe, Sarkis et al. 2006; Yanez-Munoz, Balaggan et al. 2006; Apolonia, Waddington et al. 2007). The idea of using vectors rendered to be integration-deficient stems from the fact that gene therapy trials using retroviral vectors induced T cell leukaemia in several patients. Those events showed a need for an enhanced-safety vector. Since lentivectors can infect non-dividing cells, such as dendritic cells *in vivo,* they are a promising candidate for immunotherapy based on the induction of cellular and humoral responses to antigen encoded on the vector.

The aim of the first part of this thesis (chapter 3) was to find out whether vaccination with integration-deficient vectors could be used as a successful immunization strategy. A model antigen – ovalbumin (OVA) was used in this work.

At first, vectors harbouring different mutations in either the integrase or the attachment sites were used to test the strength of the response elicited upon immunization with those vectors. IFN-γ ELISpot was performed 11 days after vaccination. Each vector was injected with 3 different doses. These experiments revealed that even though all vectors elicited specific immune responses, a much higher dose of each of the non-integrating vectors had to be used to achieve the same

outcome of vaccination as with integrating vectors. Surprisingly, vaccination using integration-deficient vectors co-expressing activators of p38 and NFKB pathways did not generate higher response at 11 days post–vaccination.

Despite the fact that NILVs elicited weaker responses than their integrating counterparts, I went on to test them in a variety of functional assays. First, I compared the duration of antigen presentation following immunization with equivalent (high) doses of the integrating vector and the DNW/2Δatt vector and found that at 5, 21 and 30 days after vaccination the expression of antigen from both vectors resulted in similar proliferation of cognate T cells injected into previously immunized mice.

Next, the integrating vector, the DNW/2Δatt, MKK6/DNW and vFLIP/DNW vectors were tested in a model where the tumour cell line EG7.OVA was first injected and immunizations followed 3 and 10 days later. As predicted, the integrating vector gave a significantly enhanced survival compared to the DNW/2Δatt vector. However, addition of vFLIP and MKK6 to the non-integrating vector strongly improved survival. Further experiments (IFN-γ ELISpot and *in vivo* bioluminescence imaging) confirmed that including a DC activator results in an improved migration of transduced dendritic cells leading to a “faster” response.

Overall, this part of my research shows that integration-deficient vectors might be engineered to elicit strong immune responses that are sufficient to eliminate tumours in mice.

5.1.2. PDL1 KNOCKDOWN FOR ENHANCEMENT OF RESPONSES TO VACCINATION WITH LENTIVECTOR

While a part of chapter 3 of this thesis dealt with providing additional activation to enhance immune responses, chapter 4 deals with blocking the inhibitory reaction following immune response. It focuses on manipulation of T cell – DCs interactions to improve responses to vaccination. To achieve this, lentivector was not only used as a tool to deliver antigen to DCs, but also as a platform to express shRNA against PDL1 thus knocking it down in infected cells.

Initially, 4 different shRNAs were chosen and cloned into a lentivector. Of those four, 1 was selected for further experiments and called P5. Transduction of dendritic cells with P5 resulted in about 70% reduction in the levels of PDL1 induced on DC surface after stimulation with LPS. It also drove DCs towards a partly matured phenotype. Vaccination with P5 cloned into a lentivector expressing OVA resulted in an increased number of pentamer specific CD8+ T cells compared to vaccination with lv.OVA, despite the fact that there was no improvement in IFN-γ responses as measured by ELISpot assay. Moreover, PDL1 knockdown did not improve survival of mice injected with EG7.OVA tumour cell line. However, in *in vitro* experiments when T cells were co-cultured with DCs transduced with lentivector expressing anti PDL1 shRNA, they proliferated more than T cells co-cultured with DCs transduced with lentivector without anti PDL1 shRNA. Most importantly, the knockdown partially blocked TCR complex degradation induced by antigenic stimulation. However, those experiments will need to be repeated in a setting where lvOVA will be replaced by vector co-expressing ovalbumin and a scramble shRNA. Even though the shRNA expression system used in these experiments is designed to retain the level of antigen expression, when I compared the levels of YFP expressed from lvYFP and lvYFP.P5 vector, lvYFP.P5 expressed only about a half of the protein quantity of lvYFP. However, since PDL1 knockdown resulted in improved T cell proliferation and it is known that higher antigen doses stimulate more proliferation, it is likely that if we achieved the same antigen expression levels the effect would be even more pronounced (Henrickson, Mempel et al. 2008; Zheng, Jin et al. 2008).

5.2. PARALLEL ADVANCES IN THE FIELD

5.2.1. INTEGRATION-DEFICIENT VECTORS

Several reports of using integration deficient vectors for vaccination have been published in the past 3 years. 1 year after I started working on this project, Negri and Cara used NILVs to deliver HIV-1 envelope sequences into BALB/c mice and managed to get long-lasting cellular and humoral responses, even if somewhat lower than those elicited by the integrating counterparts (Negri, Michelini et al. 2007).

Just around the time our data was submitted for publication Coutant and Charneau elicited robust B cell response and full protection from challenge with a lethal dose of West Nile Virus after single administration of NILV expressing the secreted form of the envelope of a virulent strain of West Nile Virus (Coutant, Frenkiel et al. 2008). Hu and Wang also used a model similar to ours and confirmed our results several months after this study was published (Hu, Yang et al. 2009). The group of Andrea Cara engineered SIV-based integrase deficient vectors (Michelini, Negri et al. 2009). EGFP was used as an antigen and single intramuscular administration elicited specific, long-lasting, poly-functional responses. Finally, the same group showed transduction of human antigen presenting cells, such as monocyte-derived DCs and marcrophages using NILVs expressing influenza matrix M1 protein (Negri, Bona et al. 2010) which resulted in induction of *in vitro* expansion of M1-primed CD8+ T cells.

5.2.2. PDL1 KNOCKDOWN

The idea of using siRNA to reduce the levels of PDL1 or PD1 has only been developed recently. Breton and Steinman electroporated PDL1 and PDL2 directed siRNA into monocytes and immature DCs which were later added to PBMCs extracted from HIV-1 infected individuals and cultured for 6 days with HIV-1 Gag p24 peptides. The knockdown resulted in only modest enhancing effect (Breton, Yassine-Diab et al. 2009).

Borkner et al. knocked down PD1 using retroviral vectors in both murine and human dendritic cells. In mice, the procedure resulted in increased proliferation and IFN-γ production. In the human model siRNA against PD1 was introduced into PBLs along with MART-1 specific 1D3 TCR. It resulted in a higher frequency of IFN-γ secreting cells and higher CD107a expression (Borkner, Kaiser et al. 2010).

At the time when I was writing this thesis, a very similar study was published using a human system (Hobo, Maas et al. 2010). SiRNA sequences were electroporated into immature DCs resulting in a knockdown of PDL1 and PDL2. This resulted in augmented IFN-γ and IL-2 production by stimulated T cells and improved proliferation and cytokine production of specific $CD4^+$ T cells.

5.3. FUTURE PERSPECTIVES AND LIMITATIONS OF THE MODEL

There is a significant interest in development of novel vaccination strategies. Lentivectors are very promising candidates as they are efficient for antigen delivery to dendritic cells – the main initiators of immune responses. This thesis has tested the potential of NILVs as a vaccination strategy. Even though they are not as potent as their integrating counterparts, I presented here that inclusion of additional molecules can make them a powerful treatment for patients with cancer. Also improvement of the level of gene expression from NILVs would enhance their efficacy. Because of their safety profile, their use does not need to be limited to treating tumours. In the future they will likely be applied to patients with AIDS or other infectious diseases.

In the second part of this thesis I showed that knocking down PDL1 in DCs influences co-cultured T cells. The *in vitro* experiments seemed promising. Nevertheless, when this strategy was applied to treat tumours in mice, it did not have an effect on the survival of used animals. However, in the experiments performed in this thesis lentivectors expressing siRNA against PDL1 were injected directly into mice. It is very likely that the effect of these immunizations would be stronger if the lentivectors were used to transduce dendritic cells *ex vivo* followed by injection of DCs. In fact, experiments performed after this thesis was submitted showed that this was indeed the case. Both IFN-gamma responses and anti-tumour responses were strongly improved compared to the ones elicited by injections of dendritic cells transduced with lentivectors epressing only the antigen (no PDL1 siRNA). This could be explained by the low number of cells transduced upon direct immunization. Even though lentivectors easily transduce dendritic cells *in vivo,* other cells also become affected. In fact, I have noticed that after direct immunization the same percentages of MHCII/CD11c+ dendritic cells and CD19+ B cells are transduced (data not presented in this thesis). This “dilutes” the effect of PDL1 knockdown in dendritic cells. The importance of PDL1 expression on B cells has not so far been investigated.

This thesis investigated the influence of PDL1 knockdown on DC phenotype. However, the effect of the lack of PD1-PDL1 signalling on T cell phenotype was not checked. As mentioned earlier, PD1 is one of the T cell exhaustion markers. Thus, it would be extremely interesting to check the phenotype of Ag-specific T cells generated

in vivo through immunization with lv-OVA.P5 and to investigate whether these cells can become exhausted *in vivo.* Likewise, it would be worth to investigate the effect of PDL1 knockdown in APCs on T cell apoptosis and proliferation *in vivo* to confirm the *in vitro* results.

One of the effects of PDL1 knockdown on the PD1-PDL1 signalling described in this thesis is a partial blockage of antigen stimulation induced TCR-CD3 down-regulation on T cells. This phenomenon has not been previously described and its effects on T cells are not known. Also, the mechanism used by PD1-PDL1 signalling to regulate TCR levels has not been described. These two questions should be investigated and the precise mechanisms of these phenomena should be elucidated.

Last but not least, it would be interesting to investigate the effect of PDL1 knockdown in APCs on T cell function in chronic infections associated with T cell exhaustion

In this thesis OVA was used as a model antigen in mice as a proof of principle for both immunization with integration-deficient vectors and with vectors knocking down PDL1 in dendritic cells. In the mouse system OVA is a foreign antigen, thus it is highly immunogenic. Therefore, it is much easier to elicit strong responses against OVA then against a self-derived antigen. Also, a successful immunization to a self-derived antigen would require a higher dose of the antigen. This could be problematic in the context of integration-deficient vectors since as shown in this thesis much higher doses of NILVs are required to obtain the same results as those obtained by using integrating vectors for immunization. Another problem that was not addressed here, but should be dealt with if using self-derived antigens, is peripheral tolerance to “self”. As PD1-PDL1 pathway is involved in tolerance, it is possible that PDL1 knockdown could result in effective immunization in such a model. However, this remains to be tested.

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