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Cancer vaccines: using lentiviral vectors to deliver antigens to dendritic cells *in vivo.*

By

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Abstract

The contention that cancers are immunogenic is still being debated: one view states that cancers evolve to evade immune surveillance, while the other claims that cancers actively induce tolerance. A unifying hypothesis is that immune surveillance operates early on in carcinogenesis, but eventually tumours adapt to shift the balance from activation to tolerance. Cancer vaccines therefore, must stimulate a tumour specific response and break active tolerance mechanisms.

Cancer vaccines were initially comprised of tumour associated antigens (TAAs), but since these were poorly immunogenic, they were replaced with dendritic cell (DC) vaccines loaded with TAAs (adopted because DC prime naïve T cells). However, such DC-based vaccines are impractical to produce and have not proved particularly effective. These disappointing results have led to the search for more innovative vaccine strategies. Viral vectors encoding TAAs are promising vaccine candidates because they can infect antigen presenting cells (APCs) *in vivo*; they may also signal "danger" to the immune system through pathogen recognition pathways.

This thesis is concerned with the development of vaccines based on lentiviral vectors (lentivectors); such vectors are readily used for gene therapy because they can stable modify non-dividing cells. The aim of this project was to assess their ability to deliver antigens to dendritic cells *in vivo* and to stimulate effective T cell responses. The findings of this thesis are that:

- Directly injected lentivectors can stimulate CD4+ (and CD8+) T cell responses to their encoded antigen if it is targeted into the endocytic pathway.
- DC stably present antigen *in vivo* following vector immunisation.
- DC can be induced to mature by including an NF-κB activator (vFLIP) in the vector.

These results support the development of lentivirus-based cancer vaccines.

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I, Helen Rowe, 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 the thesis.

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Abbreviations

AAV	Adeno-associated virus
AdV	Adenovirus
APC	Antigen presenting cell
BM	Bone marrow
cDC	Conventional DC
cFLIP	Cellular caspase-8 (FLICE)-like inhibitory protein
CMV	Cytomegalovirus
ConA	ConcanavalinA
cPPT	Central polypurine tract
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DED	Death-effector domain
DISC	Death-inducing signaling complex
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
GFP	Green fluorescent protein
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HSV	Herpes simplex virus
IFA	Incomplete Freunds' adjuvant
IFN	Interferon
Ii	Invariant chain
IKK	IκB-kinase
IL	Interleukin
IS	Immune system
KIRs	Killer cell immunoglobulin-like receptors
KSHV	Kaposi's sarcoma-associated herpesvirus
Lentivector / LV	Lentiviral vector
LB	Luria broth
LN	Lymph node
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCA	Methylcholanthrene
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLV	Murine leukaemia virus
MOI	Multiplicity of infection

MVA	Modified vaccinia virus Ankara
NF-κB	Nuclear factor of κB
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid DC
PEL	Primary effusion lymphoma
PRR	Pathogen recognition receptor
RANK	Receptor activator of NF-κB
*RHD	Rel homology domain
RPMI	Roswell Park Memorial Institute 1640 medium
SCID	Severe combined immuno-deficiency disorder
SD	Standard deviation
SFFV	Spleen focus-forming virus
siRNA	Short interfering RNA
STAT	Signal transducer and activator of transcription
TAAs	Tumour associated antigens
TAP	Transporter associated with antigen processing
TAR	Transactivation response element
TCR	T cell receptor
TfR	Transferrin receptor
TILs	Tumour infiltrating lymphocytes
TLR	Toll-like receptor
TRANCE	Tumour necrosis factor-related activation-induced cytokine
vFLIP	Viral caspase-8 (FLICE)-like inhibitory protein
VSV-G	Vesicular stomatitis G protein
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element

RCV	Replication competent virus

Chapter 1: Introduction

1.1 Cancer and the immune system

Cancer is the most common cause of death in the UK with a lifetime risk of more than one in three of developing it. Cancer is primarily a disease of old-age; usually its onset is prevented by host defence mechanisms, which are subject to deterioration throughout life. We know that both intrinsic (cell cycle regulation) and extrinsic (immune surveillance) defence programmes exist to keep cancer at bay (reviewed in (Pardoll, 2003)) and these are described below. Understanding our natural defences against cancer will help us to develop a cure.

1.1.1 Carcinogenesis

Development of cancer is a multi-step process caused by the gradual erosion of genes, in particular genes involved in guarding the genome and in cell cycle control (reviewed in (Osada and Takahashi, 2002)). It is usual for the integrity of our DNA to repeatedly become compromised by three main causes: environmental agents (such as ultraviolet light and genotoxic chemicals in cigarette smoke), normal cellular metabolism (reactive oxygen species), and spontaneous damage (deamination of cytosine to uracil for instance) (Hoeijmakers, 2001). Genetic predisposition also plays a role in some cancers. However, stringent check-points exist to ensure that any DNA errors are corrected: either the cell cycle is arrested, while DNA repair pathways operate or apoptosis ensues as a last resort when the DNA damage suffered is too severe to repair (Zhou and Elledge, 2000). The most notable tumour suppressor gene involved in these check-points is *p53*; upon DNA damage, *p53* is activated (through ATM and ATR kinases) leading to effects such as G1 / G2 cell cycle arrest, DNA repair, senescence (irreversible cell cycle arrest) or programmed cell death (Hussain and Harris, 2000). It is easy to see why *p53*, which is mutated in about 50% of all human cancers (Hussain and Harris, 2000), is known as the guardian of the genome. Bypassing *p53* is a means towards immortality, although precancerous cells are still limited by other constraints such as the progressive shortening of their telomeres (Hakim et al., 2004). The development of

malignant cancer, defined by angiogenesis and metastasis, depends on a combination of epigenetic changes, (which means that expression of genes can be dramatically altered), as well as a series of genetic changes.

Ageing is one of the biggest risk factors for cancer (Shay and Roninson, 2004) because DNA accumulates changes over time; it seems almost inevitable that genes critical for preventing oncogenesis will eventually suffer damage, leaving the individual prone to cancer. Stem cells in various tissues could be particularly vulnerable to DNA damage, due to their long life-span (Beachy et al., 2004). However, even if a renegade cell escapes DNA repair and cell cycle control, it should still be subject to attack by the immune system (IS). Or should it? The concept of immune surveillance of tumours is introduced below.

1.1.2 The "cancer immunosurveillance" hypothesis

The idea of "cancer immunosurveillance" is attributed to Paul Ehrlich, who thought that the IS continually operates to eliminate precancerous cells. The theory was formally proposed in 1967 by Burnet and Thomas, after the discovery that mice could reject tumour transplants from syngeneic mice. Their hypothesis was immediately put to the test using animal studies, and was considered to be conclusively disproved after studies by Stutman (Stutman, 1974), and then by Rygaard and Povlsen (using >1000 mice) showed no difference in the incidence of spontaneous tumours between nude and wild-type mice, over a study period of 3-7 months (Rygaard and Povlsen, 1976). In hindsight, there were a few flaws in this study: nude mice, which lack a thymus, still have a detectable population of functional T cells, as well as possessing other functional immune compartments such as NK cells, and the monitoring period was probably too short to see an increase in tumour incidence.

Since the 90s, compelling evidence (detailed below) for immune surveillance of tumours has emerged as a result of more elegant animal studies, and this has rekindled an interest in cancer immunotherapy.

1.2 Evidence for anti-tumour immunity

1.2.1 Animal experiments

Firstly, a body of data in the 90s convincingly showed that IFN-γ could protect the host against tumours (Dighe et al., 1994; Street et al., 2001). For example, in mouse models of MCA (methylcholanthrene)-induced tumours, mice that lacked either the IFN-γ receptor or STAT-1 (signal transducer and activator of transcription 1), which is involved in mediating IFN-γ receptor signalling, were 10-20 times more prone to develop tumours than wild-type mice (Kaplan et al., 1998).

Secondly, perforin-/- mice were also more prone to MCA-induced tumours than wild-type mice (Street et al., 2001; van den Broek et al., 1996); T cells and NK cells release perforin in order to lyse targets. It was next shown that RAG-2-/- mice, which lack NKT cells, T cells and B cells, due to an inability to rearrange their antigen receptor genes, were also more prone to tumour development (Shankaran et al., 2001). Finally, a series of recent experiments have shown that NKT cells, γδ T cells, NK cells, αβ T cells and IL-12 are important in tumour protection (Girardi et al., 2001; Noguchi et al., 1996; Smyth et al., 2000).

1.2.2 Clinical data

It is well known that in some patients there is spontaneous regression of melanoma and biopsies of regressing tissues have revealed the presence of lymphocytic infiltrates (Jager et al., 2000; Kawakami et al., 1998; Wang et al., 1996). Although this is not proof that regression was induced by an immune response, tumour infiltrating lymphocytes can kill tumour targets *in vitro* (Mackensen et al., 1994; Romero et al., 1998; van der Bruggen et al., 1994). Additionally, vitiligo, an autoimmune reaction against melanocytes, is known to be a sign of improved prognosis in melanoma patients (Duhra and Ilchyshyn, 1991; Nordlund et al., 1983; Romero et al., 1998).

The incidence of tumours in immunosuppressed vs. immunocompetent individuals has been documented in order to look for evidence of cancer immunosurveillance in humans. Unsurprisingly, the incidence of virally induced tumours, such as Kaposi's sarcoma, is higher in immunosuppressed individuals, but there is also some evidence for an increase in nonviral cancers, such as malignant melanoma (Sheil, 1986) and lung cancer (Pham et al., 1995).

1.2.3 Current view on the anti-tumour response

The evidence outlined above has shown that both the innate and adaptive immune systems are involved in the anti-tumour response.

Firstly, if precancerous cells avoid programmed cell death, they are likely to be faced with innate effector cells, such as NK cells, macrophages and γδ T cells. These cell types express receptors (e.g. NKG2D-DAP10) that can interact with stress-induced ligands, like MICA and Rae-1 that are induced on transformed cells (Diefenbach et al., 2000; Groh et al., 1999). Receptor ligation will lead to perforin release by NK cells and apoptosis of the transformed cell. NK cells can also kill tumour cells deficient in MHC I because interaction of MHC I with KIRs, (killer cell immunoglobulin-like receptors) on NK cells provides a negative signal (Ljunggren and Karre, 1990). NKT cells, which are a subset of T cells expressing some NK cell markers, regulate tumour cytotoxicity by secreting T helper 1 and 2 cytokines (Godfrey et al., 2000). They recognise CD1d in the context of glycolipid ligands (αGalCer) (Nakagawa et al., 1998). The NKT cell TCR is restricted to comprise an invariant Vα14-Jα28-1 TCRα chain with oligoclonal Vβ8.2, Vβ7 and Vβ2 TCRβ chains. Notably, there is evidence that NKT cells promote tumour progression (as well as anti-tumour immunity) by secreting IL-13 (Ahlers et al., 2002; Terabe et al., 2000).

Secondly, if the innate arm of immunity fails to eliminate tumour cells, they should fall prey to adaptive immunity, given the vast number of neoantigens known to arise from genetic and epigenetic changes. Such antigens could be shed from tumour cells or become exposed after lysis, leading to their processing by DC and presentation on MHC

class II or cross-presentation on MHC class I; DC should in parallel receive cytokine signals from NK cells, for instance. Both CD4+ and CD8+ T cells have been shown to be crucial for anti-tumour immunity (Gao et al., 2002; Surman et al., 2000) and more recently antibodies have also been shown to play a role (Chen et al., 1998; Montgomery et al., 2005).

It is clear that the IS is involved in keeping cancer at bay, but obviously something is missing in order for us to succumb to this disease.

1.3 Immune surveillance of cancer: why is it so ineffective?

Evidence for immunosurveillance (outlined above), along with the discovery of tumour-associated antigens (TAAs), an appreciation of the danger model (Matzinger, 2002), and a revived interest in regulatory T cells have shaped our current view of the anti-tumour response. These separate areas of research collectively show why the innate and the adaptive arms of the IS are limited in their response to tumours.

1.3.1 Tumour-associated antigens (TAAs)

Tumour antigens are known as tumour-associated antigens (TAAs), rather than tumour-specific antigens because they are not necessarily restricted to tumours, and they can be self-antigens as well as neoantigens. TAAs can be defined as antigenic changes that distinguish tumour cells from normal cells thereby eliciting an immune response. Thanks to the pioneering work of Boon and others (Barth et al., 1990; Boon, 1993; Wolfel et al., 1993), a plethora of TAAs have been discovered, which can be divided into 4 groups:

- Tissue-specific differentiation antigens, which are over-expressed on tumour cells (e.g. the melanocyte proteins: Melan-A/MART-1 and gp100, in melanoma).
- Neoantigens created due to genetic instability (e.g. a point mutation in myosin)
- Cancer-testis antigens, expressed in various cancers and during embryonic development, but not in normal adult tissues, except testis (e.g. NY-ESO-1).

- Viral antigens, expressed in viral induced cancers (e.g. the HPV, human papillomavirus proteins: E6 and E7).

TAAs are normally poorly immunogenic (because they are derived from self), but nevertheless, there are plenty of examples of cancer patients harbouring tumour-infiltrating T lymphocytes (TILs) reactive to TAAs; for example, CTL lines derived from melanoma patients were found to lyse tumour cells bearing the cancer-testis antigen, MAGE-1 *in vitro* (van der Bruggen et al., 1994). Interestingly, some melanoma patients, whose tumours regressed, were found to have a strong T cell response to certain neoantigens: for example, one patient was responding to myosin that contained a point mutation (Zorn and Hercend, 1999)and one patient was found to respond to β-catenin with a point mutation (Robbins et al., 1996). TAA-reactive CTL are normally only found in cancer patients; an exception is in the case of Melan-A specific T cells, which are also present in healthy volunteers at a lower frequency (Kawakami et al., 1994). Either way, good prognosis is associated with the presence of TAA-specific TILs, although clinical responses are only seen in some patients (Pardoll, 2003). The clinical outcome most probably depends on the balance of the anti-tumour response vs. the degree of tolerance.

1.3.2 The danger model

According to the self / non-self paradigm, the IS is ignorant of tumours because they are self (except where they have a viral cause). Therefore, the discovery of TAAs encouraged immunotherapy, because it was thought that vaccines employing these antigens could re-educate the IS to see cancer as non-self. However, results have shown that we still cannot empower the IS to effectively target cancer. There are many possibilities why immunotherapy has proved poorly effective, but an attractive one is that tumours lack "danger signals" such as pathogen-associated molecular patterns (PAMPs), which are recognised by pathogen recognition receptors (PRRs). An example of a PAMP is LPS (lipopolysaccharide), which mediates innate immunity and triggers adaptive immunity. The most important PRRs are the TLR family, described in chapter 5.

According to Matzinger's "danger" model (Matzinger, 2002), the IS is more adept at sensing danger than the difference between self and non-self. Danger signals, (such as proinflammatory cytokines, heat shock proteins and reactive oxygen intermediates), are thought to be released by stressed and necrotic cells as well as after infection; these signals are relayed to the adaptive IS through dendritic cells (DC), which up-regulate co-stimulatory molecules. The default outcome is considered to be tolerance in the absence of danger, which explains why tumours eventually succeed in inducing tolerance, if they adapt to avoid emitting danger signals.*

Wide-spread adoption of the danger model has redefined our sense of self and has re-inspired hope for cancer immunotherapy. If we can introduce the right danger signals into our vaccines, surely we can alert the IS to the danger imposed by cancer and harness it to eliminate altered self?

1.3.3 Tumours induce tolerance?

There is evidence to suggest that the IS does not react to tumours because of peripheral tolerance. This view conflicts with the evidence for tumour evasion, which implies that immunosurveillance is exerting a selective pressure on tumours, thereby driving tumour escape. An important study by Levitsky and colleagues showed that T cells specific for influenza haemagglutinin (HA) were rendered anergic upon their adoptive transfer into mice that had HA-expressing lymphomas, or HA-expressing renal carcinomas (Sotomayor et al., 2001; Staveley-O'Carroll et al., 1998). Other similar experiments have shown that T cells transferred into mice with tumours remain ignorant, or become anergic, or are deleted (Nguyen et al., 2002; Robinson et al., 2001; Wick et al., 1997). Recently, Willimsky and Blankenstein developed a mouse model, where sporadic tumours arose after rare spontaneous activation of a dormant oncogene (SV40 T antigen). In these mice, tumours were not rejected, but rather actively induced tolerance (Willimsky and Blankenstein, 2005).

One breakthrough in understanding tolerance came from the re-discovery of a population of regulatory T cells (Tregs) that could effectively block the anti-tumour

*It is important to note that the danger model is only a theory, which is based on Janeway's work on innate immunity and PAMPs (Medzhitov and Janeway, 2002).

response; incidentally their depletion can enhance vaccine-induced anti-tumour responses (Sutmuller et al., 2001). Tregs can be naturally occurring (CD4+, CD25+) or inducible, and can mediate effects through cytokines or cell/cell contact (Bluestone and Tang, 2005)). Interestingly, one study concluded that CD4+ T cells are important for anti-tumour immunity when vaccination precedes tumour injection, yet in the case of therapy of established tumours, they act to prevent the anti-tumour response, by inducing Tregs (Sutmuller et al., 2001; van Elsas et al., 2001). This highlights the role of CD4+ T cells in directing the response to danger, whilst blocking a response to self.

1.3.4 Tumours evade immunity?

Tumours would not need to escape immune recognition without immunological pressure. Therefore, the following evidence supports the cancer immunosurveillance hypothesis. It is well documented that tumours regularly lose expression of MHC class I molecules (Khong and Restifo, 2002; Pardoll, 2003) by various mechanisms (e.g. loss of β2-microglobulin (Benitez et al., 1998; Restifo et al., 1996)) for many cancers, including melanoma and breast carcinoma (Algarra et al., 1997; Cabrera et al., 1996). This does not prove immune escape, although HLA class I loss often occurs in patients who raise a partial anti-tumour response after immunotherapy (Restifo et al., 1996). Interestingly, tumours have been found to lose particular HLA alleles that present TAAs e.g. selective loss of HLA-A2, known to present most identified melanoma antigens, is well documented in melanoma patients (Kageshita et al., 1993; Natali et al., 1989). Also, HLA class I loss is associated with invasive and metastatic lesions (Garrido et al., 1997). One enigma is why NK cells fail to kill tumour cells that have lost MHC I; perhaps this can be explained away by the lack of danger again. Loss or downregulation of expression of TAAs is certain to play a role at least in vaccine-induced tumour escape (de Vries et al., 1997; Jager et al., 1996). In a vaccine trial employing transfer of Melan-A specific T cells, tumours initially regressed but then outgrew, through loss of Melan-A (Yee et al., 2002).

Another interesting finding was that the caspase-8 inhibitor, cFLIP was up-regulated in various tumours (Medema et al., 1999). cFLIP prevents caspase-8 from engaging FADD

(Fas-associated death domain), thereby blocking T cell directed Fas mediated apoptosis. cFLIP can also activate NFκB to promote cell survival (Golks et al., 2006). Additionally, tumours can overexpress PI9, a serine protease inhibitor that inactivates granzyme B (Medema et al., 2001), or they can lose expression of all TRAIL receptors to escape TRAIL-mediated apoptosis (Hersey and Zhang, 2001). Finally, tumours can release immunosuppressive signals such as VEGF, IL-10 and TGF-β, which mediate many effects including the inhibition of DC (Chen et al., 1994; Gabrilovich et al., 1996; Gorsch et al., 1992).

1.3.5 A united hypothesis

The contention that cancers are immunogenic in humans is still hotly debated: either the IS regularly removes precancerous cells, but it is less effective later in life owing to immunosenescence (Pawelec et al., 2005)), or the IS is ignorant of / tolerant to cancer and remains so throughout life. A unifying hypothesis to this apparent paradox is that cancers are subject to immune surveillance early on, but cells that adapt to evade immunity are selected to survive and establish tumours; it is at this point that they actively maintain tolerance. My opinion is that with time, knowledge about how cancers evolve and survive will enable us to harness the IS to eliminate them, whether tumour immune surveillance normally occurs or not. To date, we have learnt that a vaccine should comprise tumour antigens best suited to stimulate long-lasting CTL as well as danger signals that will switch on the IS (Belyakov et al., 2000; Klinman, 2004); we also need to employ strategies to block tolerance (Ahlers et al., 2002; Uyttenhove et al., 2003; Wang et al., 2004), for example by inhibiting Tregs (Foss, 2000).*

1.4 Cancer vaccines

Given the exquisite specificity of the IS compared to conventional cancer treatments (e.g. chemotherapy), it is no wonder that great effort is channelled into cancer vaccine development. Figure 1.1 shows a list of components desirable in a cancer vaccine, according to our understanding of cancer as discussed above.

*Also, re-designing vaccines to specifically activate innate effectors such as NK and NKT cells could swing the balance from tolerance to anti-tumour immunity. Finally, with every vaccine design, it is important to take care to avoid an auto-immune response.

Figure 1.1

	Vaccine Component	Strategy	Comments	Ref.
A) Activate a tumour response	Monoclonal antibodies (mAbs)	Some Abs block growth receptors on tumours	e.g. αHER-2 Abs (Herceptin) in use	Montgomery et al, 2005
	TAA specific T cells	Transfer T cells that lyse tumours in vitro	Has been successful in some patients	Yee et al. 2002
	TAA-loaded DC	Stimulate active immunity (CTL T cells)	Has been successful in some patients	Fay et al, 2005
	Viral vaccines encoding TAAs*	Deliver TAAs to APCs in vivo	Poxvirus vectors most common in trials	Scholl et al, 2000
	DNA plasmid expressing TAAs*	Deliver TAAs to cells by transfection / uptake	Has shown promise in animal models	King et al, 1998
B) Block tolerance	Treg inhibitors (e.g. denileukin diftitox)	Kills CD25+ leukaemia cells so may kill CD4+CD25+ Tregs	Depletion of these cells enhances responses in mice	Foss, 2000
	IDO (indoleamine-2,3-deoxygenase) inhibitor 1-methyltryptophan	Inhibits IDO, activated in dysfunctional DC (IDO+ myeloid DC)	Could show promise since IDO+ DC suppress T cells	Uyttenhove et al, 2003
	CTLA-4 blocking Abs	Prevent CTLA-4 binding to B7-1/2 that transmits a negative signal	Promising results in clinical trials	Attia et al, 2005
	Inhibit STAT-3, an oncogenic pathway (using STAT-3β)	Tumours switch on STAT-3 to survive, avoiding surveillance	Blocking STAT-3 activates pro-inflammatory signals	Wang et al, 2004
	IL-13Rα2Fc, prevents IL-13 binding its receptor	Inhibits CD4+ NKT cell IL-13 production, which suppresses responses	Improved vaccine-induced tumour protection of mice	Ahlers et al, 2002
C) Convey "danger"	Ligands for innate effectors e.g. αGalCer	Activates NKT cells, which in turn activate DC	Problems with toxicity	Nakagawa et al, 1998
	TLR agonists e.g. CpG	Triggers type 1 IFN by pDC and matures DC	Currently in clinical trials	Klinman, 2004
	Cytokines: GM-CSF IL-12	Mimic effects of danger Recruit APCs and direct Th1 immunity	Some success in patients	Belyakov et al, 2000
	Co-stimulation: CD40L and B7-1 and B7-2	Mimic effects of danger If delivered to APCs, should enhance CTL	Does not bypass CD4+ help in mouse models	Ahlers et al, 2002

* Should intrinsically supply danger

A triple action vaccine

A rational approach to vaccine design should attempt to activate tumour specific immunity, block tolerance mechanisms, and convey danger signals to the immune system. Clinical trials involving some of these strategies are shown in figures 1.2 and 1.4. My view is that triple vaccines will be more effective than their individual components have been.

1.4.1 Non-specific immunotherapy

Early cancer vaccines, designed to boost natural immunity, were non-specific because they consisted of adjuvants rather than specific antigens associated with cancer. Examples of treatments used are: Coley's bacterial toxins (Pardoll, 1993) and the BCG vaccine. Remarkably, these treatments do work in some cases, for instance, rejection of bladder cancer can be enhanced with BCG immunisation (Bevers et al., 2004). The mechanism of action is probably through innate recognition of an abundance of PAMPs.

More recently, patients have been treated with cytokines to stimulate their IS. IL-2 is one cytokine that has been used with success; in a randomised trial where high dose (HD) IL-2 was compared to low dose IL-2 plus type 1 IFN, HD IL-2 was more effective with 23.2% of patients responding (McDermott et al., 2005). Unfortunately, HD IL-2 (given daily for 4 weeks) is toxic, costly and impractical. Treatment of melanoma patients with IFNα was found to improve survival (Pfeffer et al., 1998), perhaps because type 1 IFNs are natural initiators of immune responses (Smyth et al., 2004). Other cytokines that have shown promise in cancer patients are IL-12 (De Wit et al., 1996) (suggested to promote anti-tumour immunity through NKT cells and DC (Colombo and Trinchieri, 2002)) and GM-CSF (Dranoff, 2002).

1.4.2 Antigen-specific vaccines

The discovery of tumour-associated antigens by Boon and collaborators (Barth et al., 1990; Boon, 1993; Wolfel et al., 1993) led to the development of antigen-specific cancer vaccines. The advantage of this approach over non-specific treatments is that such vaccines should harness the adaptive arm of the IS, responsible for immunity. Two important questions concerning cancer vaccine design are: "which antigens to choose?" and "how to deliver them?"

Numerous tumour antigens have been identified by biochemical, genetic, reverse immunological and serological approaches. The biochemical approach is to elute peptides from MHC molecules, using cancer cells that can be lysed by autologous CTL

(gp100 was discovered this way (Cox et al., 1994)). In the genetic approach, DNA from cancer cells is used to transfect normal cells until they are rendered susceptible to lysis by autologous CTL (in this way MAGE-1 was discovered (van der Bruggen et al., 1991)). In reverse immunology, putative peptides are tested for their binding to HLA molecules and then CTL lines are generated and tested for their ability to lyse tumour cells (used to identify epitopes within MAGE-1 (Celis et al., 1994)). Finally, SEREX (serological identification of antigens by recombinant expression cloning) employs a tumour cell cDNA library expressed in *E.Coli*. The recombinant proteins are used for western blotting with autologous serum; many new antigens have been discovered this way (e.g. NY-ESO-1 (Chen et al., 1997)).

A few of the many tumour antigens stand out as attractive vaccine candidates. One of these is the CT (cancer-testis) antigen, NY-ESO-1, of particular interest because it is expressed on various cancers, including 34% of melanomas and 30% of breast cancers, and it is relatively immunogenic, eliciting natural CD8+ and CD4+ T cell responses as well as antibodies in some patients (Gnjatic et al., 2003; Karbach et al., 2006). Another attractive CT antigen is MAGE-3 because of its tumour specificity and ability to be presented on MHC class I and II ((Chaux et al., 1999; Consogno et al., 2003; Manici et al., 1999).

As for the question of how best to deliver a tumour antigen, existing approaches either involve viruses or are non-viral based.

1.4.3 Non-viral vaccines

Non-viral vaccines are considered safer than viral vaccines and they offer the possibility to be administered repeatedly, without facing the problem of neutralising antibodies associated with viral vaccines. The main vaccines in this category are TAA peptides, cell vaccines (tumour cells/ DC or T cells) and DNA plasmids.

Peptide vaccines are only applicable in patients who express the relevant HLA allele, but one advantage is that processing is not necessary, which safeguards against the

possibility of poor processing of some peptides by the immunoproteasome (Morel et al., 2000). Some synthetic peptides have shown an effect: a Melan-A peptide enhanced the presence of corresponding CD8+ T cells in HLA-A*0201 patients, although a clinical outcome was rare even when peptides were admixed with adjuvants (Speiser et al., 2002). Other studies showed that immunisation with 2 NY-ESO-1 peptides (Dutoit et al., 2002) or a MAGE-3 peptide (Marchand et al., 1999) led to disease stabilisation in some patients. Peptide immunogenicity can be improved by modifying the amino acid sequence (Slansky et al., 2000), but it is crucial to demonstrate that the native epitope is still recognised. Overall, peptide vaccination holds promise for a few peptides, only if they are co-administered with adjuvants.

Autologous tumour cell vaccines, although impractical to produce and poorly immunogenic, can be effective if they are engineered to express co-stimulatory molecules (Cignetti et al., 2005) or to secrete cytokines, particularly GM-CSF (Soiffer et al., 1998). But nonprofessional APCs, such as tumour cells, are less efficient at presenting tumour antigens than DC (Salio et al., 2001). Autologous tumour reactive CTL (expanded *in vitro*) are regularly used in immunotherapy trials (Yee et al., 2002). Serious pitfalls with this approach are that it requires lympho-depletion beforehand, to create a niche for the infused T cells (leaving patients immunocompromised), it encourages tumour escape, T cell survival and migration are limited, and such vaccines are impractical to produce (Blattman and Greenberg, 2004).

DNA vaccines are ideal vaccine candidates because they are easy to manipulate and produce, they are not patient specific, and they intrinsically switch on the IS with their obvious CpG motifs. According to the Langerhans' cell (LC) paradigm (Banchereau and Steinman, 1998), LC (and dermal DC) should pick up antigens (including DNA plasmids) in the skin and mature and migrate to draining lymph nodes (LNs), where they should present encoded antigens to T cells and stimulate long-lasting immunity. DNA vaccines have been effective in mice (King et al., 1998) but not in patients so far (Yu and Restifo, 2002). Possible problems include the poor ability of DNA to be picked up by / penetrate cells and/or weak antigen expression.

1.4.4 DC vaccines

DC have been incorporated into vaccine designs ever since it became apparent that as "nature's adjuvants" they are more effective at stimulating immune responses than substances such as IFA (incomplete Freunds' adjuvant). DC act as sentinels, picking up antigens through phagocytosis, macropinocytosis and receptor-mediated endocytosis, and naturally processing them for presentation on MHC class I and II, their goal being to prime naïve T cells (Banchereau and Steinman, 1998). One reason why this might not occur naturally in cancer patients is because the tumour environment can suppress DC function *in vivo* (Zou, 2005). With this in mind, DC vaccines have been created by taking blood samples from patients and generating DC (usually by culturing monocytes with GM-CSF and IL-4), and then loading them with antigens for re-injection (reviewed in (Banchereau and Palucka, 2005; Figdor et al., 2004)). Antigens are loaded by pulsing DC with tumour lysate or peptides, or by transfecting them with DNA or RNA (Boczkowski et al., 1996; Figdor et al., 2004). It is important to mature DC vaccines (often with TNFα and CD40 ligand) to ensure efficient presentation, especially after the finding that immature DC can induce tolerance (Steinman and Nussenzweig, 2002).

Many DC vaccines (mainly peptide-pulsed DC) have been trialled since 1996 when Hsu and colleagues showed that 3 out of 10 B cell lymphoma patients had a clinical response to a DC vaccine (Hsu et al., 1996). The advantages are that the vaccines are safe and well-tolerated, and they can extend the life-span of many patients (Banchereau et al., 2001; Nestle et al., 1998; Thurner et al., 1999). Interestingly, in a recent trial using CD34+ progenitor-derived DC, metastatic melanoma patients who survived the longest were those who responded to more than 2 melanoma antigens (Fay et al., 2005). Still, DC vaccines are impractical to produce and have a poor success rate, maybe because only a fraction of the intradermally injected DC reach the draining lymph nodes (De Vries et al., 2003). Additional problems include the lack of specific CD4+ T cell help, and the short half-life of peptide-MHC complexes (Figdor et al., 2004). These issues may be partly solved by transducing DC with viral vectors (see section 1.5 below).

1.4.5 Vaccines in the clinic (non-viral)

Some examples of promising non-viral vaccines currently in clinical trials (Attia et al., 2005; Kirkwood et al., 2006; Lonchay et al., 2004; Shi et al., 2006a) are shown in figure 1.2. One of these trials, instigated by Yellin and colleagues has been reported on with interesting results (Attia et al., 2005). In the trial, 56 stage IV melanoma patients were vaccinated with two gp100 peptides in combination with either one of two different doses of anti-CTLA-4 antibody (Ab). Results showed that this vaccine approach caused autoimmune toxicity in 14 patients, but this correlated with a clinical response in some of these patients (autoimmune reactions were resolved). The overall objective response rate was 13%; there was no significant difference between the two different antibody doses. Previous results from the same group had shown that αCTLA-4 Ab plus peptide, and not peptide vaccination alone, induced autoimmunity and antitumour immunity. Perhaps αCTLA-4 Ab will work better in the future when combined with a more effective vaccine.

Figure 1.2

Vaccine	Phase#	Start Date	Principle Researcher & Protocol ID No.
High dose alpha interferon (IFNα-2b)	IV	Nov. 2003	Peter Mohr, MD *NCT00226408*
Two gp100 A2 peptides with αCTLA-4 antibody	III	Sep. 2004	Michael Yellin, MD *NCT00094653*
MAGE-3 A1 peptide with CpG	I/ II	Jan. 2005	Thierry Boon, PhD *NCT00145145*
NY-ESO-1 recombinant protein with two adjuvants: CpG and Montamide (ISA-51/ IFA)*	I	Mar. 2006	Nina Bhardwaj, MD, PhD *NCT00299728*
Comparison of DC vs. Montamide as adjuvants, using five melanoma peptides	I	Jul. 2005	Nina Bhardwaj, MD, PhD *NCT00124124*
Monocyte-derived DC loaded with heat treated, killed, allogeneic melanoma cells	I/ II	Jul. 2005	Anna Karolina Palucka *NCT00125749*

• Trials involve stage III/ IV melanoma patients, according to the AJCC (American Joint Committee on Cancer) staging
Phase I, assessment of safety in a small group of patients; phase II, assessment of safety and efficacy in a small group of patients; phase III, assessment of safety and efficacy in a large group of patients

* ISA-51/ IFA, incomplete Freund's adjuvant

• *Information found at: www.cancer.gov/clinicaltrials*

Example non-viral vaccines in melanoma trials
Out of these active clinical trials, the one by Yellin and colleagues has been reported on and the results are discussed in section 1.4.5. The trial initiated by Mohr and colleagues is based on the finding that IFNα reduces the risk of tumour relapse (Kirkwood et al, 2006). The Thierry Boon trial is based on the finding that MAGE-3 peptide-pulsed DC stimulated T cell responses in ~ 20% of patients, which correlated with tumour regression (Lonchay et al, 2004). The trial initiated by Palucka and colleagues is based on the finding that DC loaded with heat treated, killed melanoma cells were more effective at cross-priming than DC loaded with unheated, killed melanoma cells (Shi et al, 2006a).

1.5 Virus-based cancer vaccines

Vectors have been developed by disabling viruses for the purpose of making gene delivery vehicles. Viruses used to develop vectors include: poxviruses, measles viruses, herpes simplex viruses (HSV), adenoviruses (AdV), adeno-associated viruses (AAV), oncoretroviruses and lentiviruses (Breckpot et al., 2004; Collins and Cerundolo, 2004). Some of these are used in gene therapy e.g. to correct monogenic diseases by gene transfer, or in vaccine designs to carry antigen genes into cells. Viral vectors can be either live-attenuated (measles) or replication deficient (AdV, AAV and retroviruses) viruses, whose genomes include the "transgene(s)" of interest. Vaccinia is a live attenuated virus that is often inactivated before injection to make it replication defective. In general, vector vaccines exhibit the advantages over non-viral vaccines of more efficient delivery and expression of antigens, while conferring the added risk that patients have / might develop neutralising antibodies or other responses to the vector. An attraction of recombinant viral vectors is the potential for sophisticated vaccine design because enhancing genes, such as co-stimulatory molecules can be inserted as well as antigen genes.

1.5.1 Vectors for *ex-vivo* transduction or direct injection

Viral vectors are already widely employed in cancer vaccines: either they are used to transduce cell vaccines *ex-vivo* (e.g. to deliver antigens to DC (Brossart et al., 1997; Zhang et al., 2000) or specific TCR genes to T cells (Schumacher, 2002)) or they are directly injected. The most exciting potential of vectors is for direct immunisation because in this way, vaccines are not patient specific, but there are undeniable safety issues with this approach (Hacein-Bey-Abina et al., 2003; Marshall, 1999). Figure 1.3 summarises the advantages and disadvantages of the above mentioned vectors for vaccination in terms of safety and efficacy. Particular vector vaccines are described in more detail below.

Figure 1.3

Vaccine based on:	Genome	Advantages	Disadvantages
Adeno-associated virus (AAV)	ssDNA	•Infects DC, •Does not cause disease, •Site specific integration on chromosome 19, •No viral genes encoded	•Production and stable infection in most cells is helper dependent, •Limited insertional capacity
Adenovirus (AdV)	dsDNA	•Infects non-dividing cells e.g. DC, •Does not block maturation, •Activates DC, •Bulk production possible, •Insertion of 8kb possible	•Transient expression due to episomal genome, •Immunogenic viral proteins can hijack the response, •Can cause inflammation
Herpes simplex virus (HSV)	dsDNA	•Infects DC, •Large capacity for insertion (up to 50 kb)	•Blocks DC maturation, •Immunodominant viral epitopes, •Causes inflammation, •Attenuated vectors are helper dependent
Lentivirus e.g. HIV1	ssRNA+	•Infects non-dividing cells e.g. DC, •Stable transgene expression due to integration, •No viral genes encoded, •Insertion of 8kb possible	•Risk of insertional mutagenesis, •Does not mature DC
Oncoretrovirus e.g. MLV	ssRNA+	•Stable transgene expression due to integration, •Insertion of 8kb possible, •No viral genes encoded	•Infects only dividing cells, •Risk of insertional mutagenesis, •Does not mature DC
Paramyxovirus e.g. measles	ssRNA-	•Infects DC, •Live attenuated vaccine is known to be safe, cheap and easy to produce	•Transient expression, •Limited insertional capacity (up to 5kb), •Response may be skewed to the virus
Poxvirus e.g. vaccinia virus	dsDNA	•Infects DC, •Does not block maturation, •Matures DC, •Large insertional capacity	•Transient transgene expression, •Viral epitopes dominate immune response

* ss, single stranded; ds, double stranded; +, positive sense; -, negative sense

Virus-based vaccines

The advantages and drawbacks of using different viruses to develop vaccines are outlined here. The main vectors developed for vaccination are based on adenoviruses, retroviruses or poxviruses and these groups are described in section 1.5.

1.5.2 Poxvirus-based vaccines

Vaccinia vector (VV) is popular for vaccination because, as demonstrated by smallpox eradication in the 1970s, it is known to be safe and effective. However, as a consequence of previous vaccination against smallpox, some people will have neutralising antibodies to the vector. Repeat vaccinations with poxvirus vectors are ineffective, unless a different poxvirus is used each time, otherwise patients will quickly develop vector immunity (Scholl et al., 2000). Poxvirus vaccines have been tested in mouse models; immunisation of HLA-A2 transgenic mice with a vaccinia vector expressing multiple melanoma epitopes induced a response to several epitopes (Mateo et al., 1999), although when a similar vaccine was tested in a clinical trial, there was immunodominance towards one epitope (Melan-A) (Smith et al., 2005a). Figure 1.4 shows some promising poxvirus-based vaccines in melanoma trials (Oh et al., 2003; Rosenberg et al., 2003; Zajac et al., 2003).

The trend is to immunise with a poxvirus vector and then boost with peptides. Yet, poxviruses (particularly modified vaccinia Ankara, MVA) are best known for their efficacy at boosting DNA plasmid primed responses, to protect against infectious diseases such as malaria (McConkey et al., 2003). However, when a DNA prime / MVA boost approach was recently applied to melanoma patients, results showed that the anti-melanoma response was of a low magnitude (Smith et al., 2005a), possibly caused by viral epitopes hijacking the immune response (Smith et al., 2005b).

Figure 1.4

Vaccine	Phase[#]	Start Date	Principle Researcher & Protocol ID No.
Vaccinia vector expressing 5 minigenes: Melan-A, gp100 and tyrosinase peptides plus B7-1 and B7-2 co-stimulators	I/ II	Nov. 2002	Michel Adamina, MD *NCT00116597*
Autologous T cell transfer, followed by immunisation with fowlpox vector encoding gp100, with IL-2 injection	II	Mar. 2004	Steven Rosenberg, MD, PhD *NCT00080353*
Fowlpox vector expressing B7-1, LFA-3 and ICAM-1	II	Recruiting	Thomas Gajewski, MD, PhD *NCT00087373*

• Trials involve patients with stage IV/ recurrent melanoma, according to the AJCC (American Joint Committee on Cancer) staging
Phase I, assessment of safety in a small group of patients; phase II, assessment of safety and efficacy in a small group of patients; phase III, assessment of safety and efficacy in a large group of patients

• *Information found at: www.cancer.gov/clinicaltrials*

Example poxvirus-based vaccines in melanoma trials
Results to date from the first trial showed that in 7 out of 18 patients, disease was stabilised after vaccination (Zajac et al, 2003). The second trial is based on the previous finding that 6 out of 12 patients, who received the above stated fowlpox vaccine plus IL-2, showed tumour regressions (Rosenberg et al, 2003). The third trial follows studies in mice showing that the above stated fowlpox vector stimulates higher avidity CTL than vaccines that do not supply co-stimulatory molecules (Oh et al, 2003).

1.5.3 Adenovirus-based vaccines

Adenovirus vectors are attractive because they efficiently deliver antigens to DC and they are considered safer than retroviruses, since they do not integrate their DNA into the genome. However, as with poxvirus vectors there is a potent immune response to the viral proteins, which may interfere with the transgene response (Rosenberg et al., 1998b). It is important to stress that adenovirus vectors can be dangerous; one patient died in 1999 after being given a high dose of a second generation adenovirus vector (with the E1 and E4 genes deleted), which caused massive inflammation (Marshall, 1999). Significantly lower doses of adenoviral vectors are used in clinical trials today (Gallo et al., 2005). A new vaccine that could be effective in patients is an adenoviral vector encoding an antigen (E7 from human papillomavirus) fused to CD40L; this vaccine was shown to be effective in a mouse model (Zhang et al., 2003).

1.5.4 Retrovirus-based vaccines

The successful use of the oncoretrovirus, MLV (murine leukaemia virus) for gene therapy prompted its assessment as a cancer vaccine. For example, MLV expressing the model tumour antigen, ovalbumin was used to transduce dendritic cells *ex-vivo* and such DC were found to protect mice from ovalbumin-expressing tumours (De Veerman et al., 1999). Oncoretroviruses are limited, however, in their use as vaccine vectors because they fail to transduce non-dividing cells *in vivo*.

Also, their use is now associated with serious safety issues: in a gene therapy trial where MLV vectors were successfully used to treat children suffering from *X*-linked SCID (severe combined immunodeficiency), some patients went on to develop leukaemia (Hacein-Bey-Abina et al., 2003). Patients had been re-infused with haematopoietic stem cells that were transduced *ex-vivo* with the γ-c chain cytokine receptor, but in some patients, MLV insertion in or near the LMO2 oncogene in the re-populating cells led to oncogenesis. It has been determined that MLV tends to insert in or near promoters ((De Palma et al., 2005; Mitchell et al., 2004; Wu et al., 2003)); the task now is to make retrovirus-based gene therapy much safer. Lentivirus-based vaccines are the subject of

this thesis and since lentiviruses are also retroviruses, the lifecycle of this virus group, and lentiviruses in particular will be introduced in more detail later (see section 1.7).

1.6 Measuring cancer vaccine efficacy

The development of elegant techniques, namely tetramer staining (Altman et al., 1996), intracellular cytokine staining (Suni et al., 1998), and the ELISpot assay (Schmittel et al., 2000), has allowed the quantification of vaccine-induced CD8+ T cell responses, which in turn has enabled the direct correlation of tumour regressions with immune responses. The recent advent of MHC class II tetramers (McMichael and Kelleher, 1999) offers the opportunity to track tumour reactive CD4+ T cells, an exciting possibility considering the compelling role of CD4+ T cells in anti-tumour immunity (Gao et al., 2002).

I will briefly describe the 2 most common techniques used: tetramer staining and the ELISpot assay.

1.6.1 Pentamer staining

Pentamers, which have replaced tetramers (reviewed in (Klenerman et al., 2002)), consist of 5 MHC-peptide complexes, each biotinylated and held together with streptavidin, which is covalently linked to a fluorochrome. Blood samples can be stained directly with pentamers (along with αCD8 Ab) for flow cytometry. The advantages are that few cells are needed, antigen specific T cells can be quantified, and it can provide functional information e.g. if combined with intracellular cytokine staining. The disadvantage is that different tetramers are needed for each epitope.

1.6.2 ELISpot

The ELISpot assay (reviewed in (Letsch and Scheibenbogen, 2003)) is a modification of the sandwich ELISA, the difference being that it detects cytokine secretion by single cells; the number of cytokine secreting cells can be quantified rather than the amount of cytokine secreted, as in an ELISA. Cells are cultured in wells pretreated with a capture Ab to the test cytokine and the next day, cells are washed and the assay is developed as

an ELISA. When the colour reagent is added, each spot represents one cytokine secreting cell. The advantages of the ELISpot are that it is a functional assay, low numbers of cytokine secreting cells can be detected (particularly valuable in cancer patients), and it is performed *ex-vivo* without the need for *in vitro* re-stimulation. The disadvantage is that it does not prove the antigen specificity of the responding cells.

1.7 Lentiviral vectors

During my PhD, I have been developing lentiviral vectors as vaccines for cancer (reviewed in (Collins and Cerundolo, 2004; Dullaers and Thielemans, 2006)). The attraction of using a viral vaccine is that it can be given to all patients, the vector can be manipulated to express multiple transgenes and it may intrinsically convey "danger" to the IS. Particular advantages of lentiviral vectors are that they can transduce DC without arresting maturation, they can stably express transgenes in cells, and they do not encode viral proteins thereby limiting a response to the vector. The main disadvantage is that there are safety concerns with the use of lentivirus-based vectors, particularly HIV-1, which we are using because it is the most efficient lentivirus at transducing mouse and human DC. Safety issues are discussed later but first I will introduce retroviruses, the HIV virus, and how it has been disabled to construct a HIV vector capable of exhibiting the advantages mentioned above.

1.7.1 Introduction to retroviruses

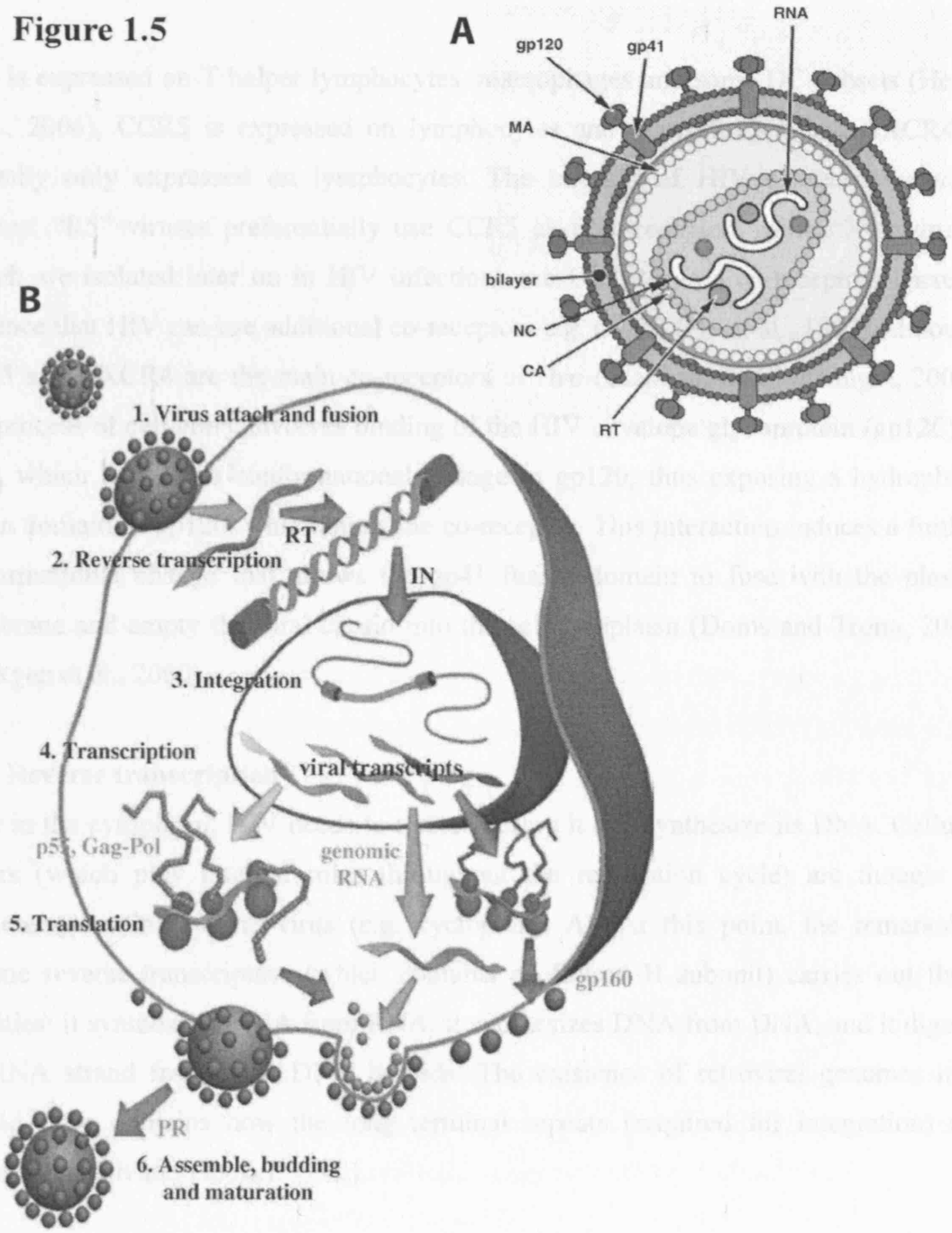
Retroviruses possess a unique enzyme, reverse transcriptase that allows them to replicate in reverse of the usual DNA-to-RNA flow of information. This attribute has enabled them both to carry an RNA genome (which mutates rapidly, favouring selection of variants that evade immunity) and to persist latently within chromosomal DNA (ensuring that a viral reservoir is maintained). The retroviridae can be divided into three groups depending on disease association: oncoretroviridae (so-called because of their oncogenic potential), spumaviridae (not known to cause disease) and lentiviridae (associated with immunodeficiency disorders).

Retroviruses (see (Yamashita and Emerman, 2006) for a review) carry two copies of their positive strand RNA genome, packaged in a capsid and surrounded by an envelope. All retroviruses encode: Gag (the structural proteins), Pol (the enzymes: reverse transcriptase/RNAse H, protease and integrase) and Env (the envelope glycoprotein). In the retrovirus lifecycle, virions enter cells by envelope fusion and they copy their RNA into double stranded DNA for insertion into the host genome; the host transcription and translation machinery is employed to produce new viral proteins and genomes, which are packaged into progeny virions that bud from the plasma membrane. The virus genome and particular stages of the lifecycle will be described in more detail for HIV-1.

1.7.2 Lentivirus attachment and entry

The structure of the mature HIV virion and the basic steps of its replication are displayed in figure 1.5. HIV, like other viruses, initially adheres to cells by interacting with various receptors in order to promote its subsequent entry into cells via its target receptor. One receptor involved in the attachment of HIV to cells is DC-SIGN (DC specific ICAM-3 grabbing non-integrin) expressed on DC, which binds HIV glycoproteins (Geijtenbeek et al., 2000; Pohlmann et al., 2001). Interestingly, coating of DC-SIGN with HIV, while allowing DC to capture the virus, primarily aids its transmission to T cells, which interact with DC (Geijtenbeek et al., 2000). The target receptor and co-receptor, neccessary for HIV entry into cells are CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and a seven-transmembrane spanning chemokine receptor (CCR5 or CXCR4, (Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996)).

Figure 1.5



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HIV structure and replication cycle

A) HIV virions consist of a nucleocapsid containing 2 identical copies of the ssRNA genome surrounded by a lipid bilayer membrane incorporating envelope glycoprotein spikes. MA, matrix protein; CA, capsid protein (p24); NC, nucleocapsid protein; RT, reverse transcriptase. Viral protease (PR), integrase (IN), *Vpu*, *Vif*, *Vpr*, *Nef* and some cellular factors are also packaged. **B)** Shows the main steps in the HIV replication cycle.

CD4 is expressed on T helper lymphocytes, macrophages and some DC subsets (Heath et al., 2004), CCR5 is expressed on lymphocytes and macrophages, and CXCR4 is normally only expressed on lymphocytes. The tropism of HIV can vary between isolates: "R5" viruses preferentially use CCR5 as a co-receptor, while "X4" viruses (which are isolated later on in HIV infection) use CXCR4 as a co-receptor. There is evidence that HIV can use additional co-receptors e.g. CCR3 (He et al., 1997), although CCR5 and CXCR4 are the main co-receptors *in vivo* (Clapham and McKnight, 2002). The process of cell entry involves binding of the HIV envelope glycoprotein (gp120) to CD4, which leads to a conformational change in gp120, thus exposing a hydrophilic fusion domain of gp120, which binds the co-receptor. This interaction induces a further conformational change that allows the gp41 fusion domain to fuse with the plasma membrane and empty the viral capsid into the cell cytoplasm (Doms and Trono, 2000; Melikyan et al., 2000).

1.7.3 Reverse transcription

Once in the cytoplasm, HIV needs to uncoat before it can synthesize its DNA. Cellular factors (which play integral roles throughout the replication cycle) are thought to promote uncoating of the virus (e.g. cyclophilin A). At this point, the remarkable enzyme reverse transcriptase (which contains an RNase H subunit) carries out three activities: it synthesizes DNA from RNA, it synthesizes DNA from DNA, and it digests the RNA strand from RNA:DNA hybrids. The existence of retroviral genomes in a diploid form explains how the long terminal repeats (required for integration) are created (Harrich and Hooker, 2002).

The cellular protein, APOBEC3G compromises the fidelity of HIV reverse transcription by causing cytidine deamination (Harris et al., 2003), but this host defence mechanism is blocked by HIV *Vif*, which prevents packaging of APOBEC3G into particles (Mariani et al., 2003). Once HIV has copied its genome into double stranded DNA, evidence suggests that it exploits the cytoskeleton to transport its DNA to the nuclear membrane (McDonald et al., 2002).

1.7.4 Integration of viral DNA

Viral DNA is termed the pre-integration complex (PIC) because of its association with cellular factors and viral proteins, such as Vpr, which allow it to traverse the nucleus through nuclear pores (Le Rouzic and Benichou, 2005; Popov et al., 1998). It is thought that members of the PIC exert some redundancy as Vpr is not necessary for HIV to infect many cell types e.g. non-dividing T cells or terminally differentiated neurons (Zufferey et al., 1997), although it plays an important role in the infection of macrophages (Eckstein et al., 2001; Zufferey et al., 1997). Viral DNA can be found in the nucleus as linear or circular forms. Integrase mediates integration of linear viral DNA by creating overhangs at each end and preparing an integration site in chromosomal DNA by breaking phosphodiester bonds; it then acts to incorporate the provirus into this site (Bushman, 2002; Van Maele and Debyser, 2005). Finally, DNA repair of the integration sites is carried out by host mechanisms (Scottoline et al., 1997). HIV targets active genes and integrates throughout transcriptional units (De Palma et al., 2005; Schroder et al., 2002; Trono, 2003). The virus exploits cellular proteins to direct integration into such regions (Ciuffi and Bushman, 2006; Jacque and Stevenson, 2006).

1.7.5 Provirus transcription and translation

Provirus transcription is directed by cellular RNA polymerase II and the promoter/enhancer in the U3 region of the 5′ LTR. This process is inefficient and only basal amounts of Tat, Rev and Nef are translated (Jordan et al., 2001). Once enough Tat has been produced, it can enhance transcription: Tat (along with the cellular factor, Cyclin T1) binds to the TAR (transactivation response element) in the R (repeat) region of newly generated mRNA transcripts. Tat binding recruits additional proteins that form a complex (called the positive transcription-elongation factor B complex), which mediates efficient transcription from the integrated provirus (Bieniasz et al., 1999; Harrich et al., 1996). Firstly, mRNAs are produced that are subject to multiple splicing, until enough Rev is generated, at which point Rev binds the Rev responsive element (RRE) in the *Env* sequence. Binding of Rev drives export of singly spliced or non-spliced RNA because Rev contains a nuclear export signal; transport into the cytoplasm

is mediated by cellular proteins, such as Crm1 (Greene and Peterlin, 2002; Neville et al., 1997; Pollard and Malim, 1998).

1.7.6 Assembly and budding of progeny virus

Singly spliced and non-spliced RNAs are translated to produce viral proteins, or used to form new genomes respectively. Gag and Pol proteins are generated as precursors that are cleaved into their constituents after virus budding: the Gag polyprotein is processed to produce the structural proteins, and the GagPol protein (which is translated due to a regularly occurring ribosomal frameshift) is processed to produce the enzymes, as well as the structural proteins (see figure 1.5). Gag and GagPol are shuttled to the surface membrane where they associate with lipid rafts (rich in cholesterol and sphingolipids) (Ono and Freed, 2001) and package the diploid RNA genome. The Gag polyprotein is thought to direct assembly (Briggs et al., 2003; Derdowski et al., 2004). Recent evidence suggests that Gag traffics to late endosomes, including multivesicular bodies at an earlier stage in the assembly process, prior to budding from the plasma membrane (Dong et al., 2005; Kramer et al., 2005).

The *Env* gene is translated into gp160, which traffics to the ER (endoplasmic reticulum) for glycosylation and subsequent cleavage (by cellular proteases) to generate gp120 and gp41 (Sheng et al., 1997). gp120 (the surface subunit, SU) associates with gp41 (the transmembrane subunit, TM) to form trimers; these spikes migrate to the cell surface and insert into the plasma membrane. Progeny virions use the plasma membrane (containing the viral spikes) to bud from the cell surface; this membrane, which now becomes the viral envelope, is rich in cellular proteins such as HLA class II (Tremblay et al., 1998). This may be a strategy to promote virus interaction with (and in turn infection of) CD4+ T lymphocytes (Arthur et al., 1992). After virus budding, HIV protease (PR) cleaves Gag and GagPol to release the structural proteins and enzymes in a process which is termed maturation.

1.7.7 Factors that determine HIV-1 virulence

HIV is a deadly pathogen that is extremely difficult to develop a vaccine against. The reasons for this are extensive but the most important ones in the context of this thesis are the abilities of various accessory and regulatory proteins to act on DC in a way that impedes the development of immunity. DC from HIV-infected patients were found to be refractory to normal maturation stimuli (e.g. LPS) and when they were co-cultured with autologous T cells, IL-10 was produced in 6 out of 10 patients (Granelli-Piperno et al., 2004). IL-10 production has been found to be induced by Nef (Tangsinmankong et al., 2000). Nef also suppresses the immune response by down-regulating CD4, MHC class I and CD28 (Arora et al., 2002; Mangasarian et al., 1999; Schwartz et al., 1996) and promotes viral spread by up-regulating DC-SIGN (Sol-Foulon et al., 2002).

Interestingly, it was recently highlighted that the inability of HIV-1 Nef (unlike SIV Nef and HIV-2 Nef) to down-regulate TCR-CD3 on T cells may partly explain its pathogenicity (Schindler et al., 2006). By reducing antigen recognition by T cells, SIV Nef is thought to prevent massive activation-induced T cell death and in turn the onset of AIDS. Tat was found to alter the array of CTL epitopes processed by exerting effects on various proteosome subunits (Gavioli et al., 2004). Tat also reprograms DC to express chemoattractants for T cells to aid virus transmission (Izmailova et al., 2003). In summary, HIV proteins render the virus poorly immunogenic; the elimination of these elements from the virus has permitted the use of lentivirus-based vectors that are able to transduce DC without exerting adverse effects on DC function. The process of attenuating the lentivirus to develop a vector is outlined below.

1.7.8 Lentivirus attenuation

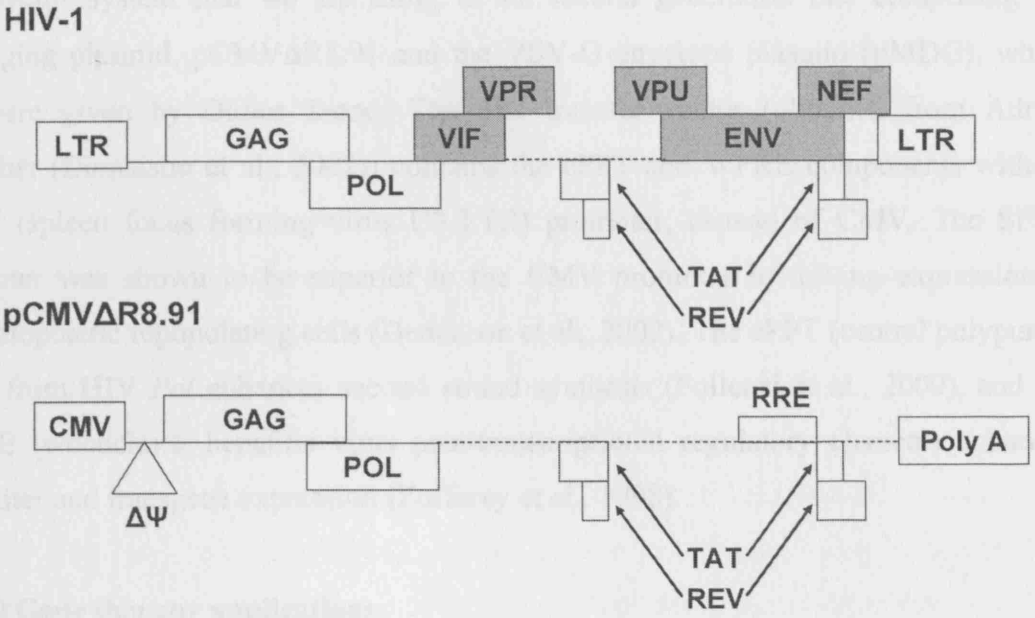
The invention of lentiviral vectors, developed by attenuating and pseudotyping lentiviruses has transformed science research and medicine. Essentially gene delivery vehicles, lentiviral vectors can be re-designed for diverse applications, including basic research and therapy of monogenic diseases. Lentiviral vectors were constructed by removing as much of the genome's coding region as possible (and replacing it with genes of interest), while leaving sequences necessary *in cis* for packaging and

integration. Deleted genes required for assembly of virus particles can be supplied *in trans.*

This system was developed in 1996 by Naldini, Trono and colleagues in the lab of I.M. Verma (Naldini et al., 1996). Vector particles were produced by 3 plasmid transfection. The packaging plasmid (pCMVΔR9), which supplied viral proteins *in trans*, was deleted for *Env, Vpu* and the packaging signal, and the 3′ LTR was substituted with a polyadenylation site. Either an envelope plasmid expressing the amphotropic envelope of MLV, or one expressing VSV-G (vesicular stomatitis virus G protein) was used; VSV-G allows vector concentration by ultracentrifugation because of its stability and it confers broad tropism (using a ubiquitous membrane phospholipid for cell entry (Barrette et al., 2000)). The third (transfer vector) plasmid contained the gene of interest, and the *cis-acting* sequences needed for packaging, reverse transcription and integration.

Zufferey, Trono and colleagues then improved vector safety by further deleting the packaging plasmid for *Vif, Vpr* and *Nef* (pCMVΔR8.91) (Zufferey et al., 1997) and this plasmid system is termed second generation, (shown in figure 1.6). The transfer vector has also been engineered to be self-inactivating (SIN) upon reverse transcription, due to a deletion of the 3′ LTR U3 region (Zufferey et al., 1998); this limits the risk of RCV (replication competent virus) emerging. Of interest, *Tat* is encoded in the packaging plasmid but it is not packaged, or expressed by the transfer vector (which is relevant to section 1.7.7 above). In the third generation system (Dull et al., 1998), the packaging plasmid is *Tat*-independent because the 5′ LTR is replaced by a strong promoter (e.g. CMV) and *Rev* is supplied on a separate plasmid so that transfection with 4 plasmids is required.

Figure 1.6



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From lentivirus to lentivector
The lentivirus genome (top) was deleted for *Env* and the accessory proteins, *Vif, Vpr*, *Vpu* and *Nef*, and further modified to construct the pCMVΔR8.91 packaging plasmid (bottom). See section 1.7.8 for details of the second generation system used to produce lentivector particles.

1.7.9 Lentiviral vector components

The vector system that we are using is the second generation one comprising the packaging plasmid, pCMVΔR8.91 and the VSV-G envelope plasmid (pMDG), which we were given by Didier Trono. The SIN transfer vector (obtained from Adrian Thrasher (Demaison et al., 2002)) contains the cPPT and WPRE components with an SFFV (spleen focus forming virus U3-LTR) promoter, instead of CMV. The SFFV promoter was shown to be superior to the CMV promoter in driving expression in haematopoietic repopulating cells (Demaison et al., 2002). The cPPT (central polypurine tract) from HIV *Pol* enhances second strand synthesis (Follenzi et al., 2000), and the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) enhances viral titer and transgene expression (Zufferey et al., 1998).

1.7.10 Gene therapy applications

One obvious application of lentiviral vectors (lentivectors) is their use for gene therapy of HIV infection e.g. they can deliver genes that block the HIV replication cycle; one example is by expressing a single chain antibody against reverse transcriptase (Mautino, 2002). With the explosion of siRNA technology, lentivectors encoding siRNAs will be used in trials to block viral replication (Morris and Rossi, 2006). Indeed, the first clinical trial with lentivectors is underway in HIV patients, using transfer of autologous CD4+ T cells expressing an *Env* inhibitor (Humeau et al., 2004). Lentivectors have been developed for the therapy of genetic diseases, such as storage deficiency diseases affecting the brain (Biffi and Naldini, 2005; Consiglio et al., 2004) and they are due to replace MLV vectors for treatment of SCID patients because they are safer and more effective (Guenechea et al., 2000).

1.7.11 Vaccine research

When I began my PhD in October 2002, reports on the use of lentivectors as vaccines were scarce. Several groups had found that lentivectors could transduce mouse and human DC with antigens *in vitro* (Chinnasamy et al., 2000; Schroers et al., 2000) and such DC were able to stimulate CD8+ T cells *in vitro* (Dyall et al., 2001; Granelli-

Piperno et al., 2000). A few reports also showed that transferred vector modified DC could stimulate CD8+ T cells in mice (Esslinger et al., 2002; Metharom et al., 2001). Several studies revealed that B cells and APCs were transduced in the spleen, following intravenous injection of a lentivector (Follenzi et al., 2002; VandenDriessche et al., 2002). Subsequent reports have demonstrated that vector modified DC can stimulate CD4+ T cells *in vitro* (Breckpot et al., 2003) and directly injected lentivectors can stimulate CTL responses in mice (Esslinger et al., 2003; Palmowski et al., 2004). Any more recent papers (published after 2004) are described in the results sections.

1.8 Important areas of lentiviral vector research

Considering that the idea of designing lentivirus-based vaccines for cancer or infectious diseases was (and still is) relatively novel, there is a plethora of topics demanding research, which are also relevant to other lentivector applications. The main areas of interest are mentioned below.

1.8.1 Safety

The most apparent concern about the use of lentivectors is the possible risk of RCV emerging. However, second generation vectors are considered safe because no accessory (virulence) genes are encoded, components required for vector production are separated between 3 different plasmids and the transfer vector is self-inactivating. It would be desirable to further attenuate the vector; a recent report showed that *Rev* can be removed and supplied as a fusion protein, with little effect on vector titer (Kowolik et al., 2003).

A greater concern is the possibility for insertional mutagenesis (as occurred in a gene therapy trial for SCID using MLV). HIV vectors presumably carry less risk of insertional mutagenesis because they preferentially integrate throughout transcriptional units instead of adjacent to promoters as is the case with MLV (De Palma et al., 2005; Mitchell et al., 2004; Schroder et al., 2002; Wu et al., 2003). Much research including work in our lab focuses on the risk of insertional mutagenesis, the engineering of site-

specific integration, and the development of non-integrating lentivectors (Montini et al., 2006; Yanez-Munoz et al., 2006).

Targeting is another important safety strategy for injected lentivectors and this has been achieved through either surface targeting (e.g. to spleen (Peng et al., 2001)) or transcriptional targeting (e.g. to hepatocytes (Follenzi et al., 2004) or endothelial cells (De Palma et al., 2003)). One project in our lab involves testing dectin-2 as a DC specific promoter (Bonkobara et al., 2001; Carter et al., 2006). Also methods have been used to inducibly express transgenes; one example is the advanced tetracycline inducible (tet-on) system (Barde et al., 2006; Vigna et al., 2005).

1.8.2 Production

Bulk production of clinical grade lentivectors is necessary for clinical applications. Stable packaging cell lines producing lentivectors have been difficult to develop, due to the toxicity of the HIV proteins (e.g. protease). Initially, inducible packaging cells were developed (Farson et al., 2001), followed by a packaging line ("STAR" cells) where HIV proteins were encoded by an MLV provirus vector (Ikeda et al., 2003). In this system, the *Gag-Pol* genes were supplied in a codon optimised form, which enhanced their transcription and improved safety because additional viral sequences could be deleted.

1.8.3 Immunogenicity

One advantage of lentiviral vaccines is the absence of pre-existing neutralising antibodies in patients. Therefore, it is important to ascertain whether or not patients develop vector neutralising antibodies, following immunisation, as is the case after injecting adenovirus- or vaccinia virus-based vectors. One group showed that mice do raise lentivector neutralising antibodies but this humoral response could be avoided if vectors are purified by sucrose gradient ultracentrifugation (Baekelandt et al., 2003). Another study showed that mice do develop some anti-vector antibodies after one vector injection but this does not prevent repetitive vaccination from inducing effective CTL responses (Esslinger et al., 2003). Other important areas of interest lacking research are:

whether or not lentivectors can stimulate CD4+ T cells specific to their transgene *in vivo* (CD4+ T cells are a key component of an effective immune response), which cell types are transduced *in vivo* and their effect on immunity, and whether or not the vector can activate DC (immature transduced DC might promote tolerance).

1.9 PhD aims

In light of the existing research on lentivectors as outlined above and the overall purpose of my PhD to develop a lentivirus-based vaccine, I set out to achieve the following goals:

1. To enhance presentation of a lentivector expressed antigen (ovalbumin) on MHC class II, to promote CD4+ T cell help.
2. To track vector modified cells *in vivo,* in order to examine stable antigen expression and presentation.
3. To introduce a dendritic cell activator into the lentivector to enhance vaccine efficacy.

Chapter 2: Materials and methods

2.1 Subcloning

2.1.1 Molecular buffers

Buffers and solutions used for subcloning are listed below:

1 x PBS (Phosphate-buffered saline)	137mM NaCl, 2mM KCL, 10mM sodium hydrogen phosphate (dibasic) and 2mM potassium hydrogen phosphate (dibasic), (pH 7.4)
TE buffer	10mM Tris.Cl (pH 8.0) and 1mM EDTA
1 x TAE (Tris-acetate-EDTA) buffer	40mM Tris (pH 7.8), 20mM sodium acetate and 1mM EDTA
Orange G loading buffer	60mM Tris (pH 7.4), 6mM EDTA (pH 8.0), 30% glycerol and 0.25% Orange G
LB (Luria-Bertani) agar	1% bacto typtone, 0.5% bacto yeast, 0.5% NaCl (pH 7.0) with 15g/L bactoagar
LB (Luria-Bertani) broth	1% bacto typtone, 0.5% bacto yeast and 0.5% NaCl (pH 7.0)
Transformation buffer	250mM PIPES, 2.5mM $CaCl_2.2H_2O$, 60mM KCL, adjusted to pH 6.7 with KOH, before adding 55mM $MnCl_2$

2.1.2 Restriction digests

DNA fragments were isolated by restriction digest in order to verify plasmids or for subcloning. All restriction enzymes were purchased from Promega. Digestions were conducted according to the manufacturers guidelines, depending on the combination of enzymes used.

2.1.3 Agarose gel electrophoresis and gel extraction

Agarose gels (normally 1%) were made by dissolving agarose (Sigma) in TAE buffer and adding 0.5µg/ml ethidium bromide (Sigma) before leaving it to set. DNA samples, including restriction digest products were mixed with Orange G loading buffer (at a ratio of 5:1) and run on agarose gels to separate DNA fragments by size. A 1Kb DNA

ladder (GibCo) was run in parallel to identify the sizes of the bands, which were visualised, using a UV lamp. DNA fragments were then isolated from gels with a scalpel and the DNA was purified from the agarose using a Gel Extraction Kit (Qiagen).

2.1.4 Polymerase chain reaction (PCR)

PCR is used to amplify a segment of DNA by using primers specific for sequences flanking the segment. The template DNA is first heated to denature it and then the reaction is cooled to allow the primers to anneal. Finally, the primers are extended with DNA polymerase. The product is amplified by repeat cycles of these three steps. PCR depends on the activity of *Taq* polymerase, a heat stable DNA polymerase extracted from *Thermus aquaticus*. PCR reactions were prepared using the reagents listed below, which were from a PCR kit (Takara).

Reagent	Quantity
10x *Taq* buffer	3µl
dNTPs	2µl (2.5mM of each)
Distilled water	18.5µl
Forward primer	2µl (100pmoles)
Reverse primer	2µl (100pmoles)
DNA template	2µl (<50ng)
Ex *Taq* pol.	0.5µl (5 units/µl)

dNTPs, deoxyribonucleoside triphosphates; Ex *Taq* pol., a mixture of standard *Taq* polymerase and pfu *Taq* (which has higher fidelity). 5% DMSO was added to some PCR reactions to reduce non-specific primer binding.

PCR was carried out using a Hybaid thermal cycler with the program: denaturation at 94°C for 2 min, followed by 35 cycles of: denaturing at 94°C for 45 sec, annealing at **60°** for 45 sec and extension at 72° for **30 sec**. A final extension step is carried out at 72°C for 5 min. Steps highlighted in bold are variable. For the extension, 30 sec is enough to extend fragments of ~ 500bp. The annealing temperature should be 5° below the lowest melting temperature (Tm) of the primer pair to be used, where:

$$Tm = 4(G + C) + 2(A + T)\ ^{o}C.$$

Primer sequences used to amplify the inserts for the transfer vector are shown below.

	Primer sequences, 5′ to 3′
OVA_F	CGGGATCCGCCACCATGGGCTCCATCGGTGCAGCAAGCATG
OVAcyt_F	CGGGATCCGCCACCATGAGCACCAGGACACAAATAAATAAGG
TfR-OVA_F	CGGGATCCGCCACCATGATGGATCAAGCTAGATCAGC
OVA_R*	
vFLIP_F	GAGGGATCCATGGCCACTTACGAGGTTCTCTGT
vFLIP_R	GAGGCGGCCGCCTATGGTGTATGGCGATAGTGTTG

2.1.5 Purification of the PCR product

PCR products were routinely purified by phenol extraction, followed by DNA precipitation. First, the reaction volume was made up to 200µl with TE buffer and then an equal volume of phenol was added. This mixture was vortexed and centrifuged at high speed (13,000rpm) for 10 min and the upper phase transferred to a new tube. Then, 1/10 of the volume of sodium acetate was added (3M) and the mixture was vortexed and spun at high speed for 10 min. Next, 2.5 volumes of 100% ethanol was added and the mixture was vortexed and left at -20°C for >30 min. The DNA was pelleted and washed with 70% ethanol before resuspending it in water and storing it at -20°C.

2.1.6 Preparation of competent bacteria

E.coli (HB101 strain, from GibCo) were grown in unselective LB (Luria-Bertani) medium for preparation of competent cells. An overnight culture was diluted 1/100 into fresh LB medium and incubated for a further 2 hours before placing the culture on ice for 10 min. Then, the bacteria were pelleted at 4°C and resuspended in 30ml ice-cold transformation buffer. The cells were pelleted again and resuspended this time in 10ml of ice-cold transformation buffer containing 10% DMSO; cells were aliquoted, snap frozen and stored at -80°C.

*

OVA_R/ OVAcyt_R :-
CGCGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTAGCTTTCTTCTTTTTAAGGGGAA

TfR-OVA_R :-
CGCGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTAAGGGGAAACACATCTGCCAAAG

2.1.7 DNA ligation and transformation

Ligation reactions were used to "paste" new DNA fragments (isolated by restriction digest) into linearised plasmids bearing the corresponding ends. This process whereby new phosphodiester bonds are formed to seal the plasmid is catalysed by DNA ligase. For ligation, the insert fragment was mixed with the vector fragment at a ratio of 3:1 in a volume of 5µl. Controls were the vector alone and the insert alone. Then, DNA ligase solution (Takara) was added at a 1:1 ratio and samples were incubated at room temperature for 1 hour. The whole of each reaction was then added to tubes on ice, containing competent bacteria. These mixtures were left on ice for 15 min, heat shocked at 42°C for 90 sec and placed back on ice. Finally, bacteria were centrifuged, resuspended in ~ 20µl LB medium and spread on ampicillin+ agar plates. Plates were incubated at 37°C upside-down, overnight. Ampicillin+ agar plates were prepared by dissolving agar (Sigma) in distilled water, autoclaving it, and adding ampicillin (50µg/ml) before pouring the agar into plates to set.

2.1.8 Purification of plasmid DNA (mini-prep)

Small scale plasmid preparation was carried out by inoculating 4 ml LB medium (ampicillin+) with a single colony overnight. The next day 1ml of the culture was used to prepare a glycerol stock (see below) and the rest was used to extract plasmid DNA using a Mini-prep kit from Marligen Bioscience. Plasmid DNA was resuspended in distilled water and verified first by restriction digest, and then by sequencing (see section 2.1.12).

2.1.9 Purification of plasmid DNA (midi-prep)

Medium scale plasmid preparation was carried out by inoculating a starter culture of 4ml of LB medium (ampicillin+) with a fresh colony or glycerol stock. This culture was then grown for 7 hours and diluted 1/200 into 100ml medium and grown overnight. Plasmid DNA was extracted, using a Plasmid Midi Kit (Qiagen), and verified by restriction digest. The DNA was then precipitated using 3M sodium acetate, as

described under section 2.1.5, and resuspended in TE buffer; the concentration was determined as stated in section 2.1.11.

2.1.10 Glycerol stocks

Glycerol stocks were made by mixing 250µl of 60% glycerol with 750µl of a fresh bacterial culture. Stocks were immediately frozen and stored at -80°C.

2.1.11 DNA quantification

The concentration of DNA was determined using either a classical spectrophotometer, by measuring the absorbance at 260nm (this value was multiplied by 50 to give the concentration in µg/ml), or using a NanoDrop ND-1000 spectrophotometer.

2.1.12 Sequencing

Sequencing of the vector inserts was carried out by I. Gerrard at the Windeyer sequencing service. DNA templates were provided in water at a concentration of 100ng/µl (7µl was required per reaction). Each template sequence was verified by 2 reactions, one using a forward primer specific for the SFFV region proximal to the insert and one using a reverse primer specific for the WPRE region adjacent to the 3′ end of the insert. Primers were obtained from Oswel DNA service and provided for sequencing at a concentration of 0.75 pmoles/µl (7µl per reaction). The integrity of each sequence was checked using Sequencher software. The primers used for sequencing are below:

SFFV_F 5′ TGCTTCTCGCTTCTGTTCG

WPRE_R 5′ CCACATAGCGTAAAAGGAGC

2.2 Lentiviral vector production

2.2.1 Transfection

293T cells were grown to 80% confluence on the day of transfection in 20cm^2 circular plates (Nunc). The protocol used for transfection was based on the one described in

(Besnier et al., 2002). For each plate, DNA plasmids were mixed as follows: 2.5μg of the packaging plasmid (pCMV8.91) with 2.5μg of the envelope plasmid (pMDG) and 3.75μg of the transfer vector plasmid, made up to 37.5μl with TE buffer. The packaging and envelope plasmids were supplied by D. Trono (EPFL, Geneva) and the transfer vector plasmid was from A. Thrasher (ICH, London). 45μl of the transfection reagent, Fugene-6 (Roche) was diluted in 500μl OptiMEM (serum free medium, GibCo) and this mixture was added to the DNA and incubated at room temperature for 15 min. Meanwhile, the medium (complete DMEM, see section 2.4.1) was changed on the cells and then the transfection mixture was added drop-wise. The following day, the medium was changed again (to remove Fugene-6).

2.2.2 Harvesting the virus

The virus supernatant was harvested 48–72 hours post transfection and passed through either a 0.2 or a 0.45μm filter, to remove any cells. The virus was concentrated (about 160x) by ultracentrifugation (twice to include a wash step) and resuspended in 1ml Hank's balanced salt solution (HBSS), aliquoted, and stored at -80 (some virus was used to determine the titer).

2.2.3 Determination of the titer

Serial dilutions of the virus were made (to contain 5, 1, 0.2, or 0.04μl of virus) and used to infect 293T cells that were plated at $2x10^5$ cells/well in 24 well plates. The cells were assayed 4-5 days post infection for expression of GFP, by flow cytometry, in order to determine the titer (the number of "infectious units", i.u. per ml). The titer for the GFP virus was usually ~$1x10^9$ i.u./ml. Also, cell pellets were washed twice and frozen ($1-5x10^6$ cells) to determine the copy number, by Q-PCR (see section 2.3). The method of titering the OVA viruses according to their copy numbers is described in chapter 3.

2.3 Q-PCR

Q-PCR samples were assayed in 96 well plates (ABgene) using an ABI prism 7000 machine. For the PCR set-up: double distilled water was used, the master mix and

carrier were prepared in a "DNA clean" room, and the samples were added to the plate in a separate "PCR set-up" room.

2.3.1 Genomic DNA extraction

Frozen cell pellets were thawed on ice and resuspended in sterile HBSS. Genomic DNA was then extracted using a DNeasy Tissue Kit (Qiagen). The concentration of DNA was determined as stated in section 2.1.11.

2.3.2 DNA carrier and master mix

The carrier solution was made-up by diluting 1mg of salmon testis DNA (Stratagene) in 10ml of water. A PCR master mix was prepared for the number of samples (run in duplicate), plus 5 standards (run in duplicate), plus 2 blanks and 2 extra. The probe (FAM-TAMRA labelled) and the primers (all from Sigma Genosys) were specific to the strong stop region of the lentiviral vector. For each PCR reaction, the following reagents were required:

Reagent
1.5µl (7.5pmols) of each primer
0.75µl (3.75pmols) of the probe
12.5µl of QuantiTect Probe PCR mastermix (Qiagen)
2.75µl of water

The sequences of the strong stop (SS) primers and the probe are:

SS_F	5′ TGTGTGCCCGTCTGTTGTGT
SS_R	5′ GAGTCCTGCGTCGAGAGAGC
Probe	5′ CAGTGGCGCCCGAACAGGGA

2.3.3 Standards

The standards were prepared by diluting the GFP transfer vector plasmid (1mg/ml) with the carrier solution. First, 2µl of plasmid was diluted in 175µl carrier and this was further diluted (1/10,000) to give the first standard, which contained 10^5 molecules per 5µl (the volume used per PCR reaction). This standard was used to make the other 4 standards by serial 1/10 dilutions in the carrier solution. Therefore, the standards covered a range of between 10^1 and 10^5 molecules per reaction.

2.3.4 Q-PCR set-up

For the standards, 19µl of master mix was added to each well, followed by 5µl of each standard. The same volume of master mix was added to 2 additional wells as blanks. Then, the carrier was added to the remaining master mix (5µl carrier per sample) and 24µl of this mixture was added to each well, followed by 2µl of each sample. At this point, the plate was sealed with a lid from Applied Biosystems and run according to the program: 94°C for 10 min, then 50 cycles of 94°C for 15 sec and 60°C for 1 min.

2.3.5 Determination of the copy number

The copy number (number of vector copies per cell) was determined by:
Mean number of molecules / Amount of DNA (pg) x 6.6 (a diploid genome contains ~ 6.6pg of DNA).

2.4 Cellular assays *in vitro*

2.4.1 Culturing cell lines

Cell lines used were 293T cells, EG7.OVA (OVA transfected EL4 cells) cells and Jurkat T cells. 293T cells are adherent cells derived from human embryonic kidney cells. They were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal calf serum (FCS), penicillin plus streptomycin (10,000 units/ml) and 2mM L-glutamine (all from GibCo). 293T cells were harvested using trypsin (GibCo) and passaged every few days. The culture medium used for EG7.OVA cells and Jurkat T cells (derived from

human leukaemic T cells) was called: Roswell Park Memorial Institute (RPMI, from GibCo) and 2-ME (2-mercaptoethanol, from Sigma) was included as an additional supplement (50μM). Since both these lines were nonadherent, they were passaged without trypsin, every few days. G418 (Invitrogen) was added to EG7.OVA cultures at a concentration of 0.4mg/ml to maintain the presence of the OVA plasmid. 293T cells were cultured in a 10% CO_2 incubator, whereas EG7.OVA cells and Jurkat T cells were cultured in a 5% CO_2 incubator, all at 37°C. Cells were frozen in FCS including 10% DMSO at a concentration of ~ 5 x 10^6 cells/ ml.

2.4.2 Generation of bone marrow-derived DC

Murine bone marrow-derived DC were prepared as previously described (Inaba et al., 1993). Briefly, the bone marrow cells were flushed out of bones using a syringe (Terumo) and needle (BD Microlance), red blood cells were lysed with Red cell lysis buffer (Sigma) and cells were washed and cultured at 6x10^5 cells/ml in IMDM (Iscove's Dulbecco's Modified Medium, from GibCo) containing 6% FCS, 2mM L-glutamine, 50μM of 2-ME, 50ng/ml GM-CSF (Peprotech), and antibiotics. Cells were fed with fresh medium containing GM-CSF on days 3 and 8. DC cultures were transduced on day 4, at MOI- 20 as stated before (Palmowski et al., 2004). DC became semi-adherent after 5 days; they were matured at the indicated time-points with 50ng/ml LPS (Sigma). DC phenotype was assessed by FACS (see section 2.4.4) before using them in ELISpot assays (see section 2.6.3), where they were plated in triplicate at between 3x10^3 and 2x10^4 cells/well, along with different dilutions of T cells (see results).

2.4.3 Expansion of transgenic T cells

OT-I and OT-II transgenic mouse spleens were a kind gift from Alistair Noble, KCL. OT-I (Clarke et al., 2000) or OT-II cells (Robertson et al., 2000)from the respective mouse strain were expanded by culturing at ~ 1.5 x10^6 cells/ml in complete medium (RPMI-based), containing the appropriate OVA peptide. The OVA class I $peptide_{257-264}$ was made at UCL and the OVA class II $peptide_{323-339}$ was ordered from Proimmune. Cultures were split every few days and at these times, IL-2 (Peprotech) was added at 10ng/ml. After 6 days, cells were washed and frozen at -80. The purity of the transgenic

T cells was assessed by tetramer staining and flow cytometry (see sections 2.4.4 and 2.6.5), using the primary antibodies: anti-CD8-FITC, αVβ5.1,5.2-biotin (both Pharmingen), anti-Vα2-FITC (Caltag), and anti-CD4-PE (eBioscience). After thawing, dead cells were removed by density separation using Lympholyte-M (Cedarlane).

2.4.4 Flow cytometry

Flow cytometry (FACS) was performed by staining cell samples with antibodies (Abs) in 96 well, U/V-bottomed plates (Nunc). All steps were carried out on ice, using ice-cold buffers, and incubations were kept in the dark. Between 10^5 and 10^6 cells were used for staining; first, cells were blocked with blocking buffer (HBSS, containing 10% goat serum (Sigma), 2% FCS and 0.1% NaAzide) for 10 min, and subsequently washed. In the case of DC, cells were then incubated with an anti-Fc receptor Ab (made at UCL) for 15 min and washed twice with wash buffer (HBSS with 2% FCS and 0.1% NaAzide). For staining, samples were resuspended in blocking buffer, containing one of the following primary antibodies: anti-CD11c, an isotype control Ab, anti-I-A^b, anti-K^b, anti-CD86 (all from Pharmingen), anti-CD80, anti-CD54 (both from eBioscience) or anti-CD40 (Serotec). All these antibodies were biotin conjugated, and diluted 1/100, except anti-CD40, which was diluted 1/10. Antibodies were incubated for 30 min, samples washed twice in wash buffer, and stained with Streptavidin-RPE Cy-5 (DakoCytomation), which was diluted 1/100 in blocking buffer. Finally, cells were washed twice and resuspended in running buffer (HBSS with 0.1% NaAzide) for direct analysis. Alternatively, samples were fixed by incubating them in ice-cold HBSS containing 3% formaldehyde for 10 min, and then washed and resuspended in running buffer for analysis the next day. FACS samples were either run on a FACScan or a BD-LSR (dual laser) flow cytometer, and results were analysed using Cell Quest software (Becton Dickinson).

2.4.5 Intracellular FACS

Intracellular FACS was carried out to detect OVA expression in vector modified DC. Briefly, DC were fixed with 2% paraformaldehyde and permeabilised with HBSS containing 0.1% TritonX100 and 2% FCS. In the following steps, 0.1% Tritonx100 was

included in the buffers: DC were blocked with blocking buffer (see section 2.4.4) and stained with an anti-OVA rabbit polyclonal Ab (from UCL), and then with a goat anti-rabbit FITC Ab (eBioscience); antibodies were diluted 1/300 with blocking buffer. Finally, the cells were washed and analysed.

2.4.6 Confocal microscopy

Cells in culture were directly viewed under a Zeiss confocal microscope with a U.V. lamp to visualise GFP expression after transfection or transduction. A laser along with Lasersharp software (Bio-Rad) was used for more sensitive fluorescence analysis and to record and save images of the cells.

2.4.7 IL-12 ELISA

IL-12p70 was detected by sandwich ELISA, using a kit from eBioscience, according to the manufacturer's guidelines. The substrate supplied was Tetramethylbenzidine (TMB), and the reaction was stopped with 1M H_3PO_4. The optical density was measured at 450nm and 570nm. The 570nm values were first subtracted from the 450nm values before plotting the results obtained for the standards on a log scale, and setting a trendline. The equation given was then used to calculate the concentration of IL-12p70 in the test samples.

2.5 Western Blotting

2.5.1 Western buffers

Buffers and solutions used for Western blotting are listed below:

RIPA lysis buffer	150mM NaCl, 50mM Tris (pH 7.5), 1% Triton, 0.5% DOC, 0.1% SDS, 1mM EDTA, 1mM EGTA, 1mM DTT, 20mM NaF, 1mM $Na_4P_2O_7$, 1mM N_3PO_4, 1mM PMSF and PIM
Protein sample buffer (6x)	125mM Tris (pH 6.8), 6% SDS, 36% glycerol, 15% 2-ME with bromophenol blue
Cytoplasmic lysis buffer	10mM HEPES (pH 7.6), 1mM EDTA, 0.1mM EGTA, 10mM KCL, 1mM DTT, 20mM NaF, 5% glycerol, 1mM PMSF and PIM
Nuclear lysis buffer	20mM HEPES (pH 7.6), 0.2mM EDTA, 0.1mM EGTA, 25% glycerol, 0.42mM NaCl, 1mM DTT, 20mM NaF, 1mM $Na_4P_2O_2$, 1mM Na_3VO_4, 1mM PMSF and PIM
Separating SDS-gel	30% Acrylamide/Bis, 125mM Tris.HCL (pH 8.8), 10% SDS, 0.1% TEMED and 1% APS
Stacking SDS-gel	15% Acrylamide/Bis, 125mM Tris.HCL (pH 6.8), 10% SDS, 0.1% TEMED and 1% APS
SDS-Running Buffer	25mM Tris (pH 8.5), 200mM glycine, 0.1% SDS
Transfer Buffer	100mM Tris (pH 8.5), 200mM glycine with 20% MeOH
PBS tween	PBS with 0.05% Tween 20
Blocking buffer	PBS tween with 2.5% semi-skimmed milk

2.5.2 Preparation of total cell lysates

Cells were harvested and washed in ice-cold HBSS before resuspending in ice-cold RIPA buffer at a concentration of 10^7 cells/ml. Samples were rotated at 4°C for 20 min and then centrifuged at high speed for 10 min. The supernatant was transferred to a fresh tube (at this point a sample was taken for determining protein concentration) and 6x sample buffer was added, along with protease inhibitors (Roche). Samples were boiled at 94°C and stored at -20°C.

2.5.3 Nuclear and cytoplasmic fractionation

Cells were washed twice in ice-cold PBS and all trace of supernatant was removed. Then the pellet was resuspended in cold cytoplasmic buffer and incubated on ice for 15 min before adding NP40 to a final concentration of 0.6%. Samples were vortexed and incubated on ice for 4 min. The lysate was then underlayered with cytoplasmic buffer, containing 30% sucrose and centrifuged for 5 min at 4°C. The supernatant was removed and labelled as the "cytoplasmic fraction". The nuclear pellet was washed twice by

overlayering and removing cytoplasmic buffer and then resuspended in nuclear lysis buffer. The nuclear proteins were extracted by 3 cycles of transferring tubes from liquid nitrogen to a 37°C water bath. Finally, samples were centrifuged for 10 min at 4°C, and the supernatant was harvested and diluted 1:2 in cytoplasmic buffer, and labelled as the "nuclear fraction". After the protein concentration was determined, 6x sample buffer was added as normal and samples were boiled before loading on a gel.

2.5.4 Bradford assay

The concentration of protein was determined using the Bradford assay (Bradford, 1976); the protein assay reagent (Bio-Rad) was first diluted 1:5 in water and 1ml of this was added to 4µl and 8µl of each sample in disposable plastic cuvettes (Bio-Rad) In parallel, BSA protein standards were prepared, in order to calculate the concentration of the test samples. All samples were incubated for 5 min before measuring the optical density at 595nm.

2.5.5 SDS-PAGE and immunoblot analysis

Denaturing SDS-PAGE (polyacrylamide) gels (10%) were made fresh and consisted of a separating gel layered on top with a stacking gel. Samples were loaded (including a Benchmark pre-stained protein ladder, Invitrogen) and gels were run in SDS-running buffer at ~150 volts and blotted onto nitrocellulose membrane (Hybond ECL membranes, from Amersham) using semi-dry transfer (Pharmacia Biotech), in the presence of transfer buffer (at 15 volts for 30 min). Membranes were blocked for 30 min with blocking buffer and probed overnight at 4°C with the primary antibody (diluted in blocking buffer). Wash steps were carried out using PBS tween (3x 10 min), before adding the appropriate horseradish-peroxidase(HRP)-conjugated secondary antibody (DakoCytomation), diluted 1:2000 in blocking buffer. Finally, membranes were washed and developed using enzyme substrate ECL reagents and Hyperfilm ECL from Amersham.

2.5.6 Primary antibodies

Primary antibodies used for Western blotting (diluted in blocking buffer) are listed below.

Antibody	Dilution	Supplier
Anti-OVA (rabbit polyclonal)	1:500	Gift: B.Chain (UCL)
Anti-p24 (sheep polyclonal)	1:100	Aalto Bio Reagents Ltd.
Anti-HA (rat monoclonal)	1:1000	Roche
Anti-p100/p52 (mouse monoclonal)	1:1000	Upstate Biotec
Anti-vFLIP (rat monoclonal)	1:100	Gift: W.Low (UCL)
Anti-p65 (rabbit polyclonal)	1:200	Santa Cruz
Anti-Tubulin (mouse monoclonal)	1:1000	Gift: S.Ley (Mill Hill)
Anti-SP1 (rabbit polyclonal)	1:200	Santa Cruz
Anti-active-p38 (rabbit polyclonal)	1:2000	Cell Signaling

2.6 Immunological monitoring *ex-vivo*

2.6.1 Mice and immunisation

C57/BL6 mice (Harlen) were immunised with 10^7 i.u. of lentivector in the tail vein (or $5x10^6$ i.u. for the experiment shown in figure 3.7, or $1x10^8$ i.u. to track transduction *in vivo*; mice did not show any sign of disease from lentivector administration. Some mice were immunised with $2x10^6$ pfu of recombinant vaccinia virus expressing OVA (kind gift from Vincenzo Cerundolo). Mice were sacrificed at stated time-points and spleens or lymph nodes were harvested for analysis.

2.6.2 Harvesting the spleen and lymph nodes

Spleens and lymph nodes (without fat) were collected in HBSS and mashed through 70μm nylon meshes (BD Falcon). Cells were then resuspended and washed in HBSS containing 2% FCS. For the spleen samples, red blood cells were lysed using Red cell

lysis buffer (Sigma), followed by two wash steps. Splenocytes or lymph node cells could then be used for the assays described below.

2.6.3 The ELISpot assay

ELISpot plates (Millipore) were coated overnight with purified anti-IFN–γ, anti-IL-4 (both from Pharmingen) or anti-IL-2 (eBioscience) antibodies at 10μg/ml in HBSS. Ex-vivo ELISpots were performed with serial dilutions of total splenocytes in triplicate, +/- the appropriate peptide. OVA peptide concentrations used were 50ng/ml for the class I peptide and 1μM for the class II peptide, or cells were pulsed with native OVA (Sigma) at 50μg/ml. In some assays, transgenic T cell responders (see section 2.4.3) were added (thawed from frozen). The mitogen, ConA (Sigma) was used at 10μg/ml as a positive control. Plates were cultured overnight for 18-20 hours and developed according to the manufacturer's directions, using reagents from Bio-Rad.

2.6.4 Depletion of CD4+ or CD8+ T cells

Ovalbumin specific CD4+ and CD8+ T cell responses were detected by ELISpot (see above) by measuring the number of spots produced in response to the respective class II or class I peptide. Since this did not directly prove which T cells were responding, we depleted either the CD4+ or the CD8+ T cell fraction before some ELISpot assays, in order to validate our results. Either fraction was depleted by negative selection using Dynabeads (Dynal Biotech); the magnetic beads specific for either CD4 (L_3T_4) or CD8 (Lyt-2) were incubated with splenocytes, according to the manufacturers guidelines, and then the bound cells were depleted in two rounds using a Dynal Magnetic Particle Concentrator (Dynal Biotech). The remaining splenocytes were washed, counted and cultured as normal.

2.6.5 Tetramer staining

Blood samples were harvested in PBS containing 10mM EDTA, and red blood cells were lysed with RBC lysis buffer (Gentra systems), washed and resuspended in 20μl PBS + 2% FCS. The tetramer H-2Kb / SIINFEKL (Proimmune) was added (1μl) for 20 min at 37°C, before washing twice and staining with anti-CD8 FITC Ab (Proimmune) at

4°C. All incubations were kept in the dark. Samples were washed and resuspended in running buffer for flow cytometry (see section 2.4.4). For staining with the newer H-2Kb / SINFEKL pentamer (Proimmune), 10μl was added per sample and incubation was restricted to 15 min at room temperature.

2.6.6 Adoptive transfer of transgenic T cells

Spleens (kind gift from Alistair Noble) from OT-I or OT-II transgenic mice were mashed to obtain fresh splenocytes; the percentage of transgenic T cells was determined by FACS so that a cell number containing 10^6 OT-Is or OT-IIs could be administered into the tail vein. OT-II adoptive transfer was performed one day before immunisation whereas OT-I's were transferred at the stated time-points. Expansion (and activation) of the transferred T cells was assessed, 6 days post-transfer by analysing harvested splenocytes with flow cytometry. For OT-II detection, 4 colour FACS was conducted using the following Abs: CD4-PE (eBioscience), CD44-APC, Vβ5.1,5.2 biotin plus 2° reagent Streptavidin PerCP (all Pharmingen) and Vα2-FITC (Caltag). For OT-I detection, pentamer staining was carried out (see above). In each case, 1μl of antibody was used to stain 10^6 fresh splenocytes after blocking with blocking buffer. Cells were analysed in running buffer (see section 2.4.4).

2.6.7 Detection of serum antibodies by ELISA

ELISA plates (Nunc) were coated overnight with native OVA (Sigma) at 10μg/ml in coating buffer (0.015M Na_2CO_3 + 0.035M $NaHCO_3$) and the following day, plates were washed (with PBS tween), blocked (with PBS containing 2% milk) and loaded with serial dilutions of mouse serum (or plasma). Plates were incubated at 37°C for 2 hours and then washed and incubated with rabbit anti-mouse Ig HRP (DakoCytomation), diluted 1/3500. The substrate, Sigma Fast OPD (Sigma) was used to develop the assay and stop solution (3M HCL) was used to stop the reaction before reading the plate at 450nm. Results were displayed as the absorbance vs. the inverse serum dilution.

2.6.8 Purification of DC using MACS beads

Spleens were mashed with HBSS containing 0.5 mg/ml of collagenase D (Sigma) and 10mM of Hepes buffer (GibCo), to obtain a high yield of DC. Then, red blood cells were lysed (see before) and splenocytes were washed and passed through a 30μm nylon mesh. Cells were counted, incubated with an anti-FcR antibody and then washed twice. CD11c positive cells could then be selected from total splenocytes using MACS beads (Miltenyi Biotec), according to the manufacturer's guidelines. The CD11c positive and negative fractions were stained for flow cytometry (see section 2.4.4), using the following antibodies: anti-CD11c-PE, anti-B220-biotin, anti-CD11b-PE, anti-CD19-PE, anti-CD3-PE (all from eBioscience), anti-I-A^b-biotin (Pharmingen) and anti-F4/80-biotin (Serotec). All antibodies were diluted 1/100 in FACS buffer, except anti-F4/80-biotin, which was diluted 1/10. Samples that included biotin-labelled antibodies were incubated with APC-conjugated streptavidin (eBioscience) before FACS analysis.

2.6.9 Tumour challenge

Mice were challenged with 1-2 x10^6 tumour cells injected subcutaneously into the flank. Tumours were visible after 4 days in unvaccinated mice and the tumour size was approximated by multiplying the diameters, measured using calipers. A χ^2 test was used to determine the significance of differences between groups, based on the proportion of animals that developed tumours. Experiments were terminated when one animal had a tumour that reached a diameter >15mm.

Chapter 3: Immunisation with a lentiviral vector stimulates both CD4+ and CD8+ T cell responses to an ovalbumin transgene

3.1 Introduction

3.1.1 Lentivirus-based cancer vaccines

Therapeutic vaccines for persistent infectious disease or cancer must reactivate an inadequate immune response. One popular approach is to inject patients with peptide-pulsed dendritic cells (DC) because they are central to initiating T cell responses. Clinical trials with various DC vaccines have shown some success; melanoma patients have undergone tumour regressions (for example (Nestle et al., 1998)) and HIV-infected patients have shown enhanced immune responses (for example (Kundu et al., 1998)). More recently, methods have been developed for expression of antigen genes in DC, using transfection or viral vectors. This will allow prolonged presentation of multiple epitopes. However, *ex-vivo* manipulation of DC is laborious, so viral vectors encoding antigens are also being used for direct immunisation. Adenovirus and vaccinia virus vectors are being tested in clinical trials, although one obstacle has been the presence of pre-existing anti-vector antibodies in patients (Rosenberg et al., 1998b; Scholl et al., 2000).

Lentiviral vectors (lentivectors) based on HIV-1 can transduce DC both *in vitro* (Chinnasamy et al., 2000; Schroers et al., 2000) and *in vivo* (Esslinger et al., 2003; Palmowski et al., 2004; VandenDriessche et al., 2002). There have been several pre-clinical studies using lentivectors to modify DC ex-vivo for re-injection (Breckpot et al., 2003; Dyall et al., 2001; Esslinger et al., 2002; Granelli-Piperno et al., 2000; Metharom et al., 2001) or to target DC *in vivo* after direct injection (Esslinger et al., 2003; Palmowski et al., 2004). The latter strategy stimulates CTL responses equal or superior to those induced by DC vaccines (Esslinger et al., 2003; Palmowski et al., 2004; Rohrlich et al., 2004).

3.1.2 CD4+ T cells in anti-tumour immunity

The presence of antigen-specific CD4+ T cells has not been demonstrated following lentivector injection, despite clear evidence for their crucial role in anti-viral and anti-tumour immunity (De Veerman et al., 1999; Schnell et al., 2000) and reviewed in (Bevan, 2004; Pardoll and Topalian, 1998). CD4+ T cells are required to maintain memory CTL (Gao et al., 2002) (as is the case for B cell memory), but can also prime CTL, probably through "licensing" of antigen presenting cells (APCs) (Wang and Livingstone, 2003). CD4+ T cells at a tumour site can also interact with NK cells and macrophages to enhance tumour destruction (Hung et al., 1998; Marzo et al., 2000). Interestingly, tumour specific CD4+ CTLs have been isolated from melanoma patients that can lyse class II positive tumours (Manici et al., 1999; Schultz et al., 2000). Overall, recent data has confirmed that CD4+ T cell help is an absolute requirement for the generation of CD8+ T cells capable of mounting recall responses to viral (Janssen et al., 2003; Shedlock and Shen, 2003), bacterial (Sun and Bevan, 2003), tumour (Surman et al., 2000) or model (Bourgeois et al., 2002a; Bourgeois et al., 2002b) antigens. Cognate, rather than non-specific T cell help is most effective (Toes et al., 1999). Therefore, vaccines able to kick-start both CD4+ and CD8+ T cell responses will result in better immunity in patients.

3.1.3 Th1 and Th2 CD4+ T cells

The CD4+ T cell response can direct either type 1 or type 2 immunity, depending on the respective polarisation towards T helper 1 (Th1) or T helper 2 (Th2) cells (reviewed in (Murphy and Reiner, 2002; O'Garra, 1998)). Type 1 immunity involves potent CTL responses, macrophage activation, and B cell isotype switching to IgG2a antibodies. In contrast, type 2 immunity involves growth of eosinophils, and growth and activation of B cells and isotype switching to IgG1, IgE, and IgA antibodies. The transcription factors T-bet (T-box expressed in T cells) and GATA-3 promote Th1 or Th2 development respectively (Szabo et al., 2000; Zheng and Flavell, 1997).* A recent paper showed that naïve T cells tend towards Th2 development, unless GATA-3 levels are down-regulated by T-bet (Usui et al., 2006). The immune outcome (controlled by the Th1/Th2 bias) is thought to be determined by the antigen dose, the state of maturation of the DC, and the

* Note that Th1 cells secrete IL-2, IFN-γ and TNFα/β, whereas Th2 cells secrete IL-4, IL-5, IL-10 and IL-13.

type of "danger signals" encountered (Boonstra et al., 2003). Type 1 immunity in particular is known to be crucial in anti-tumour immunity (Winter et al., 2003).

3.1.4 Antigen processing and presentation

CD8+ and CD4+ T cells recognise antigens presented on MHC class I or MHC class II respectively.

In the classical (endogenous) MHC class I pathway (reviewed in (Cresswell et al., 2005)), endogenous proteins are tagged with ubiquitin, which marks them for rapid degradation. Polyubiquitinated proteins are then fed into proteasomes for digestion, yielding fragments between 2 and >20 amino acids in length; these amino acids are used for protein synthesis or energy, while a fraction of peptides are channelled into the ER (endoplasmic reticulum) by TAP (transporter associated with antigen processing). MHC class I molecules (MHC I-β_2 microglobulin heterodimers) are assembled in the ER with the help of tapasin and calreticulin and then loaded with 8-9 residue peptides that may first need to be trimmed by ERAP1 (ER aminopeptidase-1). MHC I-peptide complexes then travel through the golgi apparatus to the plasma membrane.

In the endocytic MHC class II pathway (reviewed in (Li et al., 2005)), the α and β subunits of MHC II assemble in the ER and associate with the invariant chain (Ii), which prevents peptides from binding. The complex then travels through the golgi stacks to late endosomes, called MIICs (MHC class II-containing compartments), due to a targeting motif in the cytoplasmic tail of invariant chain. Proteases, including cathepsin S, then cleave the invariant chain, leaving a fragment called CLIP in the peptide binding groove. Extracellular peptides in the MIIC can then be exchanged for CLIP, a reaction catalysed by HLA-DM (H-2M in mice). MHC II-peptide complexes are then transported to the plasma membrane.

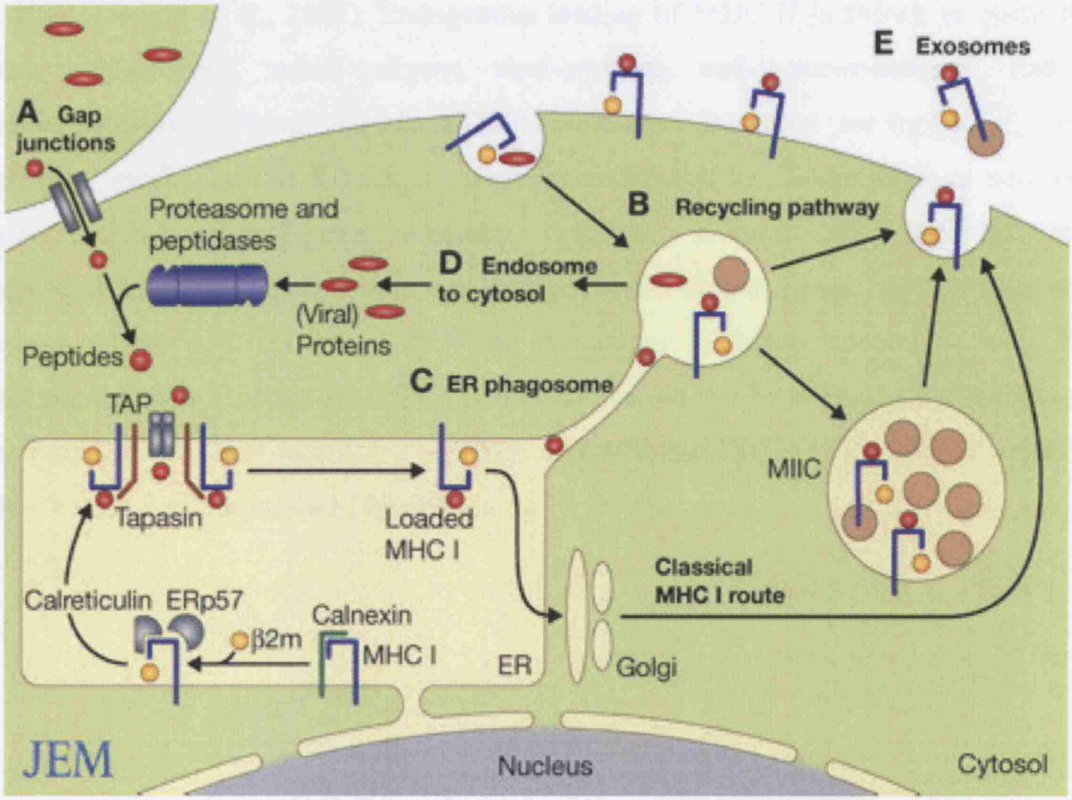
Apart from these two distinct pathways, exogenous antigens can also be presented on MHC I ("cross-presentation") and endogenous antigens can be presented on MHC II ("endogenous MHC II pathway").

Cross-presentation is an important pathway (reviewed in (Rock and Shen, 2005)) by which APCs can generate CTLs to extracellular antigens and viruses without the requirement for DC to be directly infected, (which could also compromise their function). There are 5 models leading to cross-presentation (Groothuis and Neefjes, 2005), which are described in figure 3.1.

Several groups have demonstrated that the antigenic material used for cross-priming is in fact cellular protein, and not peptides (Norbury et al., 2004; Serna et al., 2003; Shen and Rock, 2004; Wolkers et al., 2004). Generally, such exogenous proteins could follow 2 possible routes for processing and cross-priming: either they are degraded by endosomal proteases and loaded within phagosomes, or there exists a phagosome-to-cytosol transport pathway to channel antigens into the usual MHC I presentation pathway. The first scenario seems unlikely because atypical peptides could be generated by lysosomal proteases; the resulting CTL may not recognise the original peptide presented by the infected cell. However, certain antigens like soluble ovalbumin and virus-like particles (VLP) are known to be processed by endosomal proteases for cross-presentation. This so called vacuolar pathway was established after finding it to be TAP and proteasome independent, but blocked in cathepsin S deficient cells (Bachmann et al., 1995; Shen et al., 2004).

There is evidence for the alternative "phagosome-to-cytosol" pathway, (e.g. TAP / proteasome inhibitors block cross-presentation of particulate antigens (Kovacsovics-Bankowski and Rock, 1995)), although this is not definitive proof because in this case direct presentation of MHC I-peptide and therefore the recycling pathway would also be inhibited (Groothuis and Neefjes, 2005). Recent data suggesting that the phagosomal membrane could be formed in part from the ER membrane proposes that loading could occur independently of the ER (Ackerman et al., 2003; Guermonprez et al., 2003).

Figure 3.1



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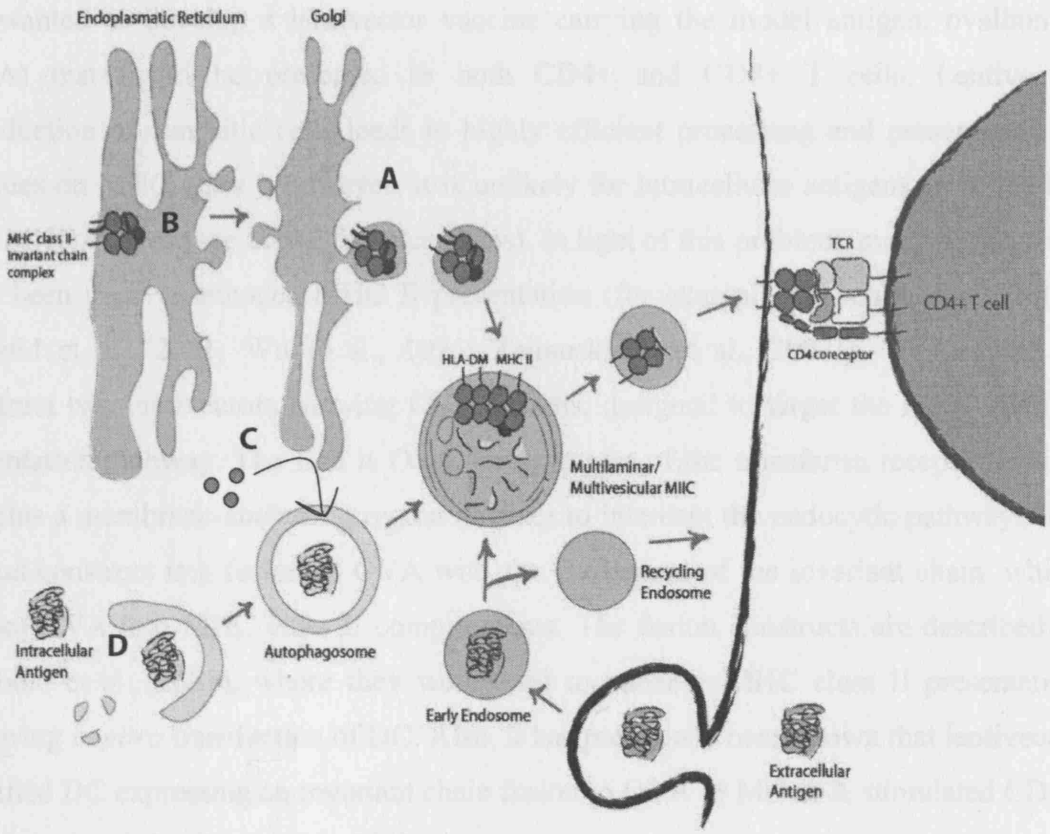
Suggested routes of cross-presentation

A) Endogenous peptides can be transported into dendritic cells that form gap junctions with infected cells and subsequently cross-presented. **B)** MHC class I molecules can exchange their endogenous peptides with exogenous ones after recycling from the cell membrane through the endocytic pathway. **C)** The phagosomal membrane may be formed in part from the ER membrane; the MHC class I loading machinery is borrowed from the ER. Exogenous antigens are transported by the ERAD system, out of the phagosome for degradation and then retro-transported back via TAP. **D)** Endosomal antigens are transported into the cytosol and subsequently follow the classical MHC class I pathway. **E)** Exosomes, which are vesicles that contain MHC I-peptide complexes can fuse with the dendritic cell and be cross-presented.

The term "endogenous MHC class II pathway" was coined when analysis of MHC II ligands revealed that > 20% of sequences were derived from cytosolic proteins (Chicz et al., 1993; Dongre et al., 2001). Endogenous loading of MHC II is known to occur for certain self-antigens, model-antigens, viral-antigens, and tumour-antigens, and 4 possible pathways (Schmid and Munz, 2005) have been described (see figure 3.2). One interesting mechanism of loading is through autophagy, an innate defence pathway against microbial pathogens whereby cytosolic proteins are engulfed into autophagosomes and digested (van der Bruggen and Van den Eynde, 2006). Recently, electron microscopy confirmed that the autophagy pathway converges with the endocytic pathway (Liou et al., 1997). It was also found that by silencing the autophagy gene (*Atg12*) with short-interfering (si)RNA, activation of EBNA1(from EBV)- specific CD4+ T cells was diminished (Mizushima et al., 1998).

Figure 3.2



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Suggested routes of endogenous MHC class II presentation
A) Transmembrane or secretory proteins that contain targeting signals can associate with MHC class II molecules and intercept the endocytic pathway. **B)** Cytosolic peptides in the ER can bind MHC class II molecules that fail to associate with the invariant chain. Peptides **(C)** or cellular proteins **(D)** (shown) can be transported into endosomes / lysosomes by a transporter, thought to be Lamp-2a (lysosome-associated membrane protein 2a, the transporter of chaperone-mediated autophagy) or by autophagy respectively.

3.1.5 Enhancing antigen presentation on MHC class II

We wanted to develop a lentivector vaccine carrying the model antigen, ovalbumin (OVA) that could be presented to both CD4+ and CD8+ T cells. Lentivector transduction of dendritic cells leads to highly efficient processing and presentation of peptides on MHC class I, however, it is unlikely for intracellular antigens to be loaded onto MHC class II (see above for exceptions). In light of this problem, many approaches have been used to enhance MHC II presentation (for example (Bonehill et al., 2003; Diebold et al., 2001; Wu et al., 1995; Zaliauskiene et al., 2002)). We decided to construct two lentivectors carrying OVA fusions, designed to target the MHC class II presentation pathway. The first is OVA fused to part of the transferrin receptor, which contains a membrane-anchoring region in order to intercept the endocytic pathway. The second construct is a fusion of OVA with the C-terminal of the invariant chain, which traffics OVA into MHC class II compartments. The fusion constructs are described in (Diebold et al., 2001), where they were used to enhance MHC class II presentation following *in vitro* transfection of DC. Also, it has previously been shown that lentivector modified DC expressing an invariant chain fusion to OVA or Melan-A stimulated CD4+ T cells *in vitro* (Breckpot et al., 2003).

3.2 Aims

The aims of this project were to:

- Construct lentivectors expressing secreted OVA, cytoplasmic OVA, or fusions of Ii-OVA and TfR-OVA
- Evaluate their relative abilities to present OVA to CD4+ and CD8+ T cells *in vitro* and *in vivo* (after direct injection into mice)
- Compare the vaccine efficacy of the panel of lentivectors using the OVA expressing tumour, EG7.OVA

3.3 Ovalbumin expressing lentiviral vectors

3.3.1 Lentiviral vector map

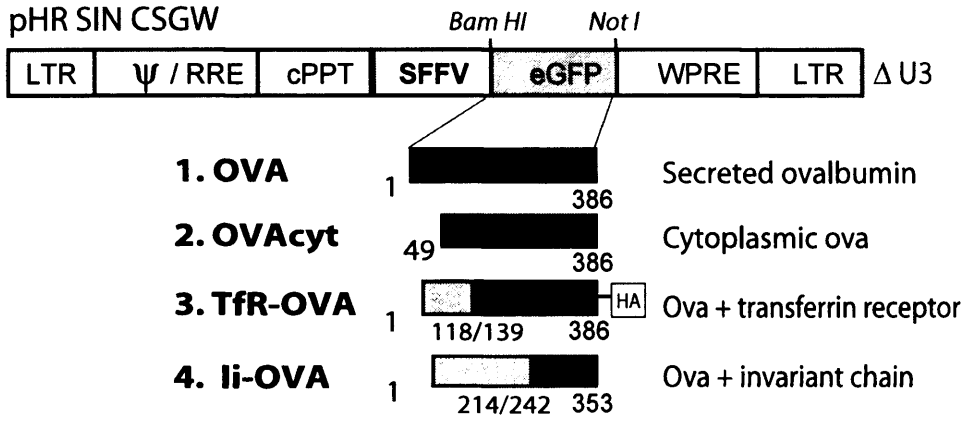
We are using the lentiviral vector pHRSIN-CSGW, (Demaison et al., 2002) (supplied by A. Thrasher), carrying an SFFV promoter because it expresses transgenes in both mouse and human DC (Neil et al., 2001; Palmowski et al., 2004). We used a GFP expressing vector (GFP LV) or a non-enveloped OVA vector (No Env LV) as controls and constructed vectors expressing various ovalbumin (OVA) transgenes (see figure 3.3). All of the OVA constructs (secreted, cytoplasmic, or targeted to the MHC class II pathway) contained the immunodominant MHC class I and II epitopes. *Bam HI* and *Not I* were used for subcloning; Ii-OVA could be directly subcloned, but the other constructs were first amplified by PCR to introduce restriction sites. The integrity of the inserts was verified by sequencing.

3.3.2 Production and titering of lentivector batches

Lentiviral vectors were produced by co-transfection of the vector plasmid with pCMVR8.91 and pMDG (see methods). The GFP vector, which was titered by FACS and Q-PCR, was used to determine the relative titers of the OVA expressing vectors according to their copy numbers. In this way, equivalent transducing units (referred to as infectious units, i.u.) of each vector could be used for *in vitro* transduction or immunisation (see figure 3.4A). We also demonstrated that the levels of p24 were similar between different vectors; supernatants from the vector batches containing equivalent numbers of i.u. were run on a gel and the Western was probed with an anti–p24 antibody (see figure 3.4B).

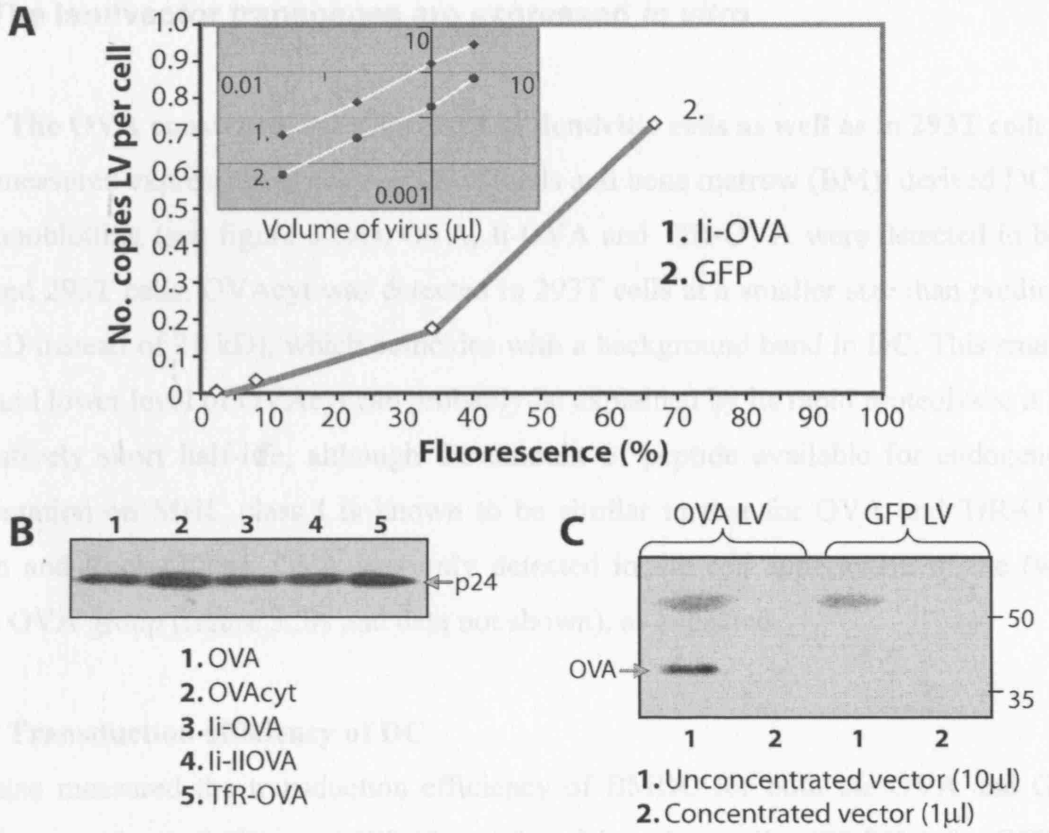
One concern was that during vector production transgene protein might become packaged or accumulate in the vector supernatant. This was checked by Western (using an anti-OVA antibody) and by Coomassie staining; OVA transgene and FCS proteins were detected in unconcentrated but not in concentrated vector preparations (see figure 3.4C).

Figure 3.3



Lentiviral vector map, showing the 4 different OVA constructs expressed. All 4 OVA constructs contain the immunodominant MHC class I and II epitopes, positioned at amino acids 245-264 and 323-339 respectively. OVA (1.) is the wild type ovalbumin construct (1, 161 bp) that is secreted. OVAcyt (2.) is cytoplasmic (1, 017 bp) because it lacks the secretory signal. TfR-OVA (3.) is a fusion of the first 118 amino acids of the human transferrin receptor, to amino acids 139-386 of OVA (1, 304 bp) (this construct also contains a HA tag). Ii-OVA is a fusion of the C-terminal end of the invariant chain with amino acids 242-353 of OVA (1, 658 bp). LTR, long terminal repeat; Ψ , packaging signal; RRE, rev response element; cPPT, central polypurine tract; SFFV, spleen focus forming virus promoter; WPRE, wood chuck post transcriptional regulatory element; Ub, ubiquitin promoter; Δ U3, deletion in U3 region (SIN vector); HA, haemagglutinin tag.

Figure 3.4



Lentivector batches were titered by Q-PCR & FACS, to ensure that the number of transducing units used was equivalent between vectors. Each vector batch was concentrated 2x by ultracentrifugation (before the 2nd spin, the vector pellet was washed each time in ~20ml HBSS) and resuspended in a final volume of 500-1000 µl HBSS. **A)** Vectors were titered by infecting $2x10^5$ 293T cells with medium containing either: 2.5,1, 0.2, or 0.04 µl of vector. 5 days post infection, expression of GFP was assessed by FACS (for the GFP vector) and genomic DNA was extracted and copy no.s measured by Q-PCR (see methods). One representative experiment is shown: the inner graph shows the Q-PCR results (volume of virus added vs. no. copies per cell) plotted on a log scale, and the outer graph shows copy no. vs. fluorescence for the GFP vector (at the 4 different vector volumes). Relative transducing units (referred to as infectious units, i.u. in experiments) could then be calculated for other vectors made in parallel from their copy no.s, using those values that corresponded to between 5 and 20% fluorescence for the GFP vector. **B)** $8x10^5$ i.u. of each conc. vector prep. was loaded per well and the gel was probed with an αp24 sheep antibody (Ab). (Ii-IIOVA is a vector that was not used in subsequent experiments, expressing only the immuno-dominant class II epitope of OVA). 1 experiment of 2 is shown. **C)** Unconcentrated and concentrated vector preps. (the titer was $3x10^9$ i.u./ml) were loaded on a gel to check for the presence of the OVA transgene (43kD). The blot was probed with a polyclonal αOVA Ab (rabbit), which also reacts to FCS proteins (probably BSA), as seen here. 1 experiment of 2 is shown. OVA and FCS were also detected by Coomassie staining, only in unconc. preps (not shown).

3.4 The lentivector transgenes are expressed *in vitro*

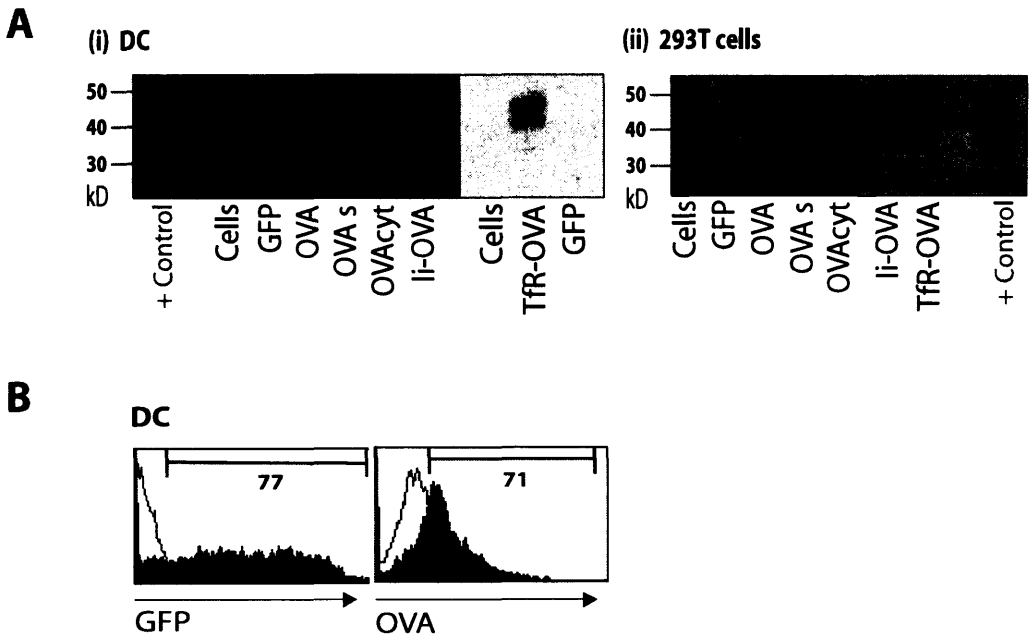
3.4.1 The OVA constructs are expressed in dendritic cells as well as in 293T cells

We measured expression of OVA in 293T cells and bone marrow (BM)- derived DC by immunoblotting (see figure 3.5A). OVA, Ii-OVA and TfR-OVA were detected in both DC and 293T cells. OVAcyt was detected in 293T cells at a smaller size than predicted (30 kD instead of 37 kD), which coincides with a background band in DC. This smaller size and lower level of OVAcyt can probably be explained by its rapid proteolysis; it has a relatively short half-life, although the amount of peptide available for endogenous presentation on MHC class I is known to be similar to that for OVA and TfR-OVA (Shen and Rock, 2004). OVA was only detected in the cell supernatant of the (wild type) OVA group (figure 3.5A and data not shown), as expected.

3.4.2 Transduction efficiency of DC

We also measured the transduction efficiency of BMDC for both the OVA and GFP lentivectors (figure 3.5B). At MOI 40, we found it to be similar (70-80%) for GFP or OVA, which was detected by intracellular FACS using the anti-OVA antibody. DC cultures were typically 70-90% CD11c positive in 5 experiments (see chapter 5 for DC phenotype following lentivector transduction), and the transduction efficiency was 50-80% at MOI 20-40 in 5 experiments; we therefore used MOI 20 for further *in vitro* experiments.

Figure 3.5



The lentivector transgenes are expressed in DC and 293T cells. A) Bone marrow (BM)-derived DC (i), or 293 T cells (ii) were transduced with the vectors at MOI- 20 and harvested for western blotting 4 days later. All 293T samples were diluted 1/5 for loading, except OVAcyt, because it has a short half-life and was otherwise undetectable. Immunoblots were probed with an αOVA polyclonal Ab, except TfR-OVA in DC, which was probed with an αHA tag Ab. All samples were cell lysates, except OVA s, showing secreted OVA (20μl supernatant from cells cultured at 10^6 cells/ml). Supernatants from the other DC and 293T cell groups, did not contain OVA, as checked by immunoblot (data not shown). + Control, 10ng of native ovalbumin. Predicted sizes: OVA, 43; OVAcyt, 37; Ii-OVA, 36; TfR-OVA, 41kD. **B)** BMDC were transduced with either the GFP or OVA vectors at MOI- 40 (in this experiment only). GFP expression was detected by FACS (left panel) and OVA by intracellular FACS (right panel), using the αOVA Ab (see methods). The numbers show the % transduction, as compared to uninfected cells (black outline).

3.5 Presentation of ovalbumin by transduced DC *in vitro*

Antigen presentation on MHC class I or II was assessed by modifying BMDC with the OVA expressing vectors and culturing them with either OT-I or OT-II transgenic T cells; OT-Is and OT-IIs recognise the respective immunodominant class I and II epitopes of OVA. Transgenic T cells were first expanded to high purity from transgenic mice (see methods); the mean purity was 91.49% for OT-Is (3 experiments) and 97.09% for OT-IIs (4 experiments).

3.5.1 OVA is processed and presented on MHC class I by vector modified DC

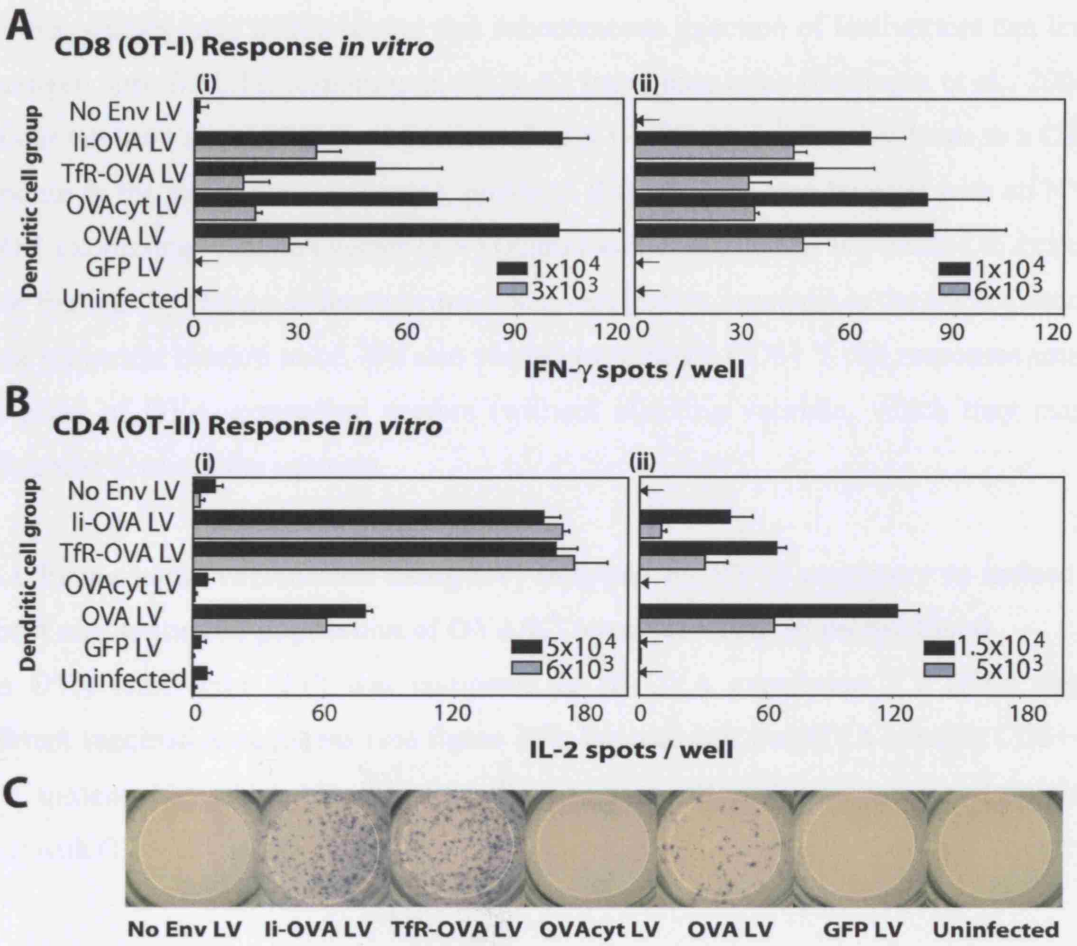
The ability of vector modified DC to stimulate a CD8+ T cell response was measured by IFN-γ ELISpot. DC expressing OVA, OVAcyt, TfR-OVA or Ii-OVA, could all present the MHC class I epitope to OT-I T cells (figure 3.6A). The response was similar using DC modified with any of the 4 vectors, although it was slightly higher with the Ii-OVA and OVA vectors. Two experiments are shown in figure 3.6A and a third is described in figure 3.6 legend. There was no response with the control groups of uninfected DC, GFP LV modified DC, or DC modified with a non-enveloped OVA LV, but all groups were able to prime a response in the presence of exogenous $OVA_{257-264}$ peptide as a positive control (data not shown).

3.5.2 OVA is processed and presented on MHC class II by vector modified DC

The same vector modified DC were cultured with OT-II T cells to measure the OVA specific CD4+ T cell response (figure 3.6B & C). We found that the OVAcyt LV modified DC could not stimulate OT-II T cells to release IL-2 (5 experiments), probably because OVAcyt did not have access to the MHC class II presentation pathway. This has been described for retrovirally modified DC expressing cytoplasmic OVA (De Veerman et al., 1999). However, Ii-OVA-, TfR-OVA- and OVA- expressing DC all induced a CD4+ T cell response. The relative response of OVA, compared to Ii-OVA and TfR-OVA varied between 3 experiments, 2 of which are shown in figure 3.6B and one of which is described in figure 3.6 legend. DC could therefore present OVA whether it was targeted into the MHC class II pathway or secreted, despite the previous finding

that uptake of soluble OVA is inefficient (Li et al., 2001). This is perhaps due to high expression levels by the lentivector; the variability in the relative magnitude of the secreted OVA response may depend on the extent to which it accumulates in the culture. All DC groups responded when pulsed with native OVA as a control (not shown).

Figure 3.6



OVA is processed and presented on MHC class I and II by vector modified DC. BMDC were transduced with the vectors at MOI- 20 and cultured with OVA transgenic T cells in ELISpot assays (see methods). OT-I T cells recognise OVA257-264, presented on MHC Kb and OT-II T cells recognise OVA323-339 on MHC I-Ab. 2 experiments are shown (i) and (ii). Each bar is the mean of triplicate wells, shown for 2 T cell dilutions (see key). No Env, non-enveloped LV. A *t* test was used to determine if differences were significant (p values < 0.05 were considered significant), taking results from the grey bars, to avoid saturation of the response. **A)** DC were plated at $3x10^3$/ well in (i) or $1x10^4$/well in (ii) with OT-I T cells. The response to Ii-OVA was significantly higher than that to TfR-OVA (p= 0.025 in (i), 0.038 in (ii)), or to OVAcyt (p= 0.015 and 0.007). The response to OVAcyt was lower than that to OVA (p= 0.03 and 0.032). **B)** The same DC were plated in parallel at $1x10^4$/well in (i) and $2x10^4$/well in (ii) with OT-II T cells. The response to Ii-OVA was significantly higher than that to OVA in (i) (p=<0.001), but the opposite was true in (ii) (p= 0.001). The response to TfR-OVA was higher than OVA in (i) (p= 0.001), but the response to OVA was higher in (ii) (p= 0.017). The response to TfR-OVA was higher than Ii-OVA in (ii) (p= 0.03). In a repeat experiment, where OT-Is responded similarly to all OVA-transduced DC, OT-IIs gave a mean of 112 spots/ well, in the Ii-OVA group, compared to 152 in the TfR-OVA group and 168 in the OVA group (DC at 10^4/ well + $5x10^4$ T cells). **C)** Typical ELISpot wells (from experiment B) (i)).

3.6 Ovalbumin specific CD8+ T cell responses *in vivo*

Previous studies have demonstrated that subcutaneous injection of lentivectors can lead to antigen specific CTL responses in HLA-A2 transgenic mice (Esslinger et al., 2003) and our lab have shown that i.v. injection of an NY-ESO-1 encoding LV leads to a CTL response in the same transgenic mice, provided that mice are also boosted with an NY-ESO-1 expressing vaccinia vector (VV) (Palmowski et al., 2004). We wanted to extend these findings by testing different prime-boost vaccination regimens in the OVA system, using syngeneic black/6 mice. We also wanted to compare CD8+ T cell responses using our panel of OVA expressing vectors (without injecting vaccinia, which may mask differences between the vectors).

3.6.1 Prime-boost vaccination using LV, followed by VV is necessary to induce a robust and sustained population of OVA/K^b tetramer+ cells in mouse blood

The OVA lentivector (*LV*) was compared to an OVA expressing VV (*VV*), using different vaccination regimens (see figure 3.7); the read-out was OVA specific CD8+ T cells, measured by taking blood samples from mice at different time-points and staining them with OVA/K^b tetramers.

Figure 3.7

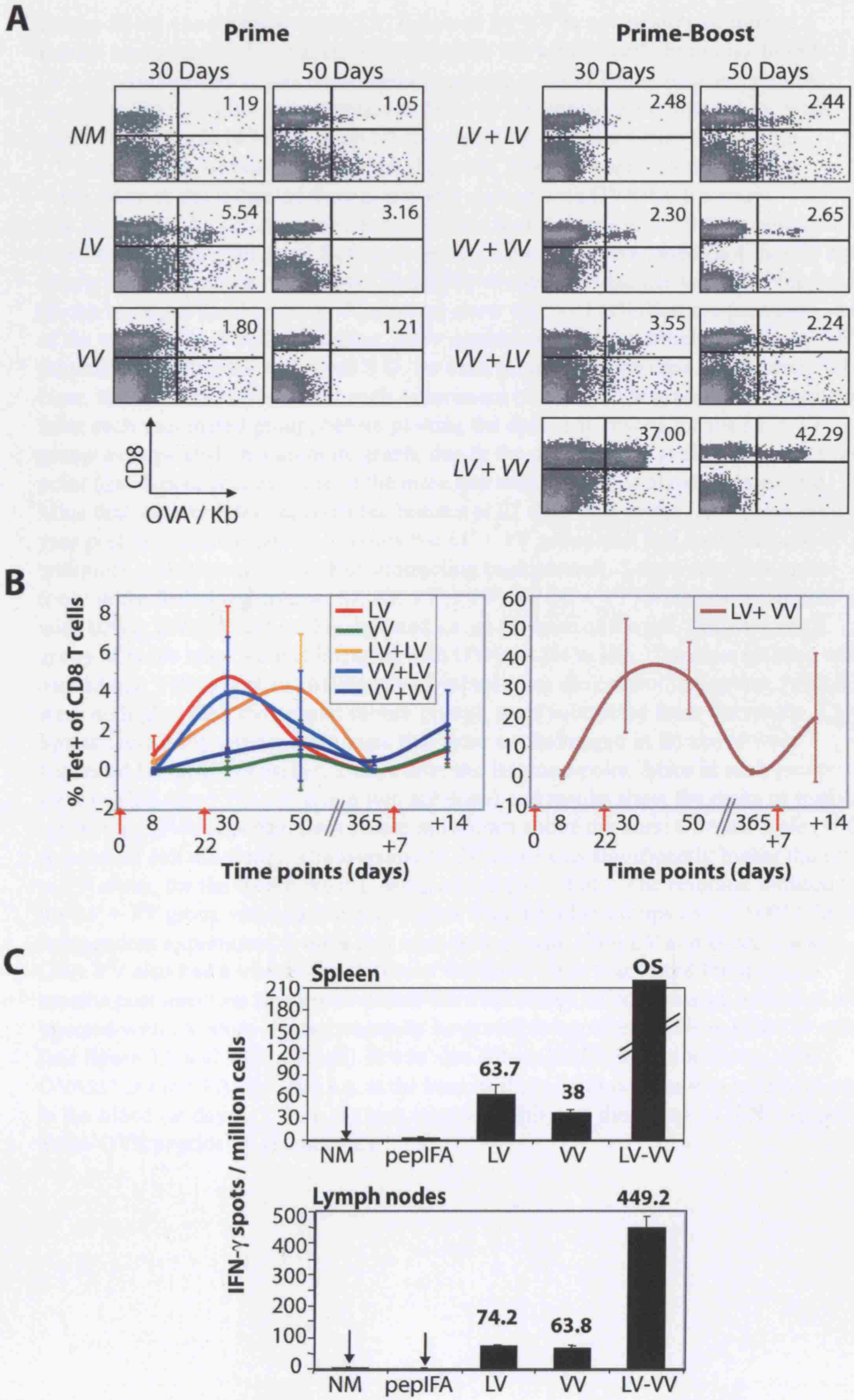

A
Prime
Prime-Boost
30 Days
50 Days
30 Days
50 Days
NM
LV
VV
LV + LV
VV + VV
VV + LV
LV + VV
1.19
1.05
5.54
3.16
1.80
1.21
2.48
2.44
2.30
2.65
3.55
2.24
37.00
42.29
CD8
OVA / Kb
B
% Tet+ of CD8 T cells
LV
VV
LV+LV
VV+LV
VV+VV
LV+ VV
Time points (days)
Time points (days)
C
Spleen
OS
IFN-γ spots / million cells
63.7
38
NM
pepIFA
LV
VV
LV-VV
Lymph nodes
449.2
74.2
63.8

Figure 3.7

Prime-boost vaccination using LV, followed by VV is neccessary to induce a robust and sustained population of OVA/Kb tetramer+ cells in mouse blood. LV (*LV*) immunisation was compared to vaccinia virus (*VV*), by measuring OVA specific CD8+ T cells, using tetramers. Mice (3 per group) were injected i.v. with either OVA LV ($5x10^6$ i.u.), or an OVA-expressing vaccinia virus ($2x10^6$ p.f.u., UV inactivated), or both, in either combination, 3 weeks apart. Blood samples were taken at the indicated time points and stained with OVA/Kb tetramers (see methods). **A)** Tetramer results for 2 of the time points (30 & 50 days post 1st injection), showing mouse 1 from each group. Live cells were gated (not shown) and results are shown as density plots (30-50,000 events recorded per sample). Numbers (in the top right-hand corners of the plots) show the % of cells that are tetramer+, out of the total CD8+ T cell population. *NM*= normal mouse (non-immunised group). **B)** Summary showing the mean and S.D. for each group at all the time points analysed. Here, the mean background for each experiment (from the *NM* group) was subtracted from each vaccinated group, before plotting the data. The results for the *LV + VV* group were plotted on a separate graph, due to the difference in scale. At the 1st time-point (day 8 post prime), none of the mice had tetramer+ cells above background. Mice that were boosted received the booster at 22 days post prime (see scale). By 1 year post prime (time-point 365) only the *LV + VV* group still had a visible pool of tetramer+ cells (mean, 1.7% after subtracting background). 7 days after this, mice (only in the following groups: *LV, VV, VV + VV and LV + VV*) were re-challenged with 100μg OVA257-264 in IFA, injected s.c. at the base of the tail. Also a control group of naive mice were challenged with OVA257-264 in IFA. Tetramer staining was carried out, 7 days post re-challenge and values from the control IFA group, (which were no higher than the normal mouse group), were subtracted from the results. **C)** Spleens and lymph nodes from mice that were re-challenged in B) above were harvested for ELISpot assays, 2 days after the last time-point. Mice in each group were pooled (the *VV + VV* group was not done) and results show the mean of triplicate wells (plus OVA peptide). Data values are shown above the bars. OS= off scale (>500 spots at all cell dilutions). The response to *LV alone* was significantly higher than that to *VV alone*, for the spleen results, using a *t* test ($p = <0.05$). The response induced by the *LV + VV* group was significantly higher than the other groups ($p = < 0.001$). In an independent experiment, 2 mice that were primed with OVA LV and boosted with OVA VV also had a visible population of tetramer+ cells that lasted for at least 3 months post injection (later time-points were not done). In comparison, mice that were injected with LV alone did not normally have visible tetramer+ cells in blood or spleen (see figure 3.8 and 3.10 legends). It was also determined that immunisation with OVA257-264 in CFA, injected s.c. at the base of the tail did not result in tetramer+ cells in the blood (at day 5, 7, 9 or 13) post injection, although there was an IFN-γ response to the OVA peptide by splenocytes.

Overall, it was difficult to detect a pool of tetramer+ cells without boosting; in the *LV alone* group (figure 3.7A, left-hand panels, and B) responses were undetectable 8 days post injection, but by 30 days, a mean of 4.82% of CD8+ T cells were OVA specific. This was substantially higher than in the *VV alone* group, which, also undetectable after 8 days, gave a mean of 0.14% after 30 days. It is important to stress that most differences between groups (including these 2 groups) were not significant (using a student *t* test) because of the high variability between mice, (although the *LV + VV* group responses were significantly higher than the *VV alone* group, p= <0.05).

However, there were general trends based on whether or not a population of tetramer+ cells was visible in mouse blood. Using this criterion, 2/3 and 1/3 mice responded in the *LV alone* and *VV alone* groups respectively (time-point: 30 days). By 50 days post injection, 0/3 and 1/3 mice still had a respective tetramer+ population in the *LV alone* and *VV alone* groups.

In the prime-boost groups, 3/3 mice in the *VV + VV*, *VV + LV* and *LV + VV* groups responded (time-point: 30 days) and maintained their tetramer+ population after 50 days (figure 3.7A, right-side). In contrast, results from the *LV + LV* group suggest this not to be an effective vaccine regimen. The response in the *LV + VV* group was substantially higher than all the other groups (see figure 3.7A, right-side, and B) and mice in this group were the only ones to sustain a visible population of tetramer+ cells over a year (a mean of 1.7% of CD8s were tetramer+ after one year). Interestingly, although the CD8+ T cell response was probably predominately vaccinia specific at day 30 (assumed because of the expansion of non-OVA specific CD8s), the proportion of CD8s that were OVA specific increased by 50 days (see figure 3.7A, right-hand panels).

Surprisingly, one year post-injection we were able to detect recall responses in mice in the *LV alone* or *VV alone* groups, after re-challenging with OVA peptide in incomplete Freunds' adjuvant (pepIFA); this vaccine does not stimulate a CD8+ response on its own (see figure 3.7B & C). It is possible that few OVA specific memory CD8+ T cells

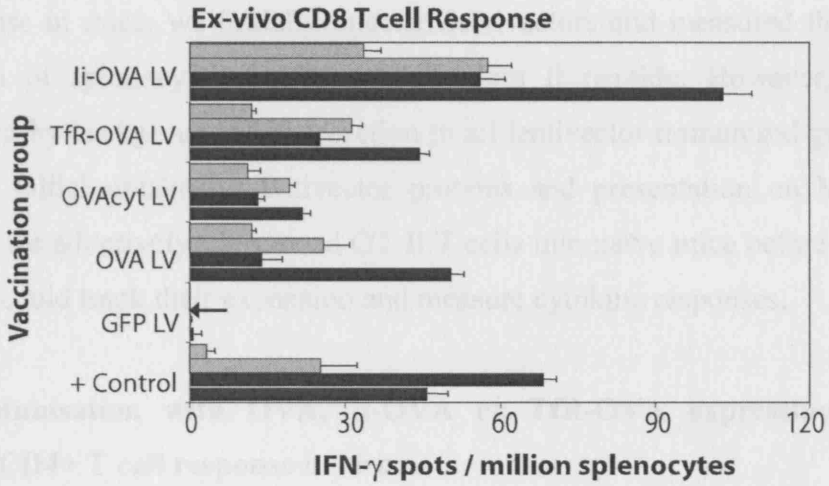
(below detection by tetramer staining) are required to mount a secondary response. Mice in the *LV + VV* group mounted a dramatic recall response to OVA re-challenge (figure 3.7B & C); ELISpot results showed that the response was > 6 times higher (an average of 449.2 IFN-γ spots / million lymph node cells) than the other groups, which was significant ($p = <0.001$).

These data suggest that lentivector vaccines should be trialled in prime-boost protocols, using LV prime and VV boost. This vaccine regimen was also effective in priming a CTL response in the NY-ESO-1 system (Palmowski et al., 2004). We propose that the lentivector is an effective vaccine for priming a CD8+ response because it expresses the antigen in DC. The response can then be boosted by vaccinia, which infects many cell types and expresses the antigen plus a range of viral proteins. This mechanism was proposed to explain the effective prime-boost immunisation seen with DNA vaccination followed by boosting with vaccinia (Schneider et al., 1999). We have shown here that memory CTL are maintained for at least a year, using this approach. Furthermore, the results illustrate the value of the more sensitive ELISpot assay over tetramer staining when measuring CD8+ T cell responses induced solely by the lentivector.

3.6.2 OVA expressing vectors prime a CD8+ T cell response *in vivo* with Ii-OVA LV being the most potent

The panel of OVA expressing lentivectors were compared for their ability to stimulate an OVA specific CD8+ T cell response, following direct injection. The response was measured by ex-vivo IFN-γ release by splenocytes after adding OVA peptide $_{257\text{-}264}$ (figure 3.8). We found that all 4 OVA expressing lentivectors induced a CD8+ T cell response; Ii-OVA LV stimulated more IFN-γ release than OVA LV, TfR-OVA LV and OVAcyt LV in 2 separate experiments. This difference was significant as determined by using a *t* test to compare Ii-OVA to the other 3 groups ($p \leq 0.001$). We then compared the 2 vectors Ii-OVA and OVA again in an 3rd experiment with duplicate mice and the Ii-OVA response was again higher e.g. both Ii-OVA mice gave >400 spots / million splenocytes, while the 2 OVA mice gave respective means of 118 and 105 spots / million splenocytes (data not shown).

Figure 3.8



OVA expressing lentivectors prime a CD8+ T cell response *in vivo* with Ii-OVA LV being the most potent. Duplicate mice were immunised (see methods) per group, in 2 separate experiments (grey bars and black bars). 14 days later, spleens were harvested and ELISpot assays done with total splenocytes, plus and minus OVA 257-264 peptide, in triplicate wells. Results of individual mice plus peptide are shown. No responses were observed in the absence of peptide. The Ii-OVA vector gave a significantly higher response than the OVA, OVAcyt and TfR-OVA vectors, for all mice ($p = \leq 0.001$). + Control, OVA peptide in complete Freund's adjuvant, injected s.c. at the base of the tail. Splenocytes from these mice were also stained with OVA/Kb tetramers; no tetramer + cells were detected in mice from any of the groups (not shown). Ii-OVA LV injected mice mounted a higher response than OVA LV mice in a 3rd experiment, described in the results; the LV dose was $3x10^7$ i.u. per mouse in this experiment only. Serum samples taken from these mice on day 14 (before harvesting spleens), showed that both OVA mice had OVA specific Abs whereas both Ii-OVA mice did not, as measured by ELISA (see figure 3.12 A).

3.7 Ovalbumin specific CD4+ T cell responses *in vivo*

To determine whether the lentivectors could also stimulate an OVA specific CD4+ T cell response in mice, we initially injected lentivectors and measured the response by stimulation of splenocytes with the OVA class II peptide. However, results were complicated by background IL-2 secretion in all lentivector immunised groups, perhaps caused by initial uptake of lentivector proteins and presentation on MHC class II. Therefore, we adoptively transferred OT-II T cells into naïve mice before immunisation so that we could track their expansion and measure cytokine responses.

3.7.1 Immunisation with OVA, Ii-OVA or TfR-OVA expressing lentivectors induces a CD4+ T cell response *in vivo*

OT-II T cell expansion results are displayed as the percentage of CD4+ T cells that were OVA specific (OT-IIs) (figure 3.9A shows typical mice). We also looked at the up-regulation of CD44 on these cells, known as a marker of antigen recognition (Camp et al., 1991) (figure 3.9A, right-hand panel). The control groups (no vaccine or GFP LV) gave rise to about 0.8-0.9 % OT-II T cells, following adoptive transfer but these cells were still naïve as shown by their low expression of CD44 (up to 21%). However, in the OVA-, Ii-OVA- and TfR-OVA- LV immunised mice, OT-IIs expanded and CD44 was up-regulated (60-90%), demonstrating that the cells were antigen experienced. Figure 3.9B gives a summary of these data. Here, OT-IIs expanded to an average of 6.7% (shown in upper graph), following immunisation with the OVA LV, compared to an average of 4.2% with the Ii-OVA LV and 2.2% with the TfR-OVA LV. In a repeat experiment, where controls gave rise to an average of 1.19%, OT-IIs, OVA LV and Ii-OVA LV induced similar expansions with averages of 3.62% and 3.92% respectively, while the TfR-OVA LV gave an average again of 2.2% OT-IIs.

In contrast, the OVAcyt LV did not stimulate OT-II T cells *in vivo* (see figure 3.9D), as shown by no expansion or CD44 up-regulation; this result was unsurprising given that OVAcyt was not presented on MHC class II *in vitro* (figure 3.6B & C).

3.7.2 Immunisation with the Ii-OVA LV stimulates the most cytokine secreting T helper cells

We measured IL-2, IFN-γ and IL-4 secretion from splenocytes (mice from figure 3.9B were pooled) in response to the OVA class II peptide. We choose to compare the OVA LV and the Ii-OVA LV, since these vectors induced the greatest expansion of adoptively transferred OT-II cells. The OVA LV group gave rise to more IL-2 spots than the Ii-OVA LV group (figure 3.9C), in agreement with the expansion data, but interestingly, the Ii-OVA LV group induced 3 times as many IFN-γ spots as the OVA LV group (this difference was significant, $p = <0.01$). This could suggest that "endogenous MHC class II presentation" favours type 1 immunity. However, we found that the Ii-OVA LV group also induced 3 times as many IL-4 spots as the OVA LV group (figure 3.9C) (this difference was significant, $p = <0.001$). These data suggested that while secreted OVA could induce an equal or slightly greater expansion of OT-II T cells, possibly owing to presentation of secreted OVA by more cells, Ii-OVA presentation resulted in more effector T cells secreting both IL-4 and IFN-γ.

Figure 3.9

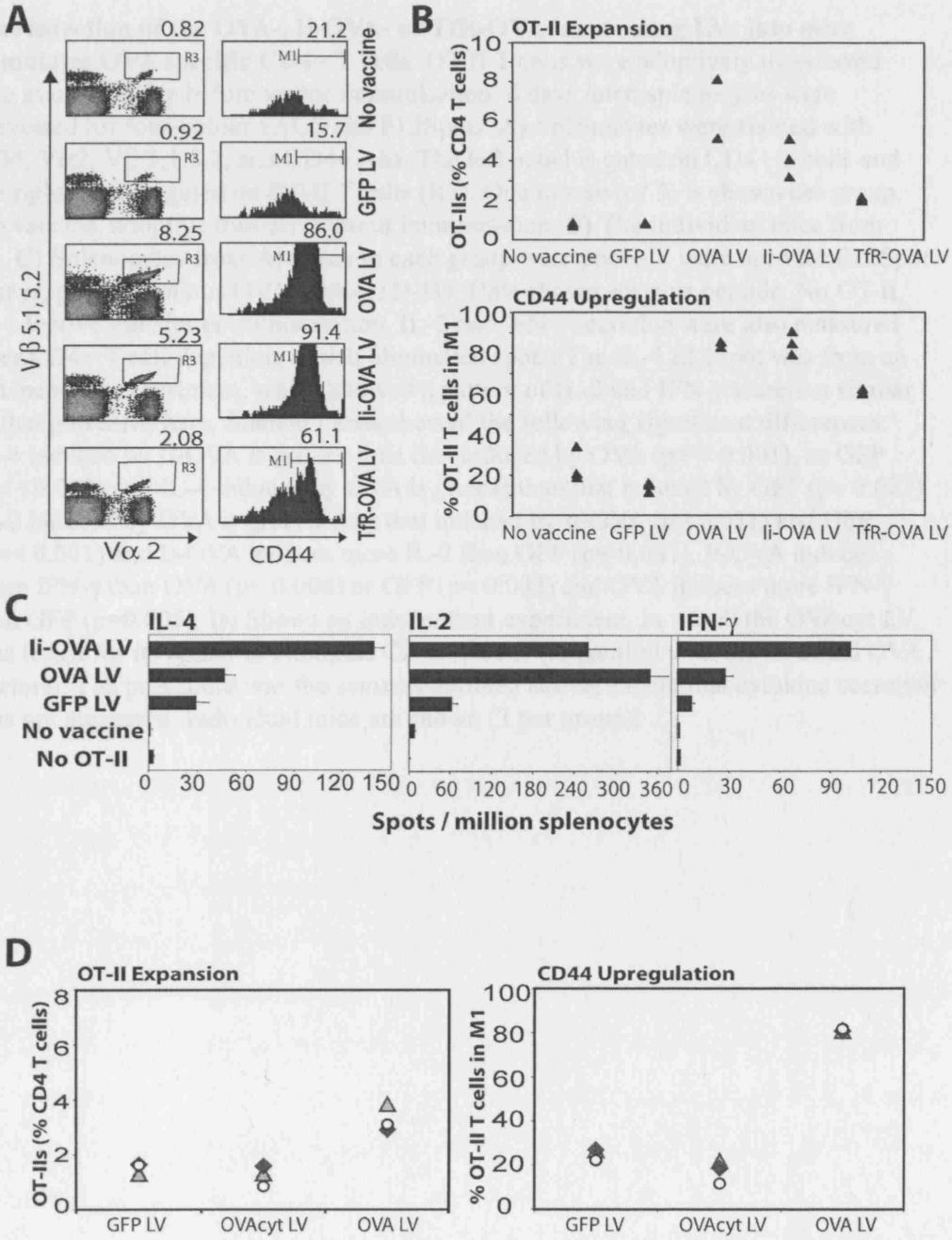

A
0.82
21.2
No vaccine
0.92
15.7
GFP LV
8.25
86.0
OVA LV
5.23
91.4
Ii-OVA LV
2.08
61.1
TfR-OVA LV
R3
M1
Vβ 5.1,5.2
Vα 2
CD44
B
OT-II Expansion
OT-IIs (% CD4 T cells)
No vaccine GFP LV OVA LV Ii-OVA LV TfR-OVA LV
CD44 Upregulation
% OT-II T cells in M1
C
IL- 4
IL-2
IFN-γ
Ii-OVA LV
OVA LV
GFP LV
No vaccine
No OT-II
Spots / million splenocytes
D
OT-II Expansion
OT-IIs (% CD4 T cells)
GFP LV OVAcyt LV OVA LV
CD44 Upregulation
% OT-II T cells in M1

Figure 3.9

One injection of the OVA-, Ii-OVA- or TfR-OVA- expressing LVs into mice stimulates OVA specific CD4+ T cells. OT-II T cells were adoptively transferred into mice, one day before vector immunisation. 6 days later, splenocytes were harvested for four-colour FACS and ELISpots. **A)** Splenocytes were stained with CD4, Vα2, Vβ 5.1,5.2, and CD44 Abs. The left panel is gated on CD4+ T cells and the right panel is gated on OT-II T cells (R3). One mouse (of 3) is shown per group. No vaccine, adoptive transfer without immunisation. **B)** The individual mice from A). **C)** Splenocytes from A) (mice in each group were pooled), were used for ELISpot assays, plus and minus OVA peptide323-339. Data shown are plus peptide. No OT-II, no adoptive transfer or immunisation. IL-2 and IFN-γ secretion were also measured after CD4+ T cell depletion, which eliminated spots. The IL-4 ELISpot was from an independent experiment, which showed a pattern of IL-2 and IFN-γ secretion similar to that presented here. Student *t* tests showed the following significant differences: IL-4 induced by Ii-OVA is greater than that induced by OVA ($p = < 0.001$), or GFP ($p = <0.001$) and IL-4 induced by OVA is greater than that induced by GFP ($p = 0.027$). IL-2 induced by OVA is greater than that induced by Ii-OVA ($p = 0.031$) and GFP ($p =< 0.001$) and Ii-OVA induces more IL-2 than GFP ($p = 0.001$). Ii-OVA induces more IFN-γ than OVA ($p = 0.006$) or GFP ($p = 0.002$) and OVA induces more IFN-γ than GFP ($p=0.005$). **D)** Shows an independent experiment, in which the OVAcyt LV was tested for its ability to stimulate CD4+ T cells (in parallel with the GFP and OVA vectors). The procedure was the same as outlined above, except that cytokine secretion was not measured. Individual mice are shown (3 per group).

3.8 Lentivector vaccine efficacy was assessed by challenging mice with an OVA expressing tumour

We examined the ability of the vaccines encoding the different OVA constructs to protect mice against tumours. We used the tumour cell line EG7.OVA (OVA transfected EL4 cells), which grows progressively when injected s.c. into C57BL/6 mice. Expression of ovalbumin in these cells was confirmed by Western (not shown). The presence of OVA specific antibodies and the CTL response were also analysed in these experiments to determine whether or not they were important in tumour protection.

3.8.1 Ii-OVA LV immunisation completely protects mice from challenge with EG7.OVA tumour cells.

Experiment 1 (figure 3.10A (i)) showed that only the Ii-OVA LV (and not the OVA, OVAcyt or TfR-OVA vectors) completely protected mice from tumour challenge. Mice in this group remained tumour free >40 days post tumour challenge. We then compared Ii-OVA LV and OVA LV (the most effective vaccines) again in experiment 2 (figure 3.10A (ii) and B). This also showed that immunisation with Ii-OVA LV completely protected mice from tumour challenge, compared to the OVA group where 2/10 mice developed tumours (note that these tumours regressed over a week). Statistical analysis of this data using a χ^2 test showed that the Ii-OVA group gave a greater significance level, compared to the controls than the OVA group did ($p=< 0.001$ and $p=0.0023$ respectively), suggesting the Ii-OVA vector to be a more effective vaccine.

Splenocytes from these mice were harvested for ELISpot assays (see below), although 4 and 3 mice were kept from the respective Ii-OVA and OVA groups. 2/4 mice in the Ii-OVA group developed tumours by day 30 post challenge, possibly due to loss of OVA expression by the tumour. The 5 remaining mice were still tumour free after 95 days, at which point they were killed.

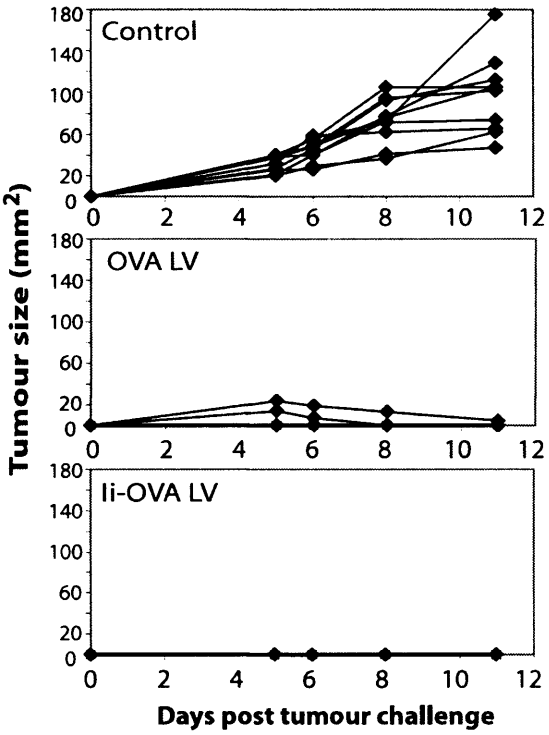
Figure 3.10

A

Mouse Group	Tumours	
	(i)	(ii)
Control	3/3	10/10
OVA LV	1/3★	2/10★
Ii-OVA LV	0/3	0/10
OVAcyt LV	1/3	ND
TfR-OVA LV	1/3	ND

★Tumours that regressed

B



Immunisation with Ii-OVA LV protects mice from challenge with EG7.OVA tumour cells. A) (i) Experiment 1. Mice were immunised twice with the different lentivectors, 3 weeks apart. 7 days later, mice were challenged with $1x10^6$ tumour cells (see methods). Tumours were measured every 2-3 days. Results show the number of mice per group with tumours. Control, unvaccinated mice. **(ii)** Experiment 2. Mice were immunised once with the lentivectors and then challenged with $2x10^6$ tumour cells, 9 days later. ND, not done. **B)** Results of individual mice from experiment 2 (A (ii)). The experiment was terminated on day 11 post-challenge, when one control mouse had a tumour with a diameter > 15mm. Differences between groups were analysed, using a χ^2 test (including Yates' continuity correction for 1 degree of freedom). The control group was significantly different from the OVA group, $\chi^2 = 10.2$, $p = 0.0023$, and from the Ii-OVA group, $\chi^2 = 16.2$, $p = < 0.001$. None of the mice from either experiment had detectable tetramer+ cells; blood samples were tested on day 11, 22 and 36 after the 1st immunisation in experiment 1 or on day 13 in experiment 2 and spleen samples were tested on day 23 in experiment 2. Blood samples were also used to measure OVA Abs (see figure 3.12). Spleens from OVA and Ii-OVA LV mice from both experiments were used in ELISpot assays (see figure 3.11).

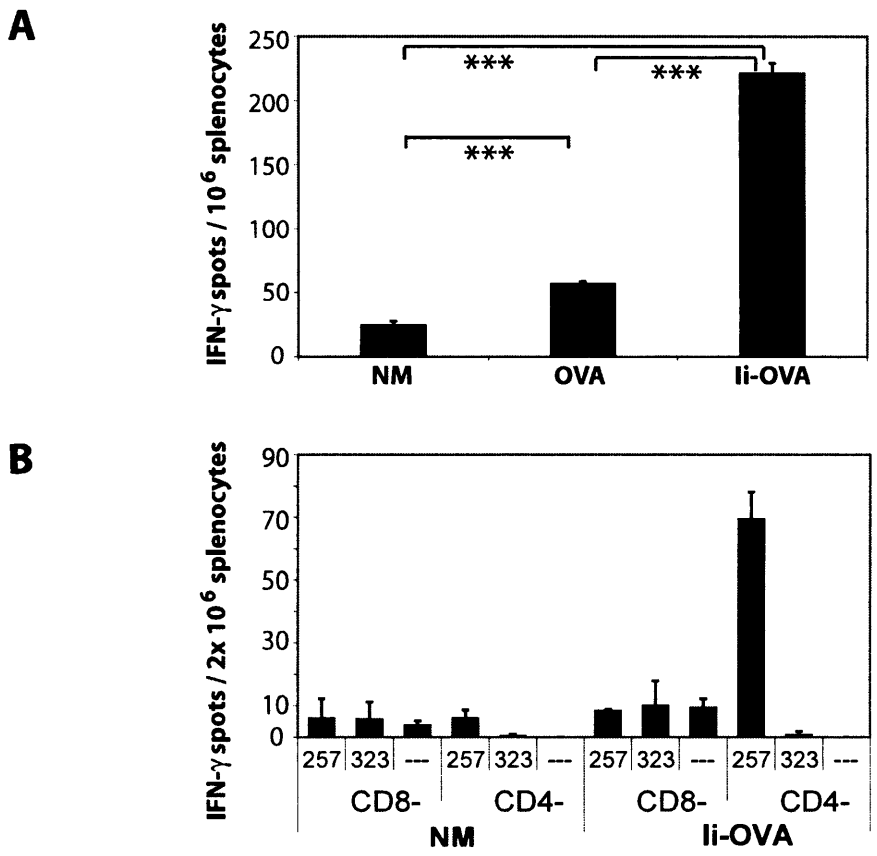
3.8.2 The Ii-OVA LV mice that survived tumour challenge exhibited a stronger CD8+ T cell response than the OVA LV mice

By harvesting spleens from the 3 groups shown in figure 3.10B, we were able to clearly demonstrate that mice in the Ii-OVA group mounted a significantly higher CD8+ T cell response than those in the OVA group ($p = < 0.001$), as measured by IFN-γ release in response to $OVA_{257-264}$ peptide (see figure 3.11A).

3.8.3 Depletion of CD8+ T cells confirmed that these cells were responsible for the OVA specific IFN-γ response

CD4+ and CD8+ T cell depletion assays were carried out to confirm that the observed IFN-γ response (see figure 3.11A) was due to CD8+ T cells. The data revealed that an IFN-γ response is only observed when the CD8+ T cell containing splenocyte fraction, from Ii-OVA mice, is cultured with the $OVA_{257-264}$ class I peptide (see figure 3.11B). Note that the OVA specific CD4+ T cell response is not apparent here probably because we did not adoptively transfer OT-II cells.

Figure 3.11



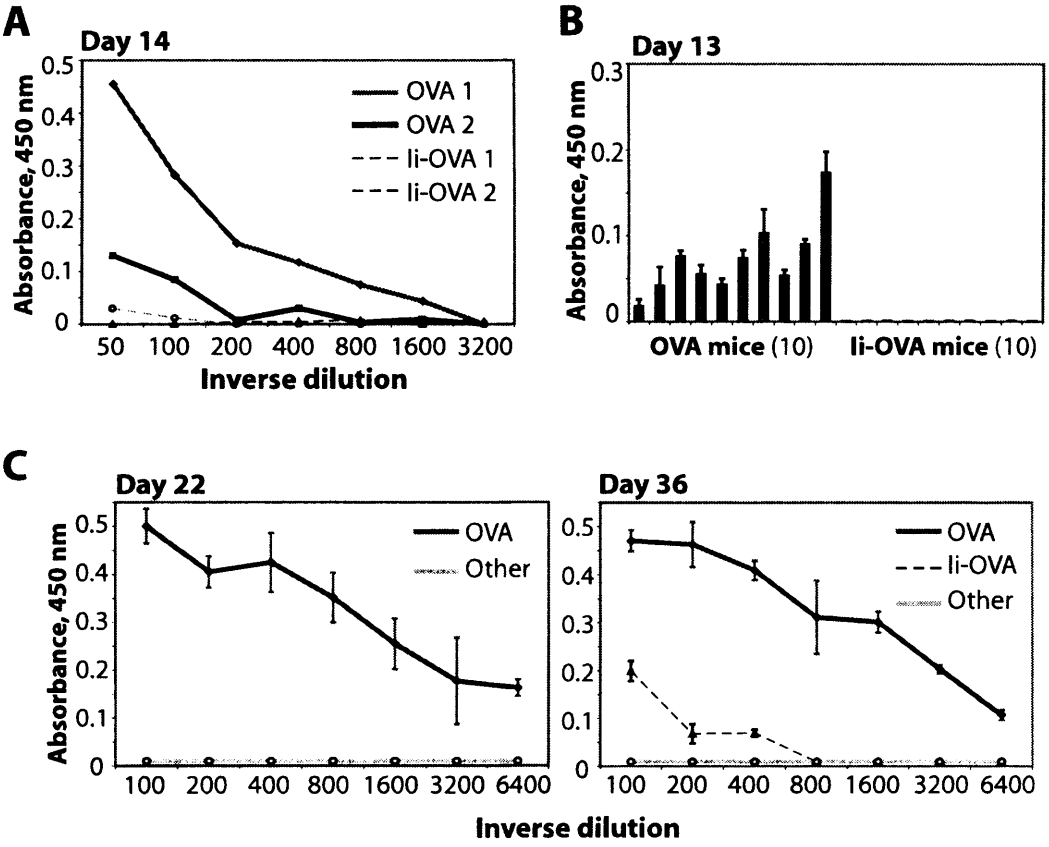
CD8+ (not CD4+) T cells are responsible for the *ex-vivo* IFN-γ response to the OVA257-264 peptide, by Ii-OVA LV immunised mice. Mice from figure 3.10 B, (immunised with either OVA or Ii-OVA LV and then challenged with the tumour) were compared for their ablity to mount a CD8+ T cell response. On day 12 post tumour challenge, spleens from 5 mice (out of 10) were pooled from the OVA group (using only mice that didn't get tumours). Spleens from the 2 OVA mice that did get tumours were pooled separately. Spleens from 5 or 6 mice were pooled for the control group (unvaccinated, "NM" here) and the Ii-OVA group respectively. Pooled splenocytes from the 4 groups were compared in an ELISpot, for their ability to secrete IFN-γ in response to OVA peptide. (The 2 OVA mice with tumours (not shown), responded equally to the other OVA mice). The results from the 3 groups (plus OVA257-264 peptide) are shown in **A)**. *** p= <0.001, using a *t* test. The minus peptide results showed only 10-30 spots (per 10^6 cells), mainly in the immunised mice. **B)** Pooled splenocytes were also depleted of either CD4+ or CD8+ T cells (see methods), before using in another ELISpot, to confirm which T cells were responsible for the observed response. The results show the number of spots per well ($2x10^6$ cells), since there were too few spots per million cells. Also, results for the OVA group are not shown, since there were too few spots. CD8- , CD8+ T cells depleted; CD4- , CD4+ T cells depleted; 257, OVA peptide257-264 added; 323, OVA peptide323-339 added; --- , no peptide added. A repeat experiment, using the OVA and Ii-OVA mice from the tumour experiment in figure 3.10 A (3 mice /group), showed similar results in both the total splenocyte ELISpot and in the depletion ELISpot (not shown).

3.8.4 OVA specific antibodies are present in OVA LV injected mice

Results initially showed that OVA specific antibodies were detected in serum from mice injected with the OVA LV but not the Ii-OVA LV, 14 days post immunisation (see figure 3.8 legend and figure 3.12A). We then found this also to be the case in mice from the tumour experiment in figure 3.10B; antibodies were measured 13 days post immunisation (see figure 3.12B). This suggested that antibodies were not required for tumour protection. This result was again repeated for mice from the tumour experiment in figure 3.10A (i); antibodies were measured 22 days post immunisation (see figure 3.12C, left-hand graph). In this experiment, antibodies were also measured after 36 days (figure 3.12C, right-hand graph), which showed that the OVA LV injected mice had a similar antibody titer as on day 22; the Ii-OVA group had also developed weak αOVA antibodies by day 36, but this could be because they were also boosted and injected with the tumour. Note that the antibody isotype(s) was not evaluated in these experiments.

Overall, these data suggest that the OVA and the Ii-OVA vectors skew the adaptive immune response towards different outcomes; the secreted OVA vector is more effective at directing an antibody response, while the targeted Ii-OVA vector drives a more efficient CTL response.

Figure 3.12



One injection of the OVA LV into mice leads to the production of αOVA Abs. OVA specific Abs were detected by ELISA (see methods), at the stated time points post injection, using either serum (A), or plasma samples (B & C). Absorbance values for the NM (normal mouse) group in each experiment were subtracted from the results before plotting them. **A)** Results from the experiment described in the legend of figure 3.8. Serial dilutions of serum were measured in duplicate. The dose of LV was $3x10^7$ i.u. (in this experiment only) **B)** Results for mice from the tumour experiment, described in figure 3.10 B. The time point shown is also 4 days post tumour challenge and the data shown is for a 1/300 dilution of plasma. **C)** Results for mice from the tumour experiment, described in figure 3.10 A (i). Plasma samples from mice in each group (3) were pooled. Other, all other groups e.g. mice immunised with Ii-OVA, OVAcyt and TfR-OVA LV's, in the case of the left-hand graph or OVAcyt and TfR-OVA in the right-hand graph. In these groups, absorbance values were ≤ background. The day 36 time-point (right-hand graph) was also 11 days post boost (given on day 25) and 4 days post tumour challenge.

3.9 Discussion

Lentiviral vectors (lentivectors) are vaccine candidates because they can deliver antigens to APCs in situ (Esslinger et al., 2003; Palmowski et al., 2004; VandenDriessche et al., 2002), potentially resulting in antigen presentation for the life-span of the transduced cell. The resulting immune response is focused on the transgene because the lentivector does not express any viral proteins. In this respect, lentivectors are similar to DNA vaccines, although they confer the advantages of more efficient antigen delivery and expression as well as offering the potential to target DC. Consequently, lentivectors might prove effective as priming vaccines for cancer or infectious disease. The aim of this study was to investigate the ability of lentivectors to stimulate an antigen specific CD4+ T cell response, since T cell help is a key component of an effective immune response (Bevan, 2004; Gao et al., 2002). We constructed lentivectors encoding OVA proteins, routed to different cellular compartments and compared their immunogenicity.

We found that all the OVA expressing lentivectors induced an endogenous CD8+ T cell response after one intravenous injection into mice. Interestingly, we found that the Ii-OVA lentivector was the most adept at priming IFN-γ secreting CD8+ T cells *in vivo,* even though it was designed for enhancing CD4+ responses. MHC class I responses to proteins routed to the endosomal compartment may occur by cross-presentation, e.g. after endosome-to-cytosol transport (Rock and Shen, 2005). The fact that the CD8+ T cell response to Ii-OVA was the most successful could suggest that fusion of Ii to 111 amino acids of OVA might yield a misfolded protein (a defective ribosomal product, DRiP (Pierre, 2005)), which is efficiently targeted to the proteasome and processed through the endogenous pathway (Diebold et al., 2001). Another explanation could be that CD4+ T cell help is supporting the generation of CD8+ T cells in this system (see below). For example, Th1 cells secreting IFN-γ are important in the generation and maintenance of anti-tumour CTL (Gao et al., 2002; Wang and Livingstone, 2003).

When we measured the OVA specific CD4+ response, we found that Ii-OVA was also the most successful at stimulating IFN-γ secreting CD4+ T cells, which could explain its

more potent CD8+ response. However, CD4+ T cells in the Ii-OVA group also secreted the most IL-4; the number of IL-2 secreting CD4+ T cells was relatively high in both OVA and Ii-OVA groups. This suggested that the Ii-OVA response (at 6 days post immunisation) is indicative of a Th0 response, due to the secretion of IL-2, IL-4 and IFN-γ. B cells have been shown to support the nonpolarised response observed initially (Moulin et al., 2000) and NKT cells are thought to contribute to early IL-4 production (Wilson and Delovitch, 2003). One way to further investigate the polarisation of the Ii-OVA response would be to measure cytokine release (IFN-γ, IL-4 and IL-10) using ELISpot assays at later time-points, in comparison to typical Th1 (e.g. OVA peptide + LPS) and Th2 (e.g. repeat i.p. injections of OVA peptide in alum (Julia et al., 2002)) vaccine regimens. Also, ELISA assays would be valuable to determine the amount of cytokines produced.

Although immunisation with the Ii-OVA vector stimulated more CD4+ T cells secreting both IFN-γ and IL-4, the secreted OVA vector induced the same or greater expansion of adoptively transferred OT-II cells. The fact that CD4+ T cells responding to secreted OVA are not so "effective" could be because uptake of secreted OVA by immature DC in the absence of adjuvant might favour expansion without differentiation (Stumbles et al., 1998), whereas presentation of intracellular Ii-OVA by DC results after transduction that might have simultaneously provided a maturation signal to the DC (see below).

When we compared the OVA and the Ii-OVA vectors in a tumour challenge model, we found that both vectors could protect mice against tumours (8/10 mice were protected in the OVA group and 10/10 in the Ii-OVA group). The fact that all mice were protected only in the Ii-OVA group correlates with the finding that this vector stimulates more "effective" cytokine secreting T cells. We did not clarify the mechanism of tumour protection, although importantly we found that the Ii-OVA mice had mounted a more potent CD8+ T cell response, whereas the OVA mice had produced OVA-specific antibodies; a lack of B cell epitopes coupled with the fact that Ii-OVA is intracellular could explain this data. Since mice in the Ii-OVA group were protected, it is likely that the CTL (and not the antibody) response played the greatest role in tumour protection.

Antibodies can mediate an array of effects against cancer such as ADCC (antibody dependent cell-mediated cytotoxicity) or CDC (complement dependent cytotoxicity). For example, α-HER2 antibodies have been found to stop tumour growth by blocking HER2 signaling through ERK (extracellular signal-regulated kinase) (Montgomery et al., 2005) or they can promote ADCC by interacting with FcRγIII on NK cells (Eccles, 2001).

Differences between the lentivectors encoding secreted or class II routed OVA antigens may be more apparent if mice were challenged with the tumour ~ 6 months post vector immunisation, because the persistence of memory CTL depends on CD4+ T cells (Janssen et al., 2003). Intracellular targeting of antigens might also prove important in a clinical setting where optimal CD4+ T cell help is more critical to prevent tumour escape. Interestingly, in a report published just after ours (Dullaers et al., 2006), CD4+ T cells were shown to be important, by their depletion prior to lentivector immunisation. Although the initial CTL response was only 2x lower in CD4+ depleted mice, the response 30 days post immunisation was 7x lower. Perhaps the vector acts as an adjuvant to "license" DC thereby partly bypassing the need for T cell help initially, although CTL might consequently be lost. The same report also claimed that lentivector immunisation (s.c.) was Th1 inducing, although cytokine release was measured after culturing lymph node cells for 3 days, which may have altered their phenotype.

We found that the OVAcyt vector did not stimulate CD4+ T cells *in vitro* or *in vivo*; the cytoplasmic protein was not delivered into the "endogenous MHC class II pathway" (Schmid and Munz, 2005). It may be that cytoplasmic peptides (the OVAcyt construct is rapidly degraded (Shen and Rock, 2004)) are not substrates for transport into endosomes / lysosomes, although more stable cellular proteins could be. It is noteworthy that in a report, where *ex vivo* LV transduced DC were transferred into mice, strong CD8+ IFN-γ responses (4 times higher than with peptide pulsed DC) were observed but relatively weak CD4+ IFN-γ responses (equivalent to peptide pulsed DC) (He et al., 2005); the antigen used was a cytoplasmic OVA construct.

We also compared lentivector immunisation to vaccinia vector and found it to be marginally better at inducing recall responses in mice, although neither vaccine induced an obvious pool of specific CD8+ T cells. In contrast, when mice were primed with lentivector and boosted with vaccinia vector, the OVA specific CD8+ T cell response was striking and could still be detected after a year, suggesting that this vaccine protocol might also be potent in the clinic. Heterologous prime-boost regimens are known to be superior to homologous vaccination (Ramshaw and Ramsay, 2000; Schneider et al., 1999); the problem of neutralising antibodies to either vaccine can be avoided and the immune response becomes focused on the epitopes common to both vaccines.

Lentiviral vectors can potentially infect many cell types including DC; these are not discussed here since chapter 4 is devoted to the characterisation of cells expressing and presenting transgene. We plan to target the vector exclusively to DC to improve safety and efficacy. With this in mind it will be critical to express fusion constructs to traffic intracellular antigens into the MHC class II pathway, to ensure the provision of T cell help. In the case of pDC, intracellular targeting of antigen is an obligatory requirement, as they are unable to present exogenous antigens (Salio et al., 2004). Targeting DC with antigens in the steady state can lead to tolerance e.g. as shown by Bonifaz et al., (Bonifaz et al., 2002) when they targeted OVA to DC via DEC-205, so it is necessary to activate DC in parallel. Since our lentivector transduces DC and primes robust immune responses, we speculate that the lentivector itself is responsible for activation *in vivo*.

There is evidence that HIV-1 activates pDC directly and myeloid DC (MDC) indirectly (Fonteneau et al., 2004). It was shown that ssRNA from HIV-1 causes pDC and MDC to release IFN-α and TNF-α and triggers up-regulation of co-stimulatory molecules through murine TLR7 (Heil et al., 2004). Also, certain viral envelope proteins are recognised by TLR4 or TLR2 (e.g. the retrovirus: MMTV, mouse mammary tumour virus), which leads to release of pro-inflammatory cytokines (Burzyn et al., 2004). Importantly, the mannose receptor was found to recognise glycosylated viral envelope proteins, such as those of VSV; the result of this was type 1 IFN release (Milone and Fitzgerald-Bocarsly, 1998). Of interest, it is unlikely but not impossible that secondary

structures in the HIV-1 genome are recognised by the cytoplasmic helicases (such as RIG-1), which are potent activators of type 1 IFN upon sensing dsRNA (Akira et al., 2006; Yoneyama et al., 2005). Overall, lentivectors, which lack HIV-1 accessory proteins that block maturation, may be potent inducers of DC maturation *in vivo*. Indeed, one study has shown that lentivectors can mature DC *in vitro* (Tan et al., 2005).

This is the first time (to our knowledge) that antigen specific CD4+ T cells (as well as CD8+ T cells) and tumour protection have been reported, following a single injection of a lentiviral vector. This draws us to conclude that a lentivector expressing an Ii fusion to an antigen would be a suitable vaccine candidate for cancer. The opposite considerations apply if lentivectors are to be injected for gene therapy applications where long-term transgene expression is required. It is noteworthy that long-term expression of the intracellular protein GFP can be achieved following i.v. injection of a lentivector (VandenDriessche et al., 2002). In contrast, long-term expression of the secreted factor IX in immunocompetent mice depends on restriction of the lentivector promoter to hepatocytes (Follenzi et al., 2004). Our results suggest that secreted proteins such as factor IX can elicit a CD4+ response, especially when the lentivector is expressed in DC, perhaps explaining the selective immunogenicity of factor IX.

Finally 2 recent reports have shown that lentivector immunisation significantly extends the life-span of mice treated in a therapeutic tumour setting (Dullaers et al., 2006; Kim et al., 2005). This finding along with our data presented here underlines the potential use of lentivirus-based vaccines in the clinic.

Chapter 4: Dendritic cells in the spleen stably present antigen

4.1 Introduction

4.1.1 Duration of antigen presentation

Sustaining antigen presentation by dendritic cells (DC) *in vivo* is a major challenge of cancer vaccines. Indeed, the problem of the rapid turnover of peptide-MHC complexes associated with DC-based cancer vaccines (Ludewig et al., 2001b; Wang and Wang, 2002) is thought to contribute to their poor efficacy; repeat vaccine injections are often required for patients to mount a detectable anti-tumour response and even then, responses are often not sustained (Fay et al., 2005). This can result in no effect on tumour load or recurrent cancers in patients who initially recovered. Even where the half-life of peptide-MHC complexes has been extended e.g. by substituting key peptide residues (Rosenberg et al., 1998a; Slansky et al., 2000), antigen presentation is still short-lived, unless the tumour peptides are encoded by the DC.

Viral vectors encoding common tumour antigens are, therefore, an attractive vaccine option because they can continuously express antigens. They can be used to transduce DC *in vitro* for re-injection (Esslinger et al., 2002; Herrera et al., 2002; Zhang et al., 2000) or more practically, they can be injected directly, leading to presentation of an array of both CD8 and CD4 epitopes *in vivo*, which might limit tumour escape (Rosenberg et al., 1998b; Scholl et al., 2000; Song et al., 1997).

Lentiviral vectors (lentivectors) are being explored as vaccines because they can deliver transgene antigens to non-dividing cells (Case et al., 1999; Naldini et al., 1996), without stimulating a potent immune response to the vector (unlike adenovirus, AdV or vaccinia virus, VV vectors). Immunisation of mice with lentivectors initially showed that they can transduce dendritic cells *in vivo* (Esslinger et al., 2003; Palmowski et al., 2004; VandenDriessche et al., 2002) and prime CTL responses (Esslinger et al., 2003; Palmowski et al., 2004). We and others have recently shown that lentivectors are also

able to stimulate antigen specific CD4+ T cells, (crucial to anti-tumour immunity (Dullaers et al., 2006; Rowe et al., 2006)) and protect mice from tumour challenge (Dullaers et al., 2006; Kim et al., 2005; Rowe et al., 2006). The use of lentiviral vaccines in the clinic might improve tumour surveillance, due to their stimulation of both CTL and CD4+ T cell responses.

Interestingly, the use of lentivectors may also solve the problem of short-lived antigen presentation, faced by current cancer vaccines. Lentiviral vectors integrate into the genome (unlike AdV and VV vectors), and can express their transgenes for the life-time of the modified cell. However, the effect of this on the immune system depends on the cell types transduced by the vector *in vivo* (whether they are antigen presenting cells (APCs) / professional APCs or non-APCs), the rate of turnover of these cells and whether or not they continue to present expressed antigens (and provide co-stimulation) to T cells *in vivo*.

The potential for stable antigen presentation and the consequence of this is one intriguing aspect of lentiviral vaccines yet to be explored. Non-DC or DC presenting antigen in the absence of activation may effectively induce tolerance (Bonifaz et al., 2002; Cong et al., 2005; Steinman and Nussenzweig, 2002). Alternatively, the lentiviral vector might intrinsically activate DC (see chapter 5), promote survival and maintain transgene presentation.

4.2 Aims

Transduction of APCs and B cells in spleen has been reported using a lentivector carrying a CMV promoter (Follenzi et al., 2002; VandenDriessche et al., 2002); similarly we found that DC and B cells are transduced using a vector carrying an SFFV promoter (Rowe et al., 2006) (vectors were injected intravenously in both cases). However, a complete characterisation of cell types transduced in spleen and most importantly their ability to present antigen has not been conducted. Therefore, the aims of this project were to answer the following questions:

1. Which cell types are transduced by the lentiviral vector *in vivo* and which cells are responsible for antigen presentation to T cells?
2. Which (if any) cell types stably express the transgene and are these cells still able to present antigen?
3. If antigen presentation is long-lived, what is the consequence on immunity?

4.3 Background

In order to understand antigen presentation in the secondary lymphoid organs, following immunisation, it is important to first discuss antigen presenting cells, and in particular dendritic cell subsets (their functions, turnover and development), as well as immune regulation. I have restricted these topics to the murine system only.

4.3.1 Antigen presenting cells

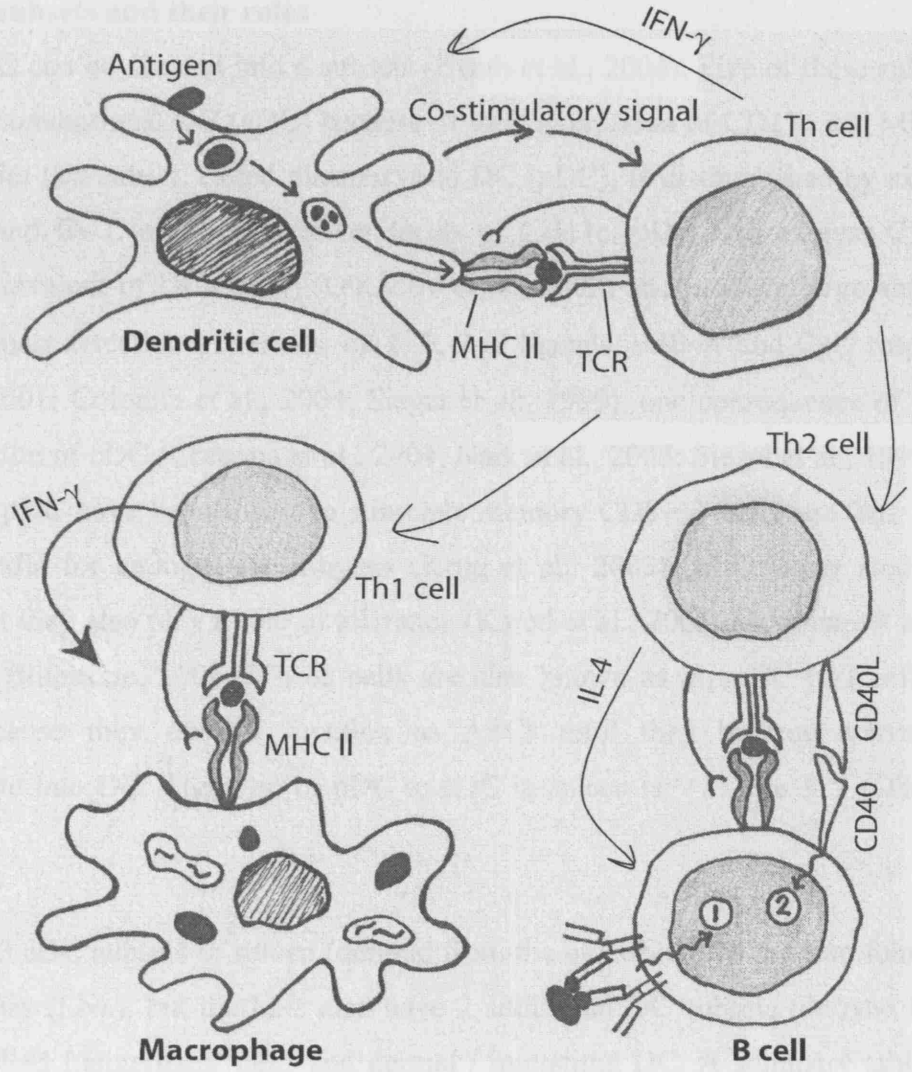
B cells, macrophages and dendritic cells are antigen presenting cells (APCs), which present processed peptides on MHC class II as well as on MHC I (see figure 4.1). Any of these cell types could potentially be transduced in spleen following tail vein administration of the lentivector (the route used to immunise mice in chapter 3), owing to the pantropic VSV-G envelope (Barrette et al., 2000).

The main role of the spleen, as the secondary lymphoid organ attached to the circulatory system, is to trap antigens from the blood, enabling lymphocytes to sample them. The lentivector can either be carried as free particles into the spleen, where transduction ensues, or it can transduce cells in the blood that migrate to the spleen. Incidentally, non-antigen presenting cells can potentially be transduced, such as T cells and NK cells; transgene presentation by these cells alone would not normally trigger immunity, due to the lack of co-stimulation, but these cells could become CTL targets in the course of an adaptive response.

For vaccine purposes, it would be preferable to transduce DC because they are "professional" APCs that can prime naïve T cells; non-professional APCs have been shown to be less efficient at antigen presentation than professional APCs (Salio et al.,

2001). T helper cells, that are primed by DC, can deliver signals to DC (to "license" them to activate CD8+ T cells) (Ridge et al., 1998), to B cells (to induce plasma cell differentiation), and to CD8+ T cells (to activate them). The balance of the cell mediated vs. the humoral response is determined by the tendency for either Th1 or Th2 effectors to develop, which in turn depends on the type and amount of antigen / danger signal and the cell types presenting/ receiving the signal (Boonstra et al., 2003). It is now apparent that there are different DC populations that can respond to different stimuli and direct different outcomes (Shortman and Liu, 2002).

Figure 4.1



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Antigen presenting cells

DC, macrophages and B cells are APCs because they can present antigens on MHC class II; they can also present antigens through the endogenous pathway on MHC class I. DC are professional APCs because they can stimulate naive T cells. Here, the DC activates the naive CD4+ T helper (Th) cell, which can then provide cytokine signals to DC (to "license" them), macrophages and B cells. Th1 cells produce IL-2, TNFα/β and IFN-γ, which direct CTL production, as well as activating macrophages (shown here) to secrete IL-12. In contrast, Th2 cells induce type 2 humoral responses, and can activate B cells through secretion of IL-4 (plus IL-2, IL-5, IL-6, IL-10 and TGFβ). Th1 cells also help B cells with class switching to IgG2a antibodies, whereas Th2 cells direct class switching to IgG1 and IgE. B cells require T helpers for linked recognition of antigens, leading to succesful B cell memory.

4.3.2 DC subsets and their roles

Murine DC can be divided into 6 subsets (Heath et al., 2004). Five of these subsets can be called conventional DC (cDC) because of their expression of CD11c and MHC class II, while the last subset, called plasmacytoid DC (pDC), is distinguished by expression of B220 and Gr-1, along with lower levels of CD11c. pDC also express CIRE (the murine equivalent of DC-SIGN) (O'Keeffe et al., 2002) and produce large amounts of IFN-α/β upon detection of virions, or TLR 7 /9 ligands, ssRNA and CpG respectively (Bjorck, 2001; Colonna et al., 2004; Siegal et al., 1999), one consequence of which is the activation of cDC (Colonna et al., 2004; Naik et al., 2005; Siegal et al., 1999). Once activated, pDC have been found to stimulate memory CD8+ T cells and Th1 CD4+ T cells, specific for endogenous antigens (Krug et al., 2003), while some studies have shown that they also play a role in tolerance (Kared et al., 2005; Moseman et al., 2004; Tang and Bluestone, 2006). These cells are also known as p-preDC (O'Keeffe et al., 2002) because they do not function as APCs until they become activated and differentiate into DC. The ratio of pDC to cDC in spleen is ~ 1: 3 to 1: 4 (O'Keeffe et al., 2002).

There are 3 cDC subsets in spleen (derived from the blood), which are also found in the lymph nodes (LNs), but the LNs also have 2 additional DC subsets (derived from the tissues) called Langerhans' cells and dermal / interstitial DC. A summary table of DC subsets is shown in figure 4.2.

The 3 conventional splenic DC subsets express either CD4, CD8α or neither, and ablation of either subset has no effect on the others (Heath et al., 2004; Kamath et al., 2002; Kamath et al., 2000), suggesting that they each develop independently. Both the CD4+ and CD4- CD8- DC reside mainly in the marginal zone of the spleen, and they are more phagocytic and less mature than the CD8α+ DC, sharing some characteristics with monocytes (adherence to glass and expression of F4/80) (Kamath et al., 2000; Leenen et al., 1998). Upon activation, they migrate into the T cell areas (De Smedt et al., 1998; De Smedt et al., 1996); there is some evidence that these 2 subsets (CD8-) are involved in Th2 responses (Maldonado-Lopez et al., 1999; Pulendran et al., 1999). In

contrast, the CD8α+ DC reside in the T cell areas of spleen (Steinman et al., 1997), are more mature (due to higher MHC II expression (Kamath et al., 2000), produce IL-12 (Steinman et al., 1997) and are involved in both cross-priming and cross-tolerance (Belz et al., 2002; Belz et al., 2004; den Haan et al., 2000; Scheinecker et al., 2002). A model of the CD8- and CD8+ subsets and their roles is shown in figure 4.3.

Figure 4.2

Table 1. DC subsets, surface phenotype, and some important properties

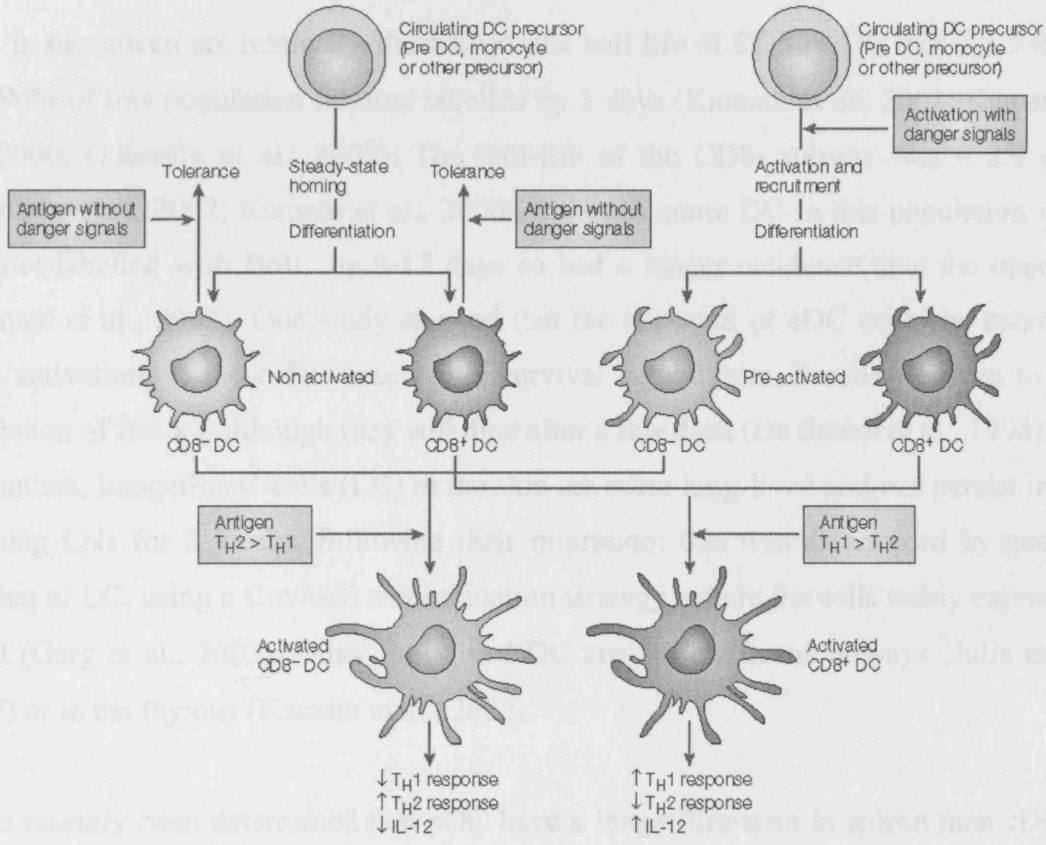
DC type	Surface phenotype						Derivation	Distinguishing properties
	CD11c	CD8	CD4	CD205	CD11b	CD45RA		
CD8 DC	+	+	–	+	–	–	Blood	High IL-12 Cross-presentation of cellular antigen Cross-priming Cross-tolerance
CD4 DC	+	–	+	–	+	–	Blood	Most numerous DCs in spleen
$CD4^+CD8^-$ DC	+	–	+	–	+	–	Blood	High IFN-γ
Langerhans' cell	+	–/low	–	Very high	+	–	Skin epithelia	Traffic to lymph node from skin Present contact sensitizing antigens
Dermal/interstitial DC	+	–	–	+	±	–	Tissue	In all tissues Traffic to draining lymph nodes Prime CD4 T-cell immunity to tissue infections
Plasmacytoid DC	Low	±	±	–	–	+	Blood/tissues	High IFN-α, do not look like DCs until stimulated

DC, dendritic cell; IFN, interferon; IL-12, interleukin-12.

Murine DC subsets

There are 6 DC subsets, plasmacytoid DC and 5 populations of conventional DC; 3 of these are blood-derived and 2 are tissue-derived.

Figure 4.3



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Model showing possible roles of CD8- and CD8+ DC
T cell responses might result from these 2 types of conventional DC already present in the lymphoid organs (left) and/or from the recruitment of circulating precursors (right). Non-activated DC may promote tolerance (in the absence of danger signals), while danger signals would activate DC to direct either a Th1 or Th2 response; it is not clear whether this is controlled by the antigen encountered, the particular DC subset or both.

4.3.3 DC turnover

A number of murine studies using continuous BrdU administration have shown that cDC in the spleen are relatively short-lived; the half life of CD8α+ DC was ~ 1.5 days, e.g. 90% of this population became labelled by 3 days (Kamath et al., 2002; Kamath et al., 2000; O'Keeffe et al., 2002). The half-life of the CD8- subsets was ~ 2.9 days (Kamath et al., 2002; Kamath et al., 2000), although some DC in this population were still not labelled with BrdU by 9-12 days so had a longer residence than the majority (Kamath et al., 2002). One study showed that the life-span of cDC could be extended after activation, if these DC received a survival signal from T cells (leading to up-regulation of Bcl-x), although they still died after a few days (De Smedt et al., 1998).
In contrast, Langerhans' cells (LC) in the skin are more long-lived and can persist in the draining LNs for 2 weeks, following their migration; this was determined by genetic tagging of LC, using a Cre/*lox*P recombination strategy, where the cells stably expressed β-gal (Garg et al., 2003). Other long-lived DC are present in the airways (Julia et al., 2002) or in the thymus (Kamath et al., 2002).

It has recently been determined that pDC have a longer life-span in spleen than cDC; it took 14 days for 90% of pDC to be labelled, after continuous BrdU administration (O'Keeffe et al., 2002). These cells probably have a longer life-span because they are non-activated DC precursors (note that they cannot differentiate into cDC (O'Keeffe et al., 2002)). A fraction of DC generated from pDC (by addition of IL-3, GM-CSF and CpG) were found to survive in culture for 4 weeks (O'Keeffe et al., 2002), which is in contrast to cDC (Kamath et al., 2000) suggesting that some activated pDC may survive *in vivo* (e.g. to maintain T cell memory).

It is accepted that DC turnover is crucial to regulate the immune response; in a study where DC apoptosis was inhibited, using a caspase-8 inhibitor, T cells continued to expand and autoimmune responses were detected (Chen et al., 2006). However, while it is known that DC are destined to die, following activation (De Smedt et al., 1998), it is not clear whether their "life is cut short" (e.g. they become CTL targets (Hermans et al.,

2000; Ludewig et al., 2001a)) or they suffer a "purposeful death" (their fate is autonomously controlled (De Smedt et al., 1998; De Trez et al., 2005)), reviewed in (Ronchese and Hermans, 2001). Some DC (dermal DC) have been found to extend their life-span by co-expressing TRANCE (tumour necrosis factor-related activation-induced cytokine) and RANK (receptor activator of NF-κB) (Cremer et al., 2002). Interestingly, CD4+ T cell help has been shown to protect against CTL mediated elimination (Mueller et al., 2006). One benefit of DC survival could be to promote the expansion of memory T cells (Ronchese and Hermans, 2001).

4.3.4 DC development

Murine pDC and cDC can be generated from either common lymphoid or common myeloid progenitors (CLPs or CMPs) from bone marrow (Ardavin, 2003), showing that the committed precursors of each population are downstream of these cells. In bone marrow, 2 DC committed precursors were identified: CD11c+ MHC II- B220- cells that differentiated into cDC (MHC II+, CD11c+, B220-), and CD11c+ MHC II- B220+ cells that differentiated into both pDC ($CD11c^{lo}$, B220+) and cDC (Diao et al., 2004). BM-derived monocytes could also differentiate into cDC in the spleen in irradiated recipients (Leon et al., 2004). In a separate study (Fogg et al., 2006), a murine bone marrow progenitor was isolated that could differentiate into monocytes, several subsets of macrophages and cDC, but not other cell types (e.g. lymphocytes or erythrocytes). This supports the hypothesis that macrophages and DC have a common precursor.
DC precursors have also been identified in murine blood; $CD11c^{lo}$. CD11b-, $CD45RA^{hi}$ cells differentiated into CD8+ DC, while CD11c+, CD11b+, CD45RA- cells differentiated into CD8- DC (O'Keeffe et al., 2003).

Interestingly, DC precursors also reside in the spleen (Quah et al., 2004). One study showed that splenic CD8α+ CD11c- cells could differentiate into CD8α+ DC after their injection into irradiated recipients (Wang et al., 2002). Very recently, splenic DC precursors have been purified ($CD11c^{int}$, $CD45RA^{lo}$ and MHC II-) that can produce all CD8+ and CD8- cDC populations found in spleen, but not pDC, and these "pre-cDC" comprise 0.05% of splenocytes (Naik et al., 2006). These precursors may be important

in generating steady-state DC that maintain tolerance. This study also confirmed that monocytes (from blood or spleen) do not differentiate into DC, except in the face of systemic inflammation.

4.3.5 Immune regulation

DC populations can maintain tolerance as well as inducing immunity, by deleting self-reactive T cells or expanding regulatory T cells (Tregs). The DC populations involved in tolerance and the exact requirements are still mysterious, although it was has been shown that stimulation of T cells by DC in the steady state induces tolerance (Bonifaz et al., 2002; Storni et al., 2003). However, injection of protein or peptide alone is not sufficient to induce tolerance (Steinman and Nussenzweig, 2002); it may be that the presenting DC must be conditioned to secrete certain cytokines such as IL-10. Notably, the CD8α+ DC subset has been implicated in cross-tolerance (Belz et al., 2002).

Treg cells have emerged as being crucial to the maintenance of tolerance, since autoimmune patients are deficient in these cells (e.g. see (Viglietta et al., 2004)). Natural Tregs express CD4, CD25 and Foxp3, which is a Treg specific transcription factor, and their specificity is skewed towards autoantigens. They are thought to function by effects on DC e.g. by blocking DC from triggering effector T cell differentiation; they have been found to interact with DC in LNs and to suppress effector responses in tissues (Belkaid and Rouse, 2005; Bluestone and Tang, 2005).

4.4 Cell types transduced by the lentivector *in vivo*

Lentiviral vectors were injected i.v. for *in vivo* transduction studies because this route of injection had been used to immunise mice (see chapter 3), but some studies were also conducted using s.c. injection, because this route has also been shown to immunise mice (Dullaers et al., 2006; Esslinger et al., 2003) and we were interested to know if the same cell types were transduced. A dose of $1x10^8$ i.u. / mouse was given to allow us to monitor transduction using a GFP transgene.

4.4.1 DC, macrophages, B cells and T cells are transduced in spleen, following i.v. injection of a lentiviral vector

In vivo transduction was analysed 5 days post i.v. injection of a GFP expressing vector to allow time for expression of the transgene. Previous biodistribution studies have already shown that gene transfer mainly occurs in liver and spleen (Follenzi et al., 2002; VandenDriessche et al., 2002) and also in bone marrow (Follenzi 2002); since we were using lentivectors for immunisation, we were mainly interested in transduction in spleen and LNs, and also in bone marrow (BM) in case a haematopoietic stem cell was transduced.

Therefore, spleens, LNs and BM were collected from mice that had been injected with either the GFP vector or PBS as a control. Spleens were further separated into the CD11c+ (DC containing) and CD11c- fractions, using MACS beads (see methods). GFP expression was analysed by FACS on unstained cell samples and the results are displayed in figure 4.4A. We found that transduced cells were present in both CD11c+ and CD11c- fractions of spleen (1.28 and 2.00% respectively). Interestingly, the MFI (mean fluorescence intensity) was ~ 3 times higher for the transduced CD11c- cells than for the transduced CD11c+ cells. This could suggest that the SFFV promoter is more efficient in non-DC cells, or that the rate of GFP protein turnover is faster in DC. Few cells were transduced in BM (0.07%) or in the LNs (0.04%), which is not surprising following i.v. injection.

Since transduction was restricted mainly to the spleen, we next wanted to know which cell types in spleen were transduced (see figure 4.4B & C). Although the percentage (%) of each cell type transduced varied between experiments, we found that the vector was able to transduce all cell types analysed. The mean %'s of each cell type transduced (out of 3 experiments) were as follows: 2.82 % of DC, 3.71 % of pDC, 1.95 % of macrophages, 1.5 % of B cells and 0.5 % of T cells. We found macrophages to reside in the CD11c+ fraction; they probably adhere to the MACS column during selection. It is unsurprising that DC (including pDC) and macrophages are transduced, considering their roles in pathogen sensing and uptake of antigens. It is possible that B cells are

readily transduced *in vivo*, due to an activation effect of the vector on these cells, (although B cells are reported to restrict lentivector transduction (Serafini et al., 2004)), while resting T cells are known to be relatively refractory to transduction (Costello et al., 2000). Although a higher percentage of DC are transduced than B cells, this will result in higher numbers of transduced B cells in the spleen, which may influence the adaptive immune response that ensues.

Figure 4.4

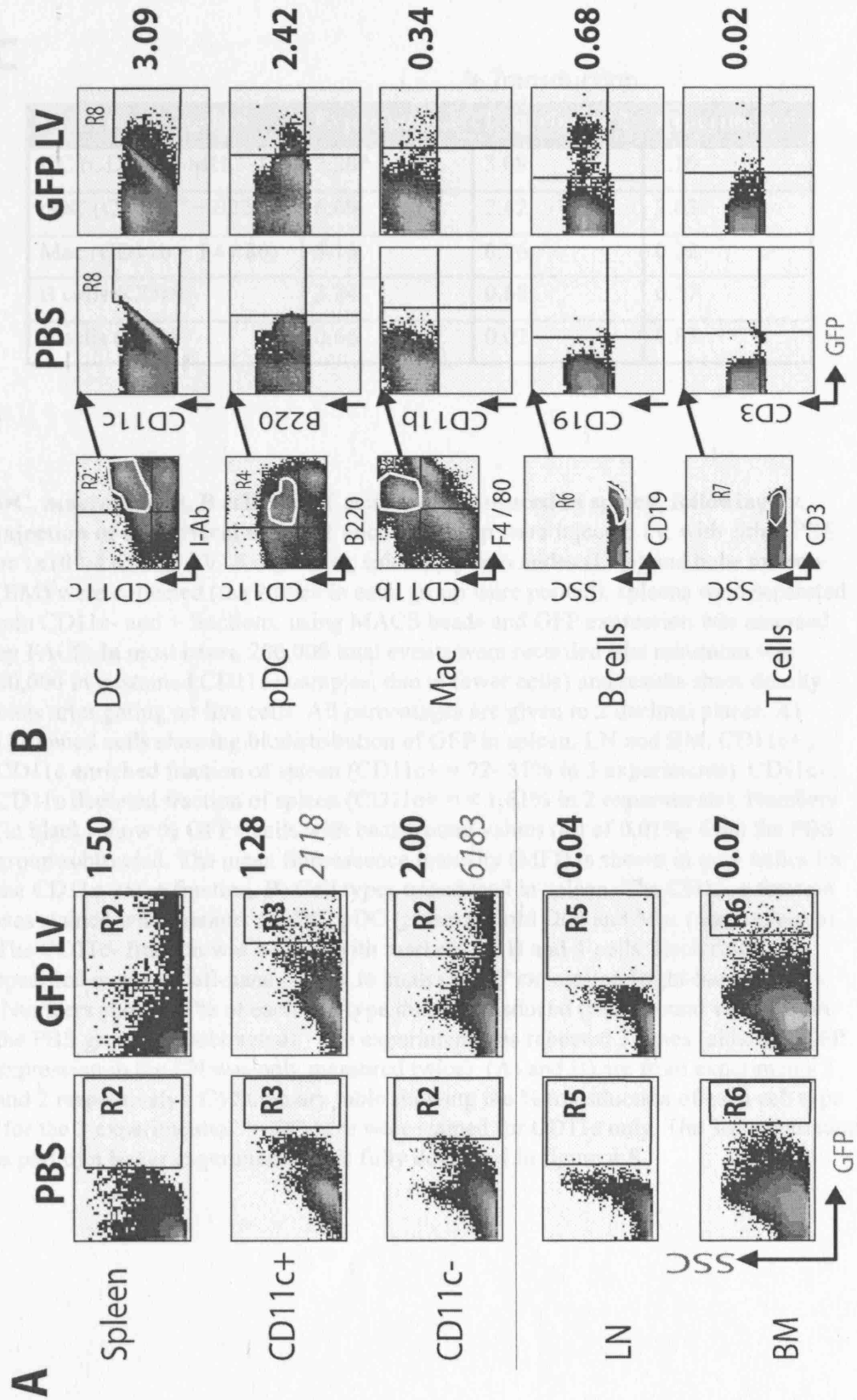

A
PBS
GFP LV
Spleen
CD11c+
CD11c-
LN
BM
1.50
1.28
2118
2.00
6183
0.04
0.07
R2
R3
R5
R6
SSC
GFP
B
DC
pDC
Mac
B cells
T cells
3.09
2.42
0.34
0.68
0.02
R8
R4
R7
CD11c
I-Ab
B220
CD11b
F4/80
CD19
CD3

Figure 4.4

C

% Transduction

Cell Type in Spleen	Experiment 1	Experiment 2	Experiment 3
DC (CD11c + MHC II)	2.20*	3.09	3.16
pDC ($CD11c^{lo}$ + B220)	6.09	2.42	2.63
Mac (CD11b + F4 / 80)	5.16	0.36	0.32
B cells (CD19)	3.24	0.68	0.57
T cells (CD3)	0.66	0.02	0.83

DC, macrophages, B cells and T cells are transduced in spleen, following i.v. injection of a lentiviral vector. 2 mice per group were injected i.v. with either PBS or $1x10^8$ i.u. GFP LV. 5 days later, spleens, lymph nodes (LNs) and bone marrow (BM) were collected (the 2 mice in each group were pooled), spleens were separated into CD11c- and + fractions, using MACS beads and GFP expression was assessed by FACS. In most cases, 200,000 total events were recorded (the minimum was 30,000 in unstained CD11c+ samples, due to fewer cells) and results show density plots after gating on live cells. All percentages are given to 2 decimal places. **A)** Unstained cells showing biodistribution of GFP in spleen, LN and BM. CD11c+, CD11c enriched fraction of spleen (CD11c+ = 72- 81% in 3 experiments). CD11c-, CD11c depleted fraction of spleen (CD11c+ = < 1.61% in 2 experiments). Numbers (in black) show % GFP+ cells with background values (all of 0.01%) from the PBS group subtracted. The mean fluorescence intensity (MFI) is shown in grey italics for the CD11c- vs. + fraction. **B)** Cell types transduced in spleen. The CD11c+ fraction was stained with markers for DC, pDC (plasmacytoid DC) and Mac (macrophages). The CD11c- fraction was stained with markers for B and T cells. Each cell type specified is gated (left-hand panel), to analyse GFP expression (right-hand panels). Numbers show the % of each cell type that is transduced (background values from the PBS group are subtracted). The experiment was repeated 3 times (although GFP expression in the LN was only measured twice). (A) and B) are from experiments 1 and 2 respectively). **C)** Summary table showing the % transduction of each cell type (for the 3 experiments). *= DC here were stained for CD11c only. The 3rd experiment is part of a larger experiment that is fully described in figure 4.8.

4.4.2 CD11c+ cells, B cells and T cells are transduced in the lymph nodes, following s.c. injection of a lentiviral vector

We next repeated the previous *in vivo* tracking experiments using s.c. injection instead so that we could compare the cell types transduced. The results are shown in figure 4.5. Detection of vector transduction in LNs, spleen and BM showed that transduced cells were undetectable in spleen or BM, which could be expected following s.c. injection (see figure 4.5A). However, transduced cells were present in both the CD11c+ and CD11c- fractions of LNs with a higher % of transduction in the DC containing fraction (2.33 vs. 0.78%), although the MFI of the transduced CD11c- cells was again higher. It is possible that these cells represent DC that have migrated to the LNs from skin after antigen capture (e.g. Dermal DC and/ or Langerhans' cells). A recent paper has reported that a lentivector injected subcutaneously transduces skin-derived DC that migrate to lymph nodes and are responsible for T cell immunity (He et al., 2006).

The CD11c- fraction of LNs was further stained for B cells and T cells (figure 4.5B), which showed both these cell types to be transduced (0.41% of B cells and 0.47% of T cells). These data imply that vector particles are drained along with lymph into the LNs. The spleen was also stained, showing that $\leq$ 0.01% of B cells, T cells or CD11c+ cells were transduced (figure 4.5B).

Figure 4.5

A
PBS
GFP LV
LN
CD11c+
CD11c-
Spleen
BM
R5
R7
R6
R2
R8
0.71
2.33
2158
0.78
4976
0.00
0.00
SSC
GFP
B
PBS
GFP LV
B cells
T cells
LN
Spleen
R10
R11
CD19
CD3
CD11c
R16
0.41
0.47
0.01
0.00
0.01

Figure 4.5

CD11c+ cells, B cells and T cells are transduced in the lymph nodes, following s.c. injection of a lentiviral vector. 2 mice per group were injected in both footpads with either PBS or $1x10^8$ i.u. GFP LV. 5 days later, spleens, BM and LNs (inguinal and popliteal - on both sides) were collected (cells from the 2 mice were pooled) and spleens and LNs were separated into CD11c- and + fractions, using MACS beads. GFP expression was assessed by FACS; 200,000 total events were recorded per sample (or 100,000 for samples in the CD11c+ fraction) and results show density plots after gating on live cells. **A)** Unstained cells showing biodistribution of GFP in total LN and CD11c- vs. + fractions of LN (upper 3 panels), or spleen or BM (bottom 2 panels). Numbers show % GFP+ cells (minus background values from the PBS group). The MFI is shown (in grey italics) for the CD11c- vs. + fractions. **B)** Cell types transduced in LN (upper 2 panels) and spleen (lower 3 panels). The CD11c- fraction of LN and spleen was stained with markers for B and T cells. Cell populations were gated (left-hand panel), to analyse GFP expression (right-hand panels). The same gates were used for spleen and LN. The CD11c+ fraction of spleen was stained for CD11c. Numbers show the % of each cell type transduced (with background values from the PBS group subtracted).

4.5 DC stably express their transgene in spleen

The previous experiments were repeated at long-term time-points (2 - 8 months) post vector injection, firstly to find out if vector modified cells could still be detected and secondly to establish which cell types (if any) remained.

4.5.1 GFP expressing DC can still be detected > 2 months after i.v. (but not s.c.) injection of a lentiviral vector.

Figure 4.6 shows the results of the long-term tracking experiments. We found that 2 months post i.v. injection of the vector, 2.11% of DC were still transduced in spleen, compared to 0.22% of macrophages and 0.01% of B cells and T cells (figure 4.6A, left-hand section). It is possible that the modified DC out-lived the lymphocytes because the latter may have been more prone to attack by the immune system. There is some evidence for long-lived DC (O'Keeffe et al., 2002), although most DC subsets turnover within a week (Kamath et al., 2002). It could be that DC are replenished from a transduced splenic precursor or BM stem cell; the latter is unlikely, especially as < 0.03% of cells in LNs and BM were transduced at this time-point. However, bone marrow transduction has been reported following intravenous injection of a lentivector (Follenzi et al., 2002; Pan et al., 2002), although the dose used was 2-10 times higher.

In contrast to these data, transduced cells were not detected 2 months (or 8 months) post s.c. injection (see figure 4.6A, right-hand section and C). In repeat experiments we found that transduced DC were consistently detected even at 7 months (see figure 4.6B), or 8 months (see figure 4.6C) post i.v. injection. Sometimes transduced B cells were also detected at long-term time-points (see figure 4.6C).

Figure 4.6

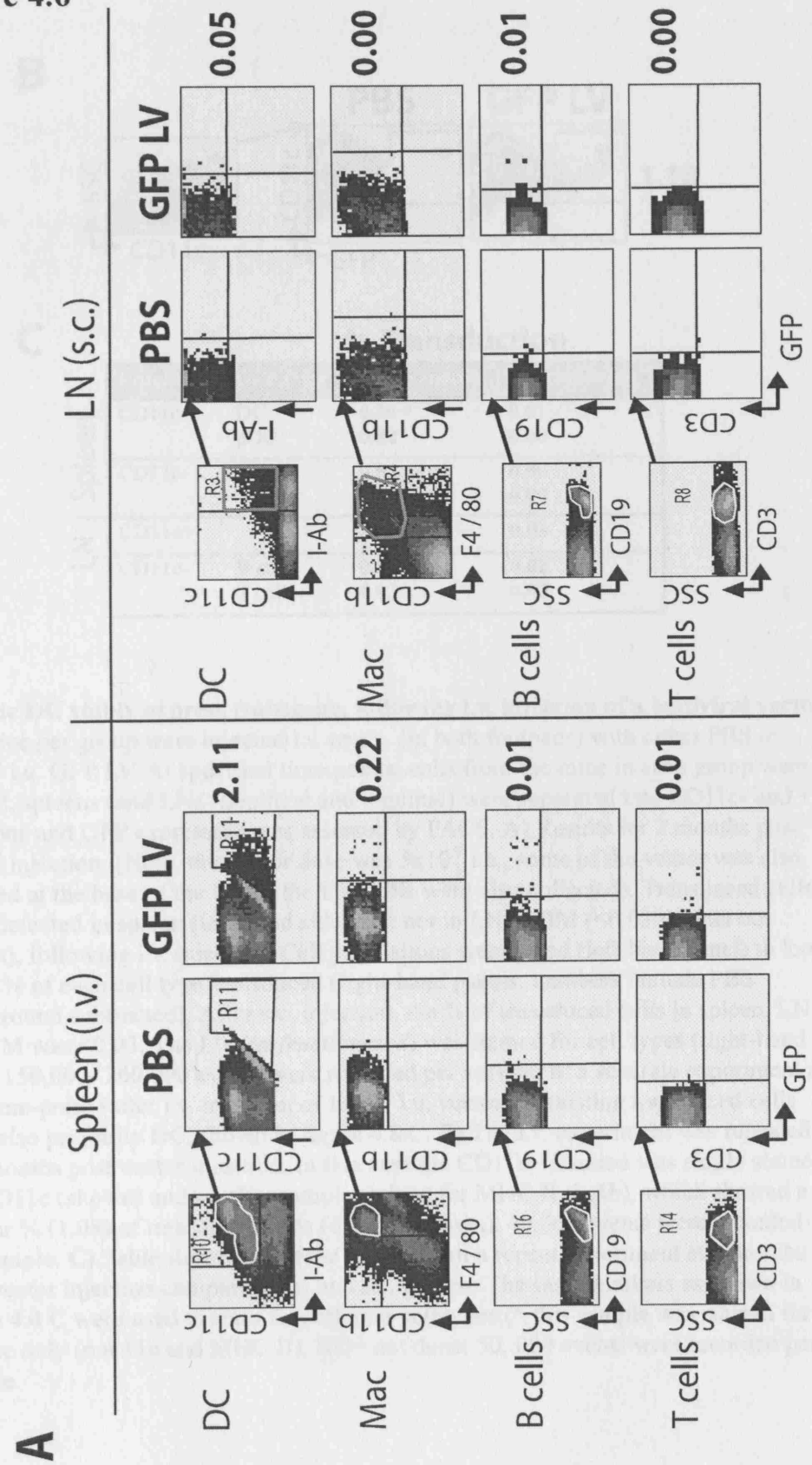
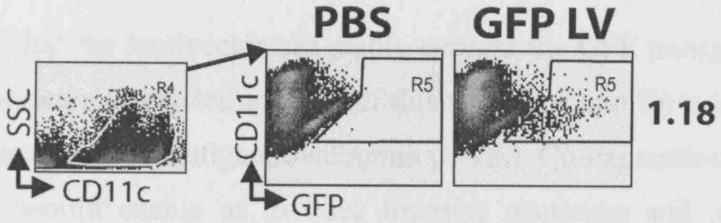

A
Spleen (i.v.)
LN (s.c.)
PBS
GFP LV
DC
Mac
B cells
T cells
2.11
0.22
0.01
0.01
0.05
0.00
0.01
0.00
R10
R11
R16
R14
R3
R4
R7
R8
CD11c
I-Ab
CD11b
F4 / 80
SSC
CD19
CD3
GFP

Figure 4.6

B



C

% Transduction

	Fraction	Cell Type	Intravenous	Subcutaneous
Spleen	CD11c+	DC	**0.30 ***	**0.01**
		pDC	**0.82**	**0.00**
	CD11c-	B cells	**0.53**	**0.00**
		T cells	**0.03**	**0.00**
LN	CD11c+		**ND**	**0.03**
	CD11c-	B cells	**0.07**	**0.02**
		T cells	**0.03**	**0.00**

Splenic DC stably express transgene, following i.v. injection of a lentiviral vector. 2-3 mice per group were injected i.v. or s.c. (in both footpads) with either PBS or $1x10^8$ i.u. GFP LV. At specified time points, cells from the mice in each group were pooled, spleens (and LNs -popliteal and inguinal) were separated into CD11c- and + fractions and GFP expression was assessed by FACS. **A)** Results for 2 months post vector injection. (Here, the vector dose was $5x10^7$ i.u., some of the vector was also injected at the base of the tail so the iliac LNs were also collected). Transduced cells were detected in spleen (left-hand side), but not in LN or BM (<0.03%- data not shown), following i.v. injection. Cell populations were gated (left-hand panel) to look at the % of each cell type transduced (right-hand panels, numbers include PBS background subtracted). After s.c. injection, the % of transduced cells in spleen, LN and BM was <0.03. The LN (***unfractionated***) was stained for cell types (right-hand side). 150,000- 200,000 events were recorded per sample. In a separate experiment at this time-point (after i.v. injection of $1x10^8$ i.u. vector), persisting transduced cells were also primarily DC, shown in figure 4.8 C. **B)** The i.v. experiment was repeated at 7 months post vector injection. In this case the CD11c+ fraction was singly stained for CD11c (shown) and another sample stained for MHC II (I-Ab), which showed a similar % (1.08) of transduced cells (data not shown). 40,000 events were recorded per sample. **C)** Table summarising the results from a repeat experiment at 8 months post vector injection comparing i.v. and s.c. groups. The same markers as shown in figure 4.4 C were used to stain for different cell types (* this sample was stained for CD11c only (not 11c and MHC II). ND= not done. 50, 000 events were recorded per sample.

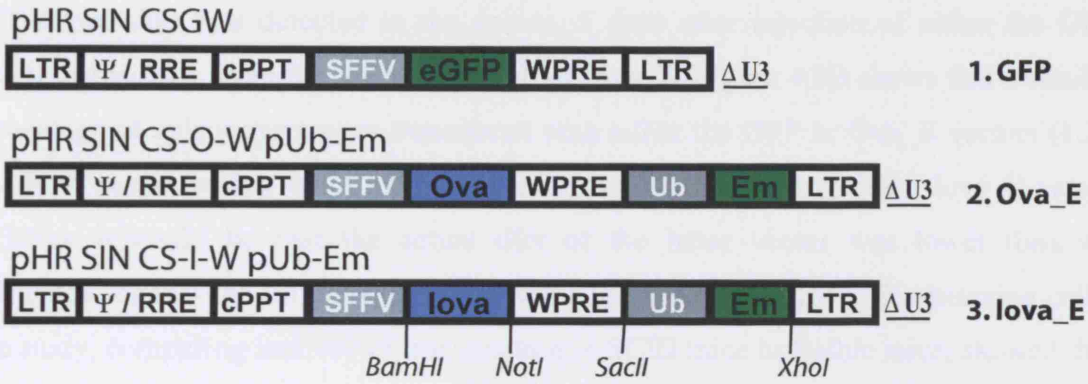
4.6 Persistence of vector modified cells *in vivo* depends on the antigen(s) expressed

Our results suggest that the lentivector can stably express the GFP transgene in splenic dendritic cells so we were interested to know if this would be true for other transgenes, such as the more immunogenic antigen, ovalbumin (OVA). Co-expression of OVA with the GFP transgene would enable us to track immune responses and transduction in parallel.

4.6.1 Construction of dual promoter vectors expressing both ovalbumin and GFP

The dual promoter lentiviral vector that we are using (kind gift from Y. Ikeda) contains the promoters: SFFV and Ub; Ub is the human polyubiquitin C promoter and it works well in a broad range of mammalian cells. Two dual promoter vectors were constructed (see figure 4.7) expressing GFP emerald (named E) under the Ub promoter, and either one of two OVA constructs under the SFFV promoter (known to work well *in vivo*, see chapter 3). The Ova_E vector expresses secreted (wildtype) OVA, and the Iova_E vector expresses the OVA immunodominant epitopes fused to the invariant chain (to enhance its presentation on MHC I and II, see chapter 3). The control vector expressing GFP alone is referred to as the GFP vector.

Figure 4.7



A dual promoter lentiviral vector was constructed to measure transduction and antigen presentation in parallel. 1. Vector map showing the original GFP vector. 2. and 3. are the dual promoter vectors, expressing either wild type ovalbumin (Ova - 1, 161 bp) or the invariant chain-ovalbumin fusion (Iova - 1, 658 bp), used before (see chapter 3). Both 2. and 3. express GFP emerald (Em -780 bp). Unique restriction sites are shown. LTR, long terminal repeat; Ψ, packaging signal; RRE, rev response element; cPPT, central polypurine tract; SFFV, spleen focus forming virus promoter; WPRE, wood chuck post transcriptional regulatory element; Ub, ubiquitin promoter; **ΔU3**, deletion in U3 region (SIN vector).

4.6.2 Comparison of long-term transduction in the spleen, after i.v. injection of the different vectors

GFP expression was detected in the spleen, 5 days after injection of either the GFP vector or the dual promoter vectors: Ova_E or Iova_E. Figure 4.8B shows that a similar percentage of splenocytes were transduced with either the GFP or Ova_E vectors (1.22 or 1.82% respectively), but a lower percentage was transduced with the Iova_E vector (0.39%). It could be that the actual titer of the latter vector was lower than we calculated, or that the immune response was already targeting Iova_E expressing cells; one study, comparing lentivector transduction in SCID mice to Balb/c mice, showed that there were less transduced cells in the Balb/c mice at 4 days post injection, suggesting the immune system to have already eliminated some cells by this time-point (VandenDriessche et al., 2002). Nevertheless, the data showed that GFP emerald was expressed from the Ub promoter *in vivo*, although the MFI was distinctly lower (1386 for Ova_E and 1235 for Iova_E) than SFFV driven GFP expression (2116).

At the 2 month time-point (see figure 4.8B), there was more decline in the % of transduced cells in the Ova_E group (to 0.08%, 22.8x lower than at day 5) than in the GFP group (to 0.36%, 3.4x lower), although this might not reflect a vector-specific difference as different mice were analysed at each time-point. The % of cells transduced also dropped notably for the Iova_E group (by 9.8x), although less cells were transduced initially. These results suggest that the GFP vector transduced cells persist longer than the Ova_E and Iova_E vector transduced cells; the presence of the OVA transgene could render transduced cells more prone to immune attack.

Q-PCR was carried out to confirm the presence of actual provirus in spleen samples (~ 1- 5 x 10^6 cells were taken for Q-PCR), using primers and probe specific for the strong stop region of the vector. Q-PCR results (see figure 4.8A) correlated with the FACS data (figure 4.8B), in that more vector copies were present in the Ova_E group at day 5, but after 2 months more copies remained in the GFP group. Less vector copies were present in the Iova_E group initially, which was consistent with the FACS data. This suggests

that GFP expression (from either the SFFV or Ub promoter) relates to the actual amount of vector copies present. The Q-PCR data also show that in this experiment, at the day 5 time-point, while CD11c+ and CD11c- fractions contained similar amounts of vector for the GFP group, the CD11c+ fraction contained more copies than the CD11c- fraction, for the Ova_E group (this trend is also shared by the Iova_E group). In all groups, more vector copies were detected in the CD11c+, than the CD11c- fraction after 2 months.* Overall, the data are consistent with the theory that transduced non-DC are more subject to immune attack (perhaps even before day 5 for the OVA vector groups).

Staining of the CD11c+ fractions for the dendritic cell population (figure 4.8C), revealed that transduced DC persisted longer in the GFP vector group; the % of transduced DC in this group appeared to go up after 2 months, although different mice were analysed at each time-point. Consistent with the Q-PCR data, the % transduced DC in the Ova_E group declined dramatically over 2 months; the % transduced DC in the Iova_E group also declined over time. These results suggest that ovalbumin expressing DC are more prone to immune attack than DC expressing GFP alone. Analysis of other cell types in spleen (see figure 4.8D), showed that initially more CD11c- cells (B cells and T cells) were transduced with the GFP vector, in agreement with the Q-PCR data; after 2 months very few transduced B cells or T cells were detected (the highest, 0.08%, was B cells in the GFP group).

*The presence of the vector in the CD11c+ fraction suggests that DC are actually harbouring vector rather than taking up GFP protein from dying cells.

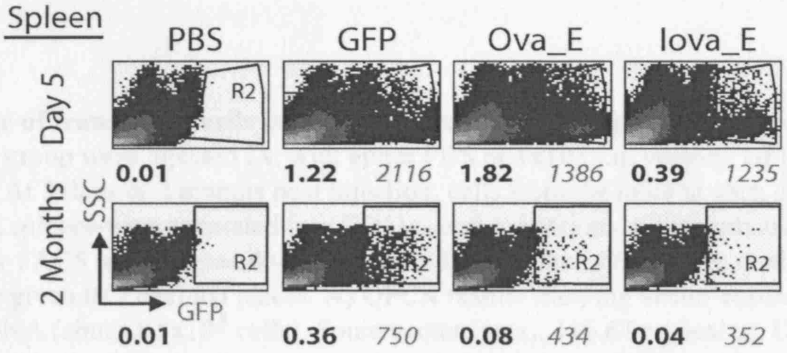
Figure 4.8

A

Vector copies / µg DNA

	Fraction of spleen	GFP	Ova_E	Iova_E
Day 5	CD11c+	2783.65	7901.33	833.68
	CD11c−	3400.86	2856.22	ND
2 Months	CD11c+	1470.74	270.08	40.03
	CD11c−	180.57	UD	UD

B



C

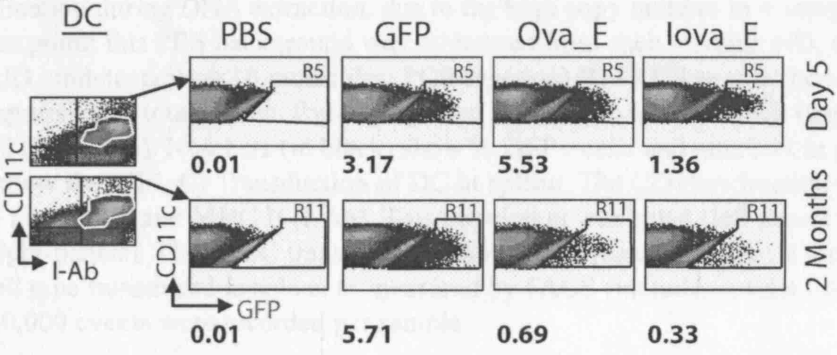


Figure 4.8

D

	GFP		Ova_E		Iova_E	
Cell Type	Day 5	2 Months	Day 5	2 Months	Day 5	2 Months
DC	3.16	5.70	5.52	0.68	1.35	0.32
pDC	2.63	4.34	7.17	1.05	2.56	0.59
Mac	0.32	0.13	0.39	0.03	0.10	0.00
B cells	0.57	0.08	0.31	0.02	0.12	0.00
T cells	0.83	0.06	0.12	0.00	0.01	0.00

Persistence of transduced cells *in vivo* depends on the transgene expressed.
2 mice per group were injected i.v. with either PBS or $1x10^8$ i.u. vectors: GFP, Ova_E or Iova_E. At 5 days or 2 months post injection, cells from the mice in each group were pooled and spleens were separated into CD11c- and + fractions. GFP expression was assessed by FACS and integrated vector detected by Taqman QPCR (see methods). Results are given to 2 decimal places. **A)** QPCR results showing vector copies/ µg genomic DNA (about $1.5x10^5$ cells). Some vector (max., 116.67 copies/ µg DNA) was detected in the PBS day 5 samples, although the controls (PCR reaction without mouse DNA) and the 2 month PBS samples were blank. This could be minor contamination during DNA extraction, due to the high copy number in + samples at this time point; this PBS background was subtracted from each + result. ND, not done. UD, undetectable (<10 molecules/ PCR reaction) **B)** FACS results showing GFP expression in total spleen. Events recorded per sample were 500,000 (day 5) or 200,000 (2 months). Numbers (in black) show % GFP+ cells and numbers in grey italics show the MFI. **C)** Transduction of DC in spleen. The CD11c+ fraction was stained for CD11c and MHC II (I-Ab). This population was gated (left panel) to detect GFP (right-panels). The % DC transduced is shown underneath. **D)** Shows the % of each cell type transduced in spleen as measured by FACS (includes results from C). 150-200,000 events were recorded per sample.

4.7 The ovalbumin antigen is stably presented in mouse spleen

Results thus far showed that splenic dendritic cells could express GFP for at least 2 months post injection of vectors that carried either GFP alone, or both OVA and GFP. The next important step was to ascertain whether OVA was stably presented in the spleen. To investigate this, we decided to adoptively transfer OT-I T cells (specific for the $OVA_{257-264}$ peptide presented on K^b) into vector injected mice at different time-points with the aim to measure expansion of these cells using pentamer staining. Results were analysed 6 days post adoptive transfer to allow time for any T cell expansion, and the presence of the GFP transgene enabled us to track *in vivo* transduced cells at the time of analysis. We decided to use the Iova_E vector for these experiments (and the GFP vector or PBS as controls) because previous results (see chapter 3) showed that the Iova construct (full name, Ii-OVA) stimulates more effective CD8+ and CD4+ T cell responses than the Ova construct. Half of the mice in each group were injected i.v. with MPL (monophosphoryl lipid A) adjuvant, one day before T cell transfer to see if this approach would enhance presentation of OVA by transduced cells. MPL, which is a non-toxic derivative of LPS, is a known adjuvant for both cell mediated and humoral immunity (Maier et al., 2005) and we found that it enhanced the CD8+ T cell response when co-injected with OVA peptide (not shown).

4.7.1 Ovalbumin is presented in mouse spleen 6 days post lentivector injection

Firstly, OT-I T cells were transferred at 6 days post vector injection and the results are shown in figure 4.9 (see legend for experimental details). Figure 4.9A shows that in the non-MPL groups (− MPL), OT-I's expanded to an average of 9.14% (of total CD8+ T cells) in the Iova_E vector injected group, compared to 0.77% and 0.5% in the respective GFP vector and PBS injected groups. In the MPL injected groups (+ MPL), results were similar with a mean of 6.77% in the Iova_E vector group, and 1.07% and 0.84% in the respective GFP vector and PBS groups; MPL seemed to increase the background in the control groups but not the expansion in the Iova_E vector group. It could be that the vector intrinsically acts as an adjuvant at this early time-point. Interestingly, when the Iova_E vector was injected at the same time as the OT-I T cells,

the expansion of these cells was substantially greater with a mean of 43.60% of CD8+ T cells being OVA specific. This could result from the vector effectively activating DC and other APCs at this time-point.

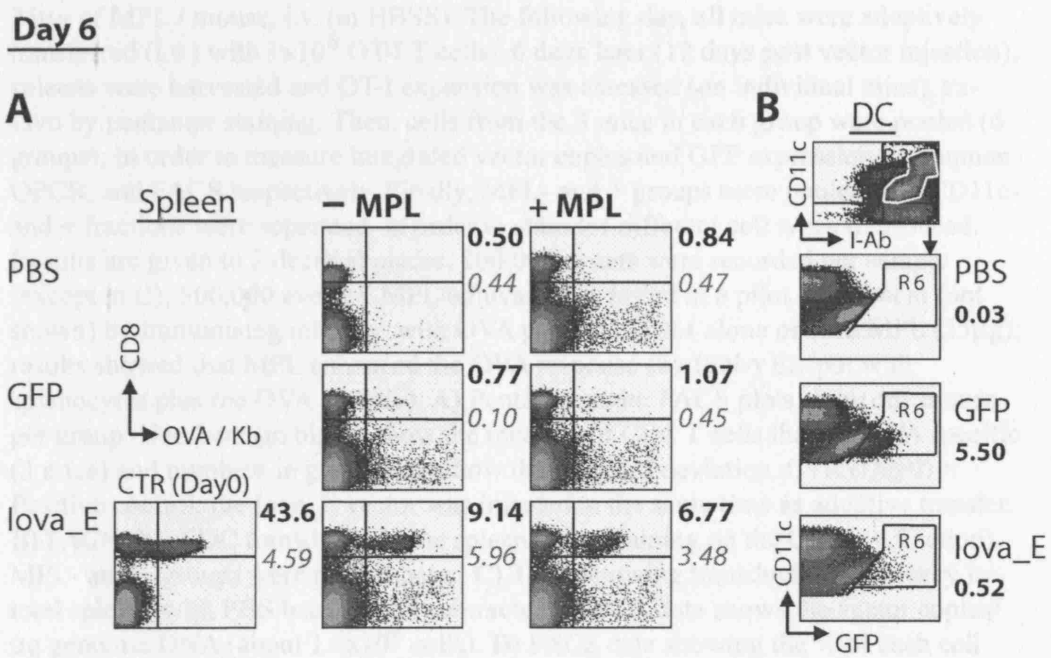
Transduced DC were detected in these mice by separating the CD11c+ fraction from spleen and staining for DC (see figure 4.9B). At this time-point (day 12 post vector injection), we found that the % of transduced DC was ~ 10x higher in the GFP vector group than the Iova_E vector group. It is possible that more Iova_E expressing DC were killed than GFP expressing DC, due to the transferred T cells as well as the endogenous immune response. Nevertheless, we still found 0.52% of DC to be transduced.
Q-PCR results confirmed the presence of vector provirus in total spleen samples by using strong stop primers and probe. Results are shown in figure 4.9C, along with the % transduced cells in spleen, measured by FACS; FACS values reflect the Q-PCR data in that the actual number of vector copies is much lower in the Iova_E group, than in the GFP group.*

FACS analysis of transduced cell types in spleen (figure 4.9D) showed that mainly DC were transduced for both vector groups; in comparison few or no lymphocytes were transduced for the respective GFP or Iova_E vector groups. This might also be attributed to immune killing. We also found that 0.85% of plasmacytoid DC were transduced in the Iova_E mice, while transduced macrophages were undetectable (data not shown). The fact that transduced DC (and not other APCs) were detected in these mice implicates DC in the role of antigen presenters to transferred T cells.

In summary, these data suggest that transferred (OT-I) T cells are responding to OVA presented on MHC class I by DC in the spleen, 6 days post vector injection; the confirmation of transgene expression by dendritic cells (and not other APCs) supports this hypothesis. However, we can't rule out the presence of endogenous OVA specific CD8+ T cells, although previous data (see chapter 3) have shown that endogenous CD8+ T cells are rarely detected in spleen or blood by OVA/ K^b tetramer staining, unless mice are also boosted with an OVA expressing vaccinia virus (see chapter 3).

*These data can also be used to calculate the number of vector copies in the spleen (which contains ~$1x10^8$ cells) to see if there is a correlation with the % of GFP expressing cells as measured by FACS. We found that there is a correlation and therefore it is unlikely that uptake of transduced dying cells contributes to the % of GFP+ cells measured.

Figure 4.9



C

	– MPL		+ MPL	
Vector	%GFP (FACS)	LV copies/µg DNA (QPCR)	%GFP (FACS)	LV copies/µg DNA (QPCR)
GFP	0.40	7989.28	0.81	6135.80
Iova_E	0.02	138.22	0.03	81.55

D

MPL +/- Pool

Vector	DC	B cells	T cells
GFP	5.47	0.12	0.01
Iova_E	0.49	0.00	0.00

Figure 4.9

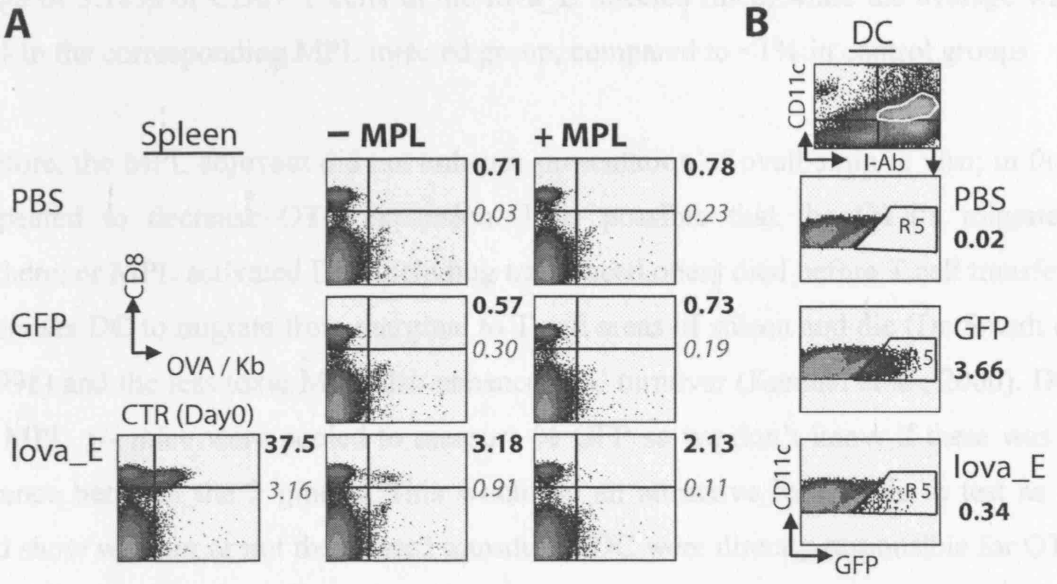
Ovalbumin is presented in mouse spleen, 6 days post lentivector injection.
6 mice per group were injected i.v. with either PBS, or $1x10^8$ i.u. GFP or Iova_E vectors. 5 days later, each group was divided into 2 and one of them injected with 25μg of MPL / mouse, i.v. (in HBSS). The following day, all mice were adoptively transferred (i.v.) with $1x10^6$ OT-I T cells. 6 days later (12 days post vector injection), spleens were harvested and OT-I expansion was assessed (on individual mice), ex-vivo by pentamer staining. Then, cells from the 3 mice in each group were pooled (6 groups), in order to measure integrated vector copies and GFP expression by Taqman QPCR, and FACS respectively. Finally, MPL- and + groups were pooled, and CD11c- and + fractions were separated, in order to stain for different cell types transduced. Results are given to 2 decimal places. 200,000 events were recorded per sample (except in C), 500,000 events). MPL adjuvant was tested in a pilot experiment (not shown) by immunising mice i.v. with OVA peptide 257-264 alone or with MPL (25μg); results showed that MPL enhanced the OVA response (by IFN-γ Elispot with splenocytes plus the OVA peptide). **A)** Pentamer stain: FACS plots show one mouse per group. Numbers (in black) show the mean % of CD8 T cells that are OVA specific (3 mice) and numbers in grey italics show the standard deviation. CTR (Day0) = Positive control, the Iova_E vector was injected at the same time as adoptive transfer. **B)** FACS: % of DC transduced in the spleen (after staining on the CD11c+ fraction). MPL- and + groups were pooled here. **C)** Table showing transduction efficiency in total spleen (with PBS background subtracted). QPCR data shows the vector copies/ μg genomic DNA (about $1.5x10^5$ cells). **D)** FACS data showing the % of each cell type (minus PBS background) transduced in spleen (includes data from B).

Figure 4.10

Ovalbumin is presented in mouse spleen, one month post lentivector injection.
The same experiment as described in figure 4.9 was repeated here, except that in this case, the mice were left for one month, inbetween vector injection and the MPL step. **A)** Pentamer stain. See figure 4.9 A for description. **B)** The % of DC transduced in the spleen. See figure 4.9 B for description. **C)** Table showing transduction efficiency in total spleen. Refer to figure 4.9 C. UD, undetectable (<10 molecules vector / PCR reaction). **D)** The % of each cell type transduced in spleen. Refer to figure 4.9 D.

Figure 4.10

One Month



C

	− MPL		+ MPL	
Vector	%GFP (FACS)	LV copies/µg DNA (QPCR)	%GFP (FACS)	LV copies/µg DNA (QPCR)
GFP	0.28	1216.21	0.21	285.89
Iova_E	0.02	UD	0.01	UD

D

MPL +/- Pool

Vector	DC	B cells	T cells
GFP	3.64	0.06	0.02
Iova_E	0.32	0.00	0.01

4.7.2 Ovalbumin is presented in mouse spleen one month post lentivector injection

The previous experiment was repeated but with adoptive transfer at one month post vector injection. Results (see figure 4.10A) showed that there was a modest expansion of transferred T cells at one month; in the non-MPL group, OT-I T cells expanded to an average of 3.18% of CD8+ T cells in the Iova_E injected mice, while the average was 2.13% in the corresponding MPL injected group, compared to <1% in control groups.

Therefore, the MPL adjuvant did not enhance presentation of ovalbumin *in vivo*; in fact it appeared to decrease OT-I expansion. It is possible that the OT-I's migrated elsewhere, or MPL activated DC (including transduced ones) died before T cell transfer; LPS causes DC to migrate from marginal to T cell areas of spleen and die (De Smedt et al., 1998) and the less toxic MPL also enhances DC turnover (Kamath et al., 2000). DC from MPL +/- mice were pooled to measure % GFP so we don't know if there was a difference between the 2 groups. This would be an attractive hypothesis to test as it would show whether or not the deleted transduced DC were directly responsible for OT-I expansion.

Injection of the Iova_E vector at the same time as adoptive transfer led to a similar OT-I expansion as in figure 4.9 e.g. here, a mean of 37.5% of CD8+ T cells were OVA specific.

Dendritic cell expression of transgene was confirmed by FACS (see figure 4.10B), with 0.34% of DC transduced in the Iova_E group (this was 10x lower than the GFP group again). In contrast a much lower % of transduced cells was detected by FACS in total spleen ($\leq$ 0.02%) for the Iova_E group (see figure 4.10C) and vector copies were below detection by Q-PCR; It was also determined that $\leq$ 0.01% of B and T cells were transduced in the Iova_E group.

In summary, these data suggest that transferred (OT-I) T cells are responding to OVA presented on MHC class I by DC in the spleen, one month post vector injection; in support of this proposal, dendritic cells (and not B cells) were found to express

transgene at this time-point. The next section provides further evidence for this hypothesis.

4.7.3 The CD11c+ fraction of spleen is responsible for antigen presentation

The isolated CD11c+ and CD11c- fractions of spleen from the one month experiment described above (3 groups, PBS, GFP and Iova_E) were cultured with OT-I T cells (thawed from frozen) overnight in an ELISpot assay, the results of which are shown in figure 4.11A & B.

We found that the CD11c+ fraction (and not the CD11c- fraction) in the Iova_E group was able to present OVA to OT-I T cells, resulting in IFN-γ secretion; this result was independent of whether peptide was added in the assay. The control CD11c+ groups did not present OVA even when it was added in the assay; perhaps the DC, which were still bound to the MACS beads, were unable to present the exogenous peptide or the added OT-I's were lacking co-stimulation that was provided in the Iova_E group, due to the previous activation of DC by T cells *in vivo*. A future control would be to include a maturation step with LPS.

The CD11c- cell fraction from all groups (which included the transferred OT-I's) did not present OVA (figure 4.11A & B), but could respond when ConA was added (not shown). The finding that isolated CD11c+ cells from the Iova_E group could present antigen *in vitro*, further implicated DC in the role of antigen presenters to transferred OT-I's *in vivo*.

CD11c+ and CD11c- fractions from the positive control group (where the Iova_E vector was injected at the same time as adoptive transfer) were also included in the ELISpot, and the results are shown in figure 4.11C & D. It was clear from this group that this CD11c+ fraction was also primarily responsible for antigen presentation to OT-I's *in vitro*, suggesting that DC (and not B cells) are the main antigen presenting cells *in vivo*, despite both cell populations being transduced initially.

Figure 4.11

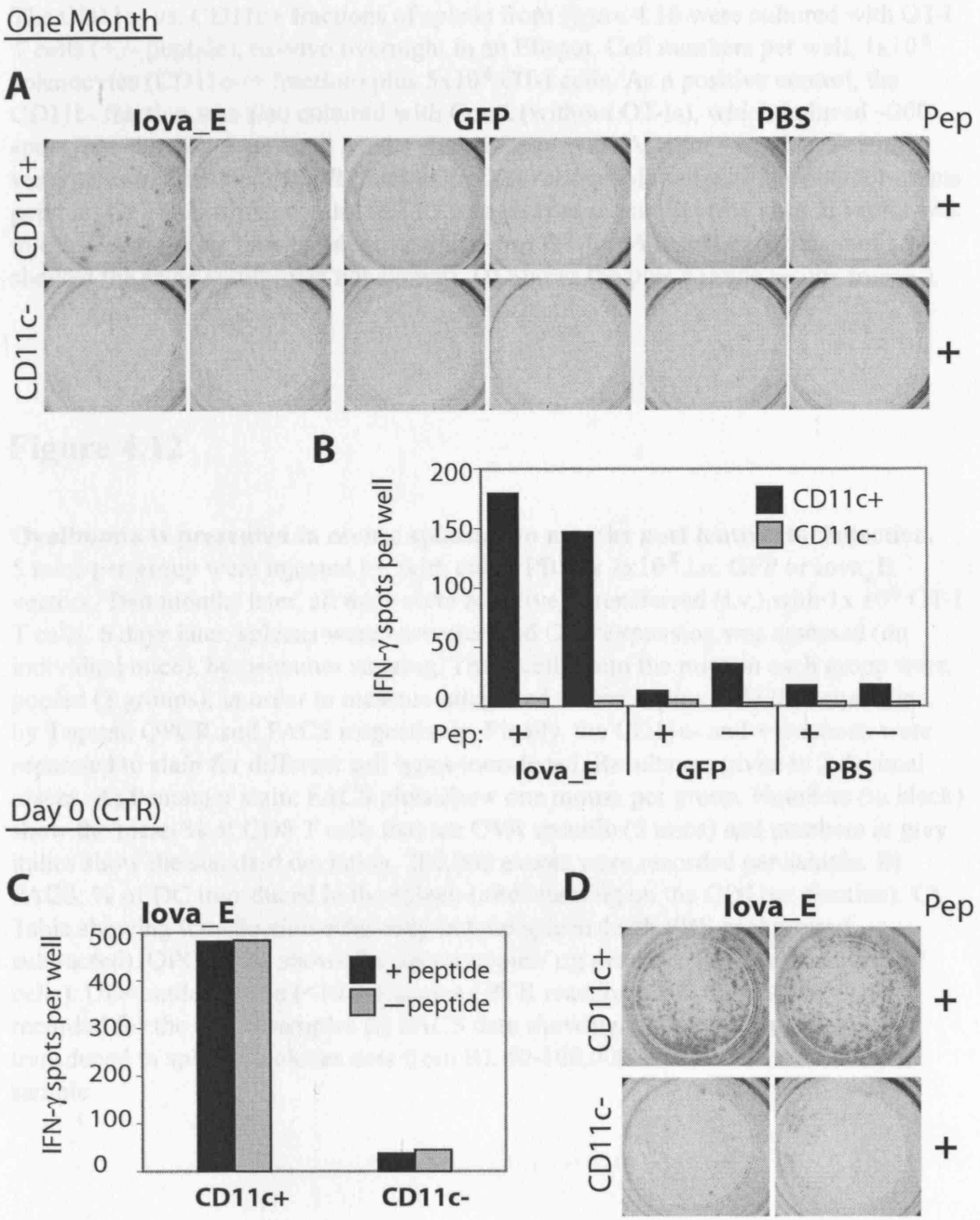

One Month
A
Iova_E
GFP
PBS
Pep
CD11c+
CD11c-
+
+
B
IFN-γ spots per well
200
150
100
50
0
CD11c+
CD11c-
Pep: + — + — + —
Iova_E
GFP
PBS
Day 0 (CTR)
C
Iova_E
IFN-γ spots per well
500
400
300
200
100
0
+ peptide
—peptide
CD11c+
CD11c-
D
Iova_E
Pep
CD11c+
CD11c-
+
+

Figure 4.11

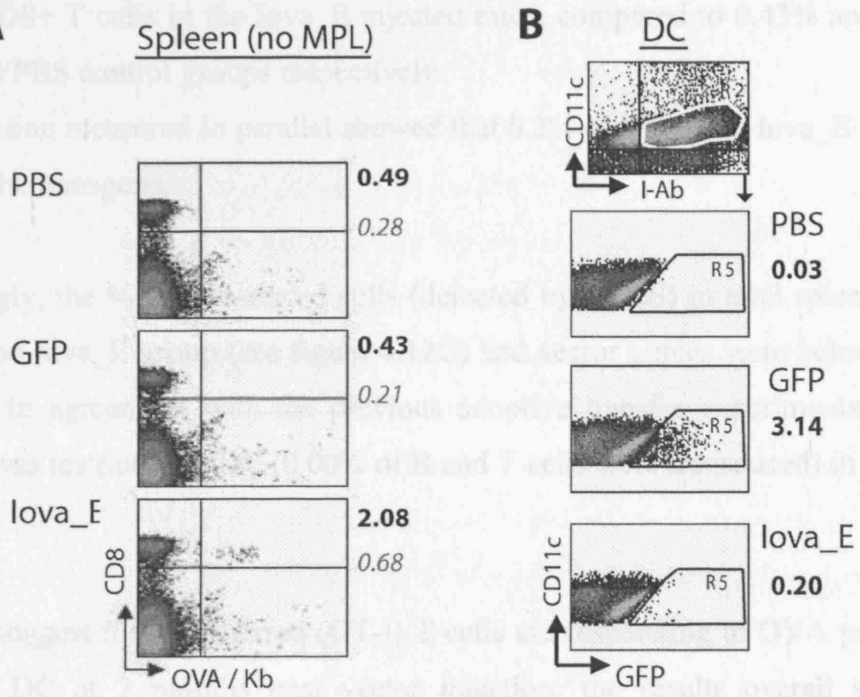
The CD11c+ fraction of spleen is responsible for stable antigen presentation. The CD11c- vs. CD11c+ fractions of spleen from figure 4.10 were cultured with OT-I T cells (+ /- peptide), ex-vivo overnight in an Elispot. Cell numbers per well, $1x10^6$ splenocytes (CD11c-/+ fraction) plus $5x10^4$ OT-I cells. As a positive control, the CD11c- fraction was also cultured with ConA (without OT-Is), which induced ~200 spots (not shown). **A)** Elispot results shown for plus OVA 257-264 peptide. Samples were measured in duplicate. **B)** Results from A) above, plotted with the data for minus peptide. **C)** The positive control (CTR) was assayed in parallel (the Iova_E vector was injected at the same time as adoptive transfer of OT-Is). A repeat experiment of C) showed the same result (data not shown). **D)** Shows the plus peptide results from C).

Figure 4.12

Ovalbumin is presented in mouse spleen, two months post lentivector injection. 5 mice per group were injected i.v. with either PBS or $1x10^8$ i.u. GFP or Iova_E vectors. Two months later, all mice were adoptively transferred (i.v.) with $1x\ 10^6$ OT-I T cells. 6 days later, spleens were harvested and OT-I expansion was assessed (on individual mice), by pentamer staining. Then, cells from the mice in each group were pooled (3 groups), in order to measure integrated vector copies and GFP expression, by Taqman QPCR and FACS respectively. Finally, the CD11c- and + fractions were separated to stain for different cell types transduced. Results are given to 2 decimal places. **A)** Pentamer stain: FACS plots show one mouse per group. Numbers (in black) show the mean % of CD8 T cells that are OVA specific (5 mice) and numbers in grey italics show the standard deviation. 200,000 events were recorded per sample. **B)** FACS: % of DC transduced in the spleen (after staining on the CD11c+ fraction). **C)** Table showing transduction efficiency in total spleen (with PBS background subtracted). QPCR data shows the vector copies/ µg genomic DNA (about $1.5x10^5$ cells). UD= undetectable (<10 molecules / PCR reaction). 500,000 events were recorded for the FACS samples **D)** FACS data showing the % of each cell type transduced in spleen (includes data from B). 50-100,000 events were recorded per sample.

Figure 4.12

Two Months



C

Spleen

Vector	% GFP (FACS)	LV copies/µg DNA (QPCR)
GFP	0.20	214.82
Iova_E	0.01	UD

D

Cell types in spleen

Vector	DC	B cells	T cells
GFP	3.11	0.03	0.04
Iova_E	0.17	0.00	0.00

4.7.4 Ovalbumin is presented in mouse spleen two months post lentivector injection

Adoptive transfer was then repeated, but at two months post vector injection (there was no MPL injected group in this experiment). Results (see figure 4.12A) showed that there was a detectable OT-I expansion at this time-point, to an average (5 mice per group) of 2.08% of CD8+ T cells in the Iova_E injected mice, compared to 0.43% and 0.49% in the GFP and PBS control groups respectively.
DC transduction measured in parallel showed that 0.2% of DC in the Iova_E group were expressing the transgene.

Unsurprisingly, the % of transduced cells (detected by FACS) in total spleen was only 0.01% for the Iova_E group (see figure 4.12C) and vector copies were below detection by Q-PCR. In agreement with the previous adoptive transfer experiments, transgene expression was restricted to DC (0.00% of B and T cells were transduced) in the Iova_E group.

These data suggest that transferred (OT-I) T cells are responding to OVA presented on MHC I by DC at 2 months post vector injection; the results overall support the hypothesis that lentivector encoded antigens are stably presented by dendritic cells in mouse spleen. However, future experiments (outlined in the discussion) should enable us to confirm these results.

4.8 Discussion

One desirable trait of a cancer vaccine is the ability to sustain antigen presentation by dendritic cells *in vivo*, to avoid the need to repeatedly immunise patients. Lentiviral vaccines might exhibit this trait because they stably express genes in non-dividing cells, such as DC *in vivo*. However, their ability to direct stable antigen presentation and the possible consequence of this on immunity has not previously been explored.

Here, we show that splenic dendritic cells in particular stably express their transgene up to 8 months post systemic administration of a GFP expressing lentiviral vector, even though initially a range of cell types in spleen are transduced. In contrast, transduced cells do not persist after immunising mice with the same vector subcutaneously, although again a range of cells are transduced initially, this time in draining lymph nodes.

We used a vector co-expressing ovalbumin (OVA) and GFP to show that the CD11c+ (and not the CD11c-) fraction of spleen is primarily responsible for antigen presentation. This explains why lentivectors successfully stimulate CTL and CD4+ T cell responses (Dullaers et al., 2006; Esslinger et al., 2003; Palmowski et al., 2004; Rowe et al., 2006) and suggests that transduced B cells do not play a big role in immunity. Furthermore, we have provided evidence to support our proposal that stably modified DC can still present antigen for at least one month post immunisation; this was determined by *in vivo* expansion of OT-I T cells, transferred one month and two months post immunisation, and by the finding that CD11c+ cells isolated after one month could stimulate OT-I's *in vitro*.

This is the first time (to our knowledge) that the presence of stably modified DC has been demonstrated, following i.v. injection of a vaccine. Our data suggest that these cells can persistently present antigen, however, there are still many questions to be answered concerning the nature of these DC, why they persist, and what effect they exert *in vivo*.

As to the nature of the transduced DC, our data clearly show that both conventional DC (cDC) and plasmacytoid DC (pDC) in the spleen stably express the transgene (GFP was expressed by MHC II+, CD11c^{hi} / CD11c^{lo} cells, which were analysed together and by pDC, which were analysed separately). The fact that modified DC numbers decrease over time suggests that non-dividing DC precursors or long-lived DC are transduced.
It could be that pDC are targeted by the vector (perhaps inducing IFNα/β secretion that would contribute to the primary immune response), but they do not receive the

appropriate signals to differentiate into DC because they are confronted with a vector, not a virus (or they do not receive the appropriate signals to die). They could, therefore, be the long-lived DC precursors harbouring the vector. If stably modified DC were mainly pDC, it would explain why MPL did not activate them; pDC express TLRs 7 and 9 but ~~not~~ low TLR4 (Naik et al., 2005).

Alternatively, pDC (or cDC) could become activated upon picking up the vector, differentiating into DC that persist, due to a survival signal delivered by the vector and/or through interaction with antigen specific T cells (e.g. inducing expression of TRANCE and RANK (Cremer et al., 2002) or Bcl-2 (Nopora and Brocker, 2002)). There is evidence that the lentivector can activate DC (see chapter 3 discussion). Indeed, we have found that the vector can exert modest activation effects on BMDC in vitro (see chapter 5).

It is possible that some transduced DC are non-activated CD8- cDC in marginal zones, a fraction of which could be long-lived (Kamath et al., 2002), or that a blood-derived DC precursor (O'Keeffe et al., 2003) or BM-derived stem cell/DC precursor (Diao et al., 2004; Fogg et al., 2006) is transduced, although it is unlikely that BM is transduced (see results section 4.5.1). The most obvious explanation, in light of a recent study (Naik et al., 2006) is that a splenic DC committed precursor is directly transduced, because a high proportion of vector particles traffic straight to the spleen after i.v. injection. Such precursors, which are $CD11c^{int}$ and MHC II- can give rise to the 3 splenic cDC subsets, which are all CD11c+ and MHC II+ (like the stably transduced DC that we have analysed). The next interesting step will be for us to characterise the stably transduced cells further to see if one or more DC subsets are modified.

Another enigma is the continued survival of the transgene expressing DC / DC precursors, when they should be subject to CTL killing as is normally the case (Hermans et al., 2000). Possible explanations are the expression of a survival gene (see above), or perhaps CTL are deleted / anergic or ignorant due to lack of danger signals/ limited antigen presentation. There was a lower % of transduced DC in the Iova_E vector

injected mice, compared to the GFP vector injected mice (see figures 4.8, 4.9, 4.10 and 4.12), suggesting that endogenous and/ or transferred T cells were killing DC in the Iova_E mice.

We cannot at this time conclusively prove that these stably modified DC are presenting antigen *in vivo* because although transferred OT-I T cells expanded in the Iova_E mice, results could be complicated by circulating endogenous OVA specific CD8+ T cells; the endogenous population cannot normally be detected without boost vaccinations, but their presence cannot be ruled out. Experiments are ongoing to resolve this matter by repeating the one month and two month time-points with and without adoptive transfer.

Another idea is to transfer the isolated DC containing fraction into naïve mice (plus OT-I transfer) or into OT-I transgenic mice to see if they can stimulate OVA specific CD8+ T cells; this will complement the experiments of culturing the isolated fractions with T cells *in vitro* that we did here. Finally, we plan to look at the activation status of the modified DC; OT-1 cells can expand without co-stimulation (Heath et al., 2004; Storni et al., 2003) so our results do not inform us of the phenotype of the presenting DC. It will be useful to test the effector function of expanded OT-I's in parallel (e.g. by intracellular cytokine staining). Injecting an adjuvant at the same time or just after T cell transfer may boost T cell responses; inactivated influenza virus is a potent pDC activator (O'Keeffe et al., 2002) and CpG activates all DC populations.

The question as to the effect of stably presenting DC on immunity is still puzzling. The most likely consequence is the induction of tolerance to the encoded antigen, since the presenting DC should not be activated; otherwise they would be programmed to die (Kamath et al., 2000). However, the finding that mice can mount recall CD8+ T cell responses to the transgene one year post lentivector injection (see chapter 3) suggests the opposite and so this question remains unanswered.*

Our results and ongoing research in this area will have important implications in the design of lentivirus-based cancer vaccines. Also, gene therapists should take measures

* It would be informative to do in vivo killing assays or to challenge mice with tumours at later time-points, to investigate this question further.

to avoid transduction of DC and their precursors in spleen when injecting vectors i.v., to avoid unwanted transgene responses (Follenzi et al., 2002). Alternatively, injection of lentivectors i.v. could prove an ideal way to induce tolerance, provided that the vectors are prepared to minimise any DC activation.

Previous data from our lab has shown that i.v. injected lentivectors induce transgene specific CTL and CD4+ T cell responses and protect mice from tumour challenge (Palmowski et al., 2004; Rowe et al., 2006); the i.v. route is, therefore, immunogenic rather than tolerogenic in this system. We speculate that the persistence of vector modified DC may render lentiviral vectors more potent cancer vaccines than those in use, by preventing tumour out-growth, especially if stably modified DC can be repeatedly mobilised by injecting adjuvants. It could also be possible to target the vector (by transcriptional or surface targeting) to DC or particular DC subsets / precursors that could be activated by specific adjuvants. This novel vaccine approach may prove effective in other chronic diseases such as HIV infection, as well as against cancer.

Chapter 5: Expressing a DC activator in the lentivector to enhance vaccine efficacy

5.1 Introduction

5.1.1 Developing the ideal vector vaccine

It is clear from cancer immunotherapy trials that the ideal vector vaccine is not currently in use, although it is hopefully within reach (reviewed in (Breckpot et al., 2004; Collins and Cerundolo, 2004)). One major difficulty has been to develop a potent vaccine vector that will both activate dendritic cells (DC) *in vivo,* and direct a type 1 adaptive immune response solely towards the antigen(s) expressed, and not against the vector. Repeat immunisations of vector could then be administered without the problem of neutralising antibodies (Breckpot et al., 2004; Rosenberg et al., 1998b), or immunodominance of anti–vector T cells over transgene-specific T cells (Smith et al., 2005b). Instead of loading DC with antigens *in vitro*, the vector could be manipulated to deliver antigen genes specifically to DC (through surface or promoter targeting (Bonifaz et al., 2004; Bonkobara et al., 2001; Carter et al., 2006)) *in vivo*.

Achieving this goal could provide a breakthrough in therapeutic vaccines not only against cancer, but also against chronic infectious diseases in which pathogens (such as hepatitis B and C viruses, HBV / HCV, or human papillomaviruses, HPV) successfully evade immunity. Among the candidate viruses from which to develop such a vaccine, lentivirus-based vectors offer some advantages. Importantly, they do not block DC maturation (as in the case of herpes simplex virus, HSV (Salio et al., 1999)) or activate DC to present primarily vector peptides (because unlike adenovirus, AdV and vaccinia virus, VV vectors they do not express viral proteins (Naldini et al., 1996; Zufferey et al., 1997)). There is also the interesting prospect of stably modified DC persistently presenting the antigen of choice (see chapter 4 for preliminary data on this topic).

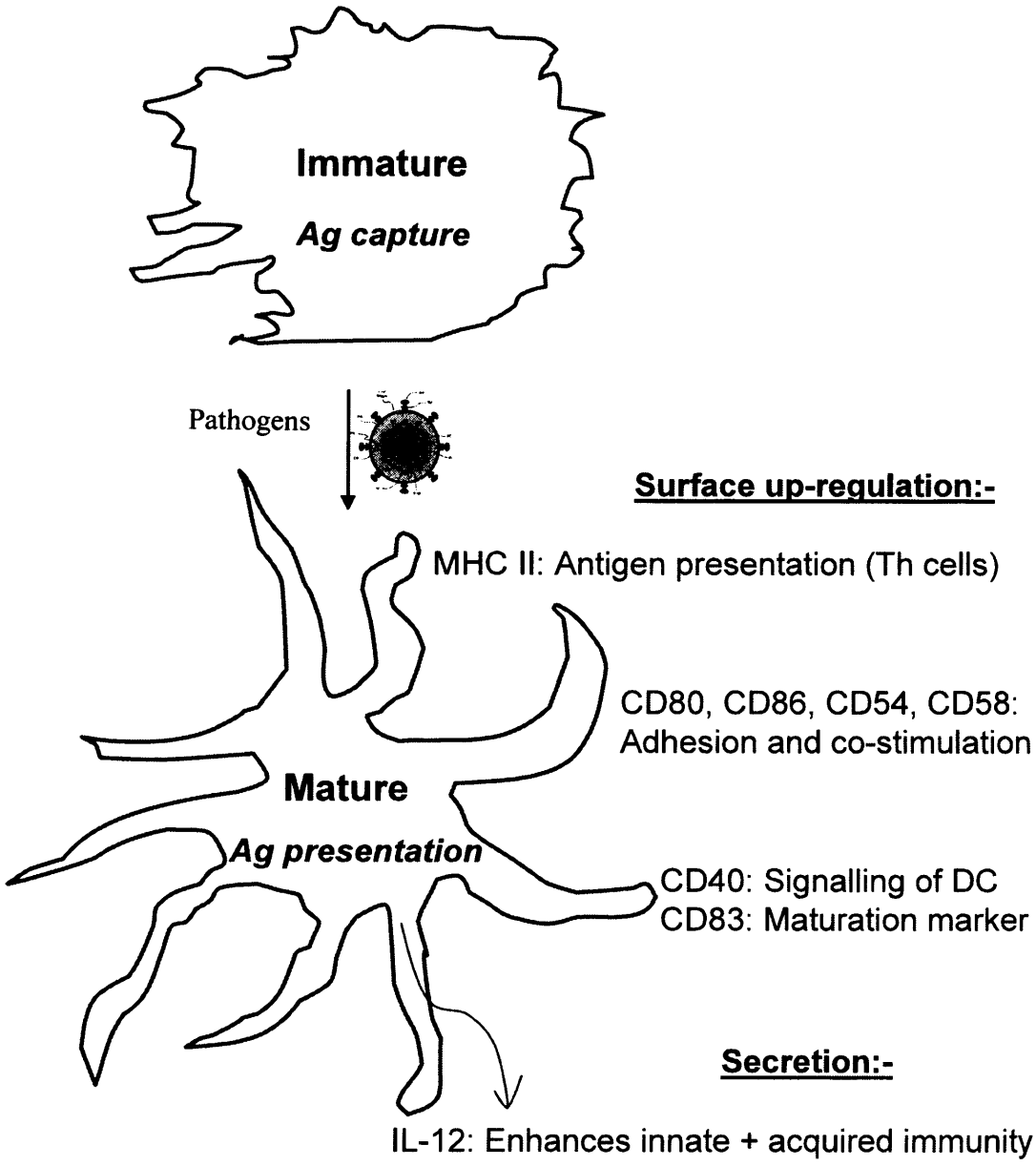
However, development of such an appealing vaccine vector relies on activating DC *in vivo*. Lentiviral vectors (lentivectors) are predominantly used in gene therapy, the main attraction being their ability to integrate into the genome, but also because they do not mature dendritic cells (Breckpot et al., 2003; Esslinger et al., 2002; Veron et al., 2006); Indeed, there is some interest into their use for tolerance induction (Veron et al., 2006). Yet our immunisation results (see chapter 3) clearly show that lentivectors can be immunogenic. In agreement with our results, mounting data on viral recognition pathways suggests the consensus view, that lentivectors do not activate DC to be outdated. One group reported that human DC were activated by lentivectors *in vitro* (Tan et al., 2005), although an extremely high MOI (500) was used. Another study showed that lentivectors cause downregulation of co-stimulatory markers on human DC (Chen et al., 2004). Possible activation effects of the lentivector on DC are discussed in chapter 3.

However, the fact remains that in comparison to other viral vectors (based on vaccinia, measles, herpes simplex or adenoviruses (Klagge et al., 2004; Thomas et al., 2003)), lentiviral vectors exert little effect on DC maturation. The aim of the project described in this chapter, therefore was to introduce a DC activator into the lentivector to enhance vaccine efficacy.

5.1.2 DC maturation

Dendritic cells bridge the innate and adaptive immune systems through innate sensing of pathogens, and antigen presentation and provision of activation signals to naïve T cells. Immature DC exist as different subtypes (see chapter 4 and (Heath et al., 2004)) and since their role is to capture antigens, it follows that they are located at sites of pathogen entry, such as in the skin (Langerhans' cells), or at sites where antigens are trapped (plasmacytoid DC in spleen). According to the Langerhan cell paradigm, DC capture antigens and then mature and migrate to lymph nodes, where they prime naïve T cells (Banchereau and Steinman, 1998). The main changes that DC undergo to become mature are shown in figure 5.1.

Figure 5.1

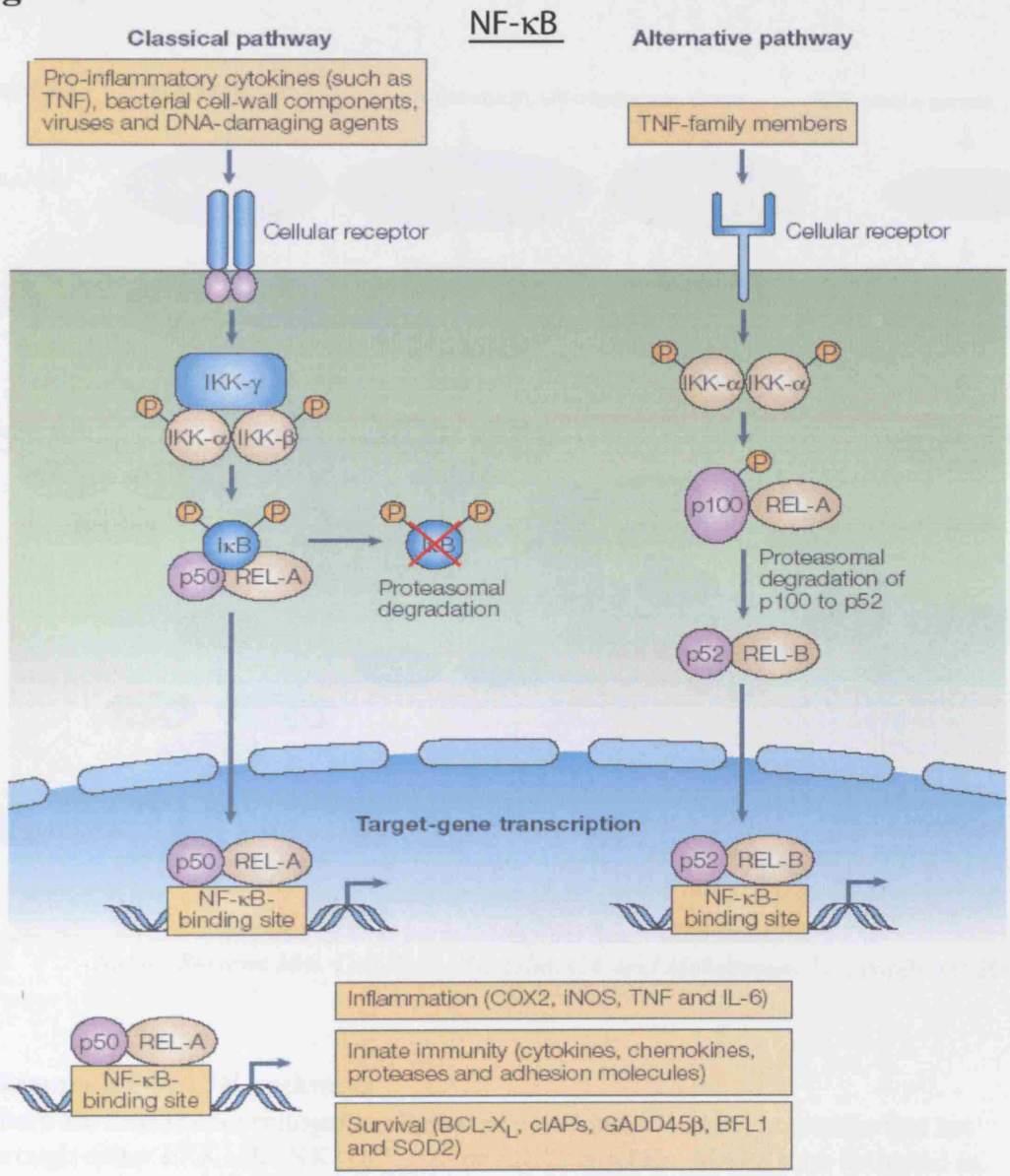


Characteristics of DC maturation. Immature dendritic cells, which are unable to stimulate naive T cells, are specialised in antigen (Ag) capture; once they encounter a danger signal (e.g. pathogens or pro-inflammatory cytokines), they undergo maturation, migrating to secondary lymphoid organs to stimulate adaptive immune responses. Information from: *Banchereau and Steinman, 1998* was used to construct this figure.

DC are the sentinels of the immune system, maintaining tolerance in the absence of "danger" (Steinman and Nussenzweig, 2002), and controlling the type of adaptive immune response that results (through their interaction with T helper cells), upon sensing "danger"(Matzinger, 2002).

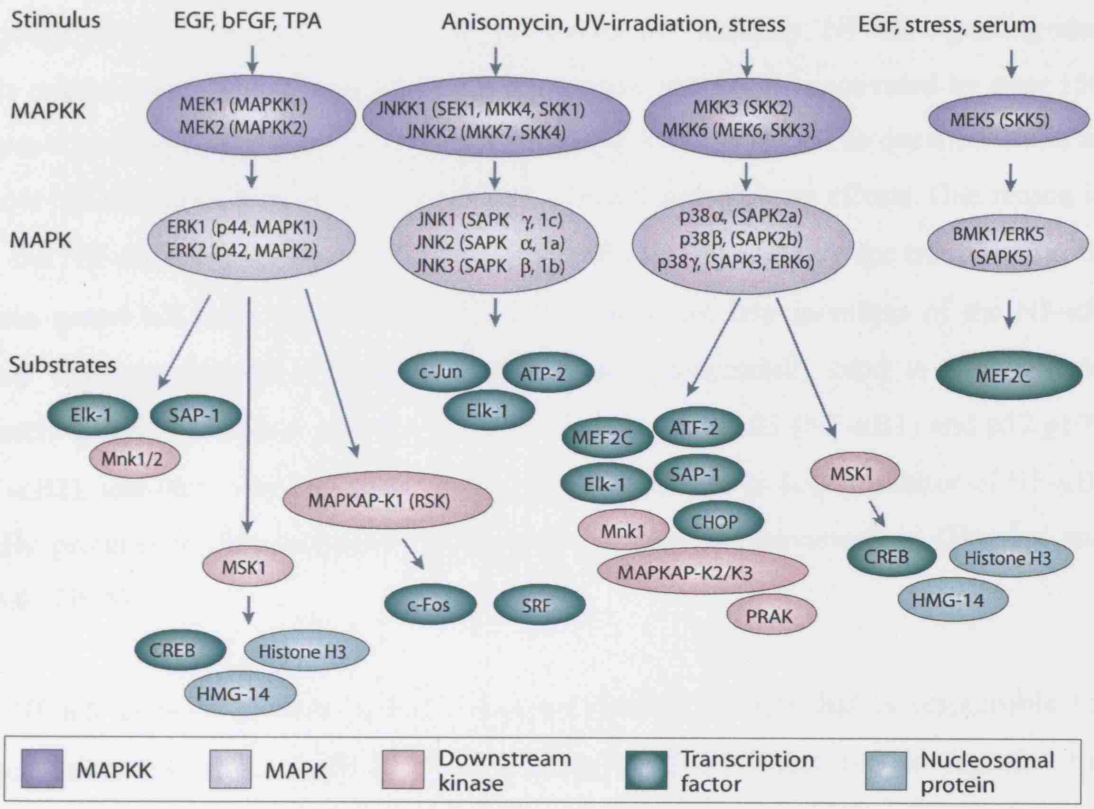
The minimal signaling pathways required to induce DC maturation are still being elucidated. However, it is known that LPS (lipopolysaccharide, from Gram negative bacterial cells walls), which fully matures DC within a day, activates both the nuclear factor (NF)-κB transcription factor and mitogen-activated protein kinase (MAPK) cascades (p38, ERK and JNK) (Hofer et al., 2001; Rescigno et al., 1998). These pathways are described in figures 5.2 and 5.3 respectively.

Figure 5.2



NF-κB is activated through either the classical or the alternative pathway.
The classical pathway, triggered by LPS, for example, involves the phosphorylation of IκB (the inhibitor of (NF)-κB) by IKK-β (inhibitor of κB kinase β), after activation of the IKK complex. Phosphorylated IκB is degraded by the proteasome, which releases NF-κB dimers (mostly of p50-RelA) that translocate to the nucleus and activate genes through κB binding sites. Some of the consequences of the classical pathway are described in the boxes.
The alternative pathway, triggered by certain TNF-family members, involves IKK-α dependent phosphorylation of p100 that induces degradation of its C-terminal half, releasing p52-RelB dimers.

Figure 5.3



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Mammalian MAPK pathways

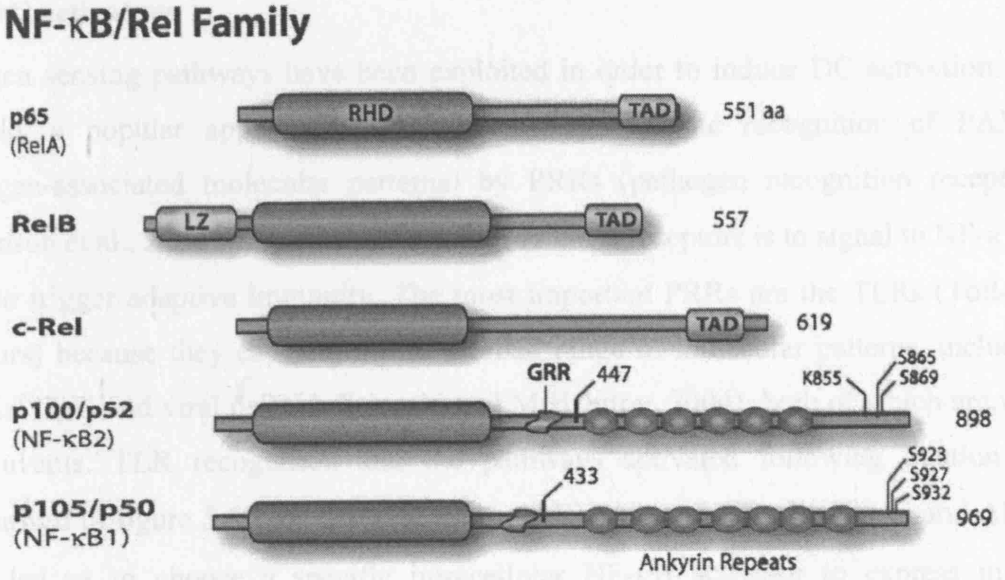
There are four known mitogen-activated protein kinase (MAPK) cascades that act through either ERK1/2, JNK1/2/3, p38 or ERK5 MAPKs. MAPKs are activated in the cytoplasm in response to stress signals and they translocate to the nucleus to modulate gene expression, through activation of transcription factors, for instance (e.g. c-jun and c-fos). However, MAPKs also act on cytoplasmic targets to exert post-transcriptional regulation. In each cascade, members of the MAPKK (MAPK kinase) family phosphorylate a MAPK, which activates its target substrates. In fact the MAPKKs depend on activation by MAPKKKs that are initially activated by small GTP-binding proteins for instance. The effects of MAPK cascades are diverse and include roles in cell proliferation, cell survival and in the immune response.

5.1.3 The NF-κB transcription factor

Although its most significant role is in the control of immunity, NF-κB signaling also plays critical roles in development and cell survival; indeed, it is activated by over 150 known stimuli and modulates over 150 target genes (Pahl, 1999). The question arises as to how NF-κB gene modulation is regulated to exert such diverse effects. One reason is that the NF-κB response is cell type specific; NF-κB is necessary for transcription of certain genes but may not be sufficient. Also, there are five members of the NF-κB family that can respond to different stimuli and preferentially bind to and activate different genes. These are: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), and they exist as homo- or heterodimers, bound to IκB (inhibitor of NF-κB) family proteins in the cytoplasm, when they are inactive (reviewed in (Hayden and Ghosh, 2004)).

All NF-κB proteins possess a Rel homology domain (RHD) that is responsible for dimerization, interaction with IκB and binding to DNA. When IκB is degraded the dimers can translocate to the nucleus and act on target genes. Figure 5.4 shows a diagram of the NF-κB proteins. Some target genes activated are: chemokines, cytokines, adhesion molecules and co-stimulatory molecules.

Figure 5.4



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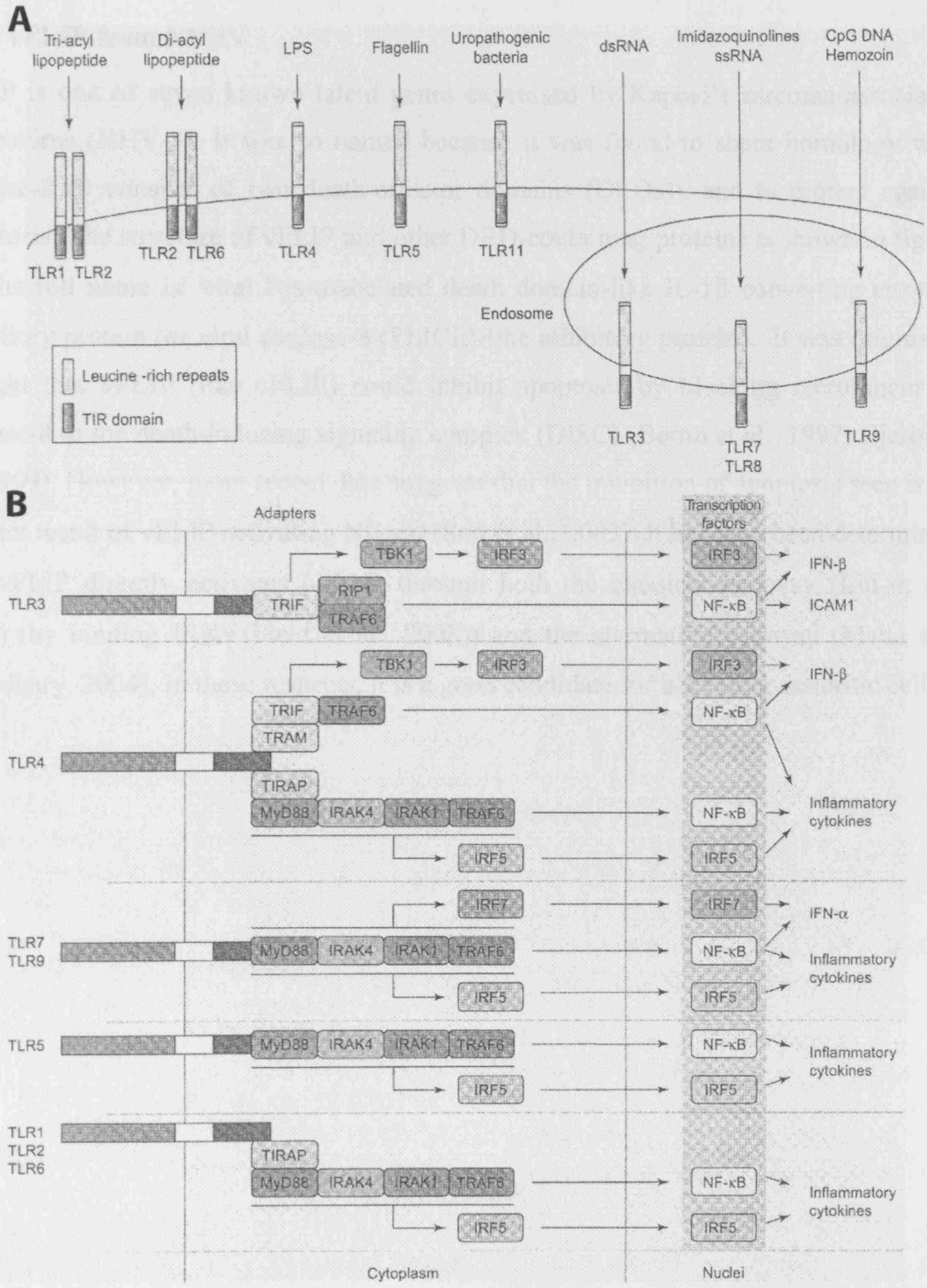
Members of the NF-κB protein family

All the members have a Rel homology domain (RHD) that includes the nuclear localization signal (NLS), as well as DNA binding and dimerization domains; IκB binds this region to sequester NF-κB in the cytoplasm. However, NF-κB1 and NF-κB2 exist as p100 and p105 precursors respectively that contain IκB activity, due to the ankyrin repeats (these motifs are conserved throughout the IκB family). Therefore, p52 and p50 are released by respective p100 or p105 processing (the C-terminal is degraded), whereas RelA, RelB and c-Rel are released, following phosphorylation and degradation of IκB. The NF-κB proteins can form homodimers or heterodimers that direct different outcomes. RelB does not homodimerize - it partners with p52 or p50, while RelA usually partners with p50. TAD, transactivation domain. LZ, leucine zipper.

5.1.4 DC activators

Pathogen sensing pathways have been exploited in order to induce DC activation. For example, a popular approach is to mimic natural innate recognition of PAMPs (pathogen-associated molecular patterns) by PRRs (pathogen recognition receptors) (Stevenson et al., 2004). One result of ligation of these receptors is to signal to NF-κB in order to trigger adaptive immunity. The most important PRRs are the TLRs (Toll-like receptors) because they can respond to a broad range of molecular patterns, including bacterial CpG and viral dsRNA (Iwasaki and Medzhitov, 2004), both of which are used as adjuvants. TLR recognition and the pathways activated following ligation are summarised in figure 5.5. The fact that every TLR signals to NF-κB (Kawai and Akira, 2005) led us to choose a specific intracellular NF-κB activator to express in the lentivector.

Figure 5.5



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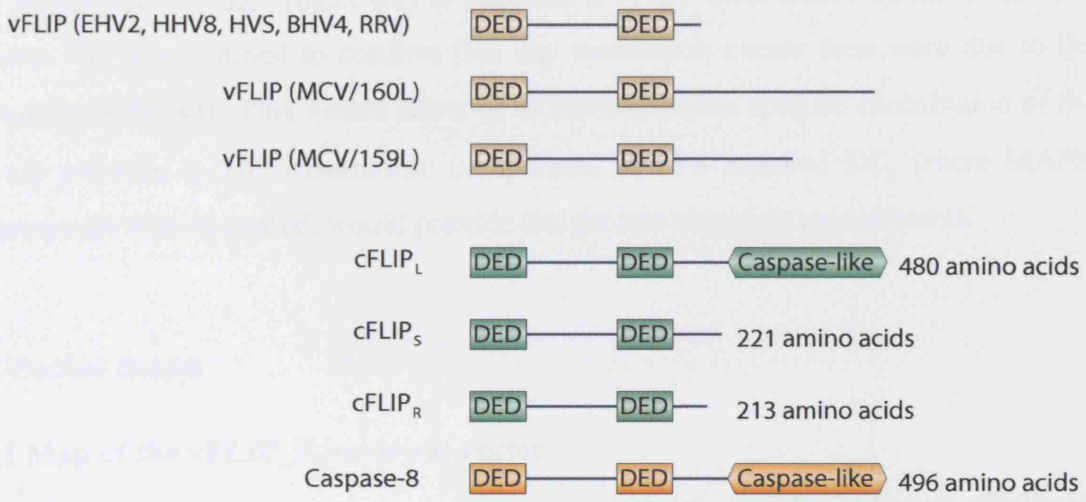
TLR ligation (see A)) leads to signaling through NF-κB (shown in B)).

5.1.5 vFLIP from KSHV

vFLIP is one of seven known latent genes expressed by Kaposi's sarcoma-associated herpesvirus (HHV-8). It was so named because it was found to share homology with caspase-8 (it consists of two death-effector domains (DEDs)), and to protect against apoptosis. The structure of vFLIP and other DED-containing proteins is shown in figure 5.6. Its full name is: viral Fas-associated death domain-like IL-1β converting enzyme inhibitory protein (or viral caspase-8 (FLICE)-like inhibitory protein). It was originally thought that vFLIP (like cFLIP) could inhibit apoptosis by blocking recruitment of caspase-8 to the death inducing signaling complex (DISC) (Bertin et al., 1997; Djerbi et al., 1999). However, more recent data suggests that the inhibition of apoptosis seen is an indirect result of vFLIP activating NF-κB (Sun et al., 2003). It has now been determined that vFLIP directly activates NF-κB through both the classical pathway (Liu et al., 2002) (by binding IKKγ (Field et al., 2003)) and the alternative pathway (Matta and Chaudhary, 2004). In these respects, it is a good candidate for activating dendritic cells.

Figure 5.6



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vFLIP and proteins it shares homology with

vFLIP (viral caspase-8 (FLICE)-like inhibitory protein) contains 2 death-effector domains. vFLIPs are expressed by γ-herpesviruses, including KSHV (HHV8), equine herpesvirus-2 (EHV-2), herpesvirus saimiri (HVS), bovine herpesvirus 4 (BHV4) and rhesus rhadinovirus (RRV). The other variants are expressed by MCV (human molluscipoxvirus). Cellular FLIP (cFLIP) blocks recruitment of caspase-8 to the DISC (the death inducing signalling complex) thereby inhibiting apoptosis; it can also activate caspase-8 and directly activate NF-κB. Therefore, it functions to control cell proliferation and death. The long variant of cFLIP (cFLIP$_L$) contains an inactive caspase-like domain. The short variant (cFLIP$_S$) and a variant cloned from the Raji B cell line (cFLIP$_R$) lack this caspase-like domain. vFLIP from KSHV directly activates NF-κB through both classical and alternative pathways but it has not been proven to inhibit caspase-8 mediated apoptosis in the same way as cFLIP.

5.2 Aims

The primary aim of the project was to establish if vFLIP from KSHV could mature DC *in vitro*. We also wanted to confirm that any maturation events seen were due to the activation of NF-κB. This would allow us to investigate the specific contribution of the NF-κB pathway to DC maturation; comparison to LPS matured DC, where MAPK pathways are also activated, would provide insight into signaling requirements.

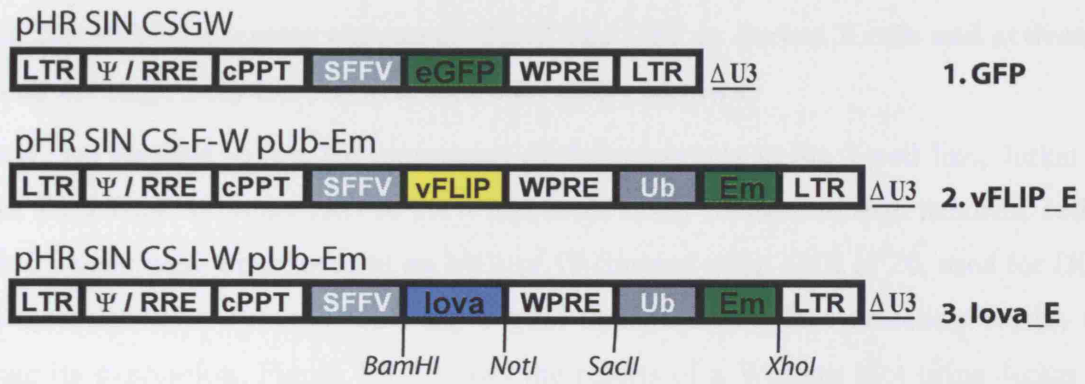
5.3 Vector maps

5.3.1 Map of the vFLIP_E lentiviral vector

Figure 5.7 shows the map of the dual promoter vector, into which vFLIP was subcloned. vFLIP was inserted under the stronger SFFV promoter, and emerald GFP, expressed from the weaker human polyubiquitin C (Ub) promoter, since GFP was only used as a marker for transduction. We knew from the results of chapter 4 that the Ub promoter works well in murine dendritic cells, although GFP fluorescence is about one log lower, compared to GFP expression driven by SFFV, as seen by the MFI (mean fluorescence intensity). vFLIP (567bp) was amplified by PCR from the pcDNA3.1 vector plasmid, using primers designed to introduce a *Bam HI* and a *Not I* site at the 5′ and 3′ ends respectively. The vFLIP dual promoter vector is referred to as the vFLIP_E vector. The Iova_E vector (expressing an ovalbumin construct and described in chapter 4) was used as a control in most experiments (or sometimes the GFP vector) to rule out any chance that activation effects seen could be due to the vector or GFP expression.

During vector production, 293T cell supernatants were filtered through 0.2μm filters (instead of 0.45μm filters as was the case in chapter 3). This was to obtain cleaner vector preparations to reduce any possible activation of DC, due to contaminating proteins e.g. VSV-G structures. Also, vector pellets were always washed in 20ml of HBSS in between the 1st and 2nd round of ultracentrifugation, to remove FCS and any transgene proteins in the prep (see chapter 3, figure 3.4).

Figure 5.7



A dual promoter lentiviral vector was constructed in order to express vFLIP and detect transduction. The vFLIP_E vector (2.), used to activate NF-κB, expresses vFLIP (567 bp) and GFP emerald (Em, 780 bp). The original GFP vector (1.), or a vector expressing the invariant chain-ovalbumin fusion (Iova, 1,658 bp), (see chapter 3) plus Em (3.), were used as controls. Unique restriction sites are shown. LTR, long terminal repeat; Ψ , packaging signal; RRE, rev response element; cPPT, central polypurine tract; SFFV, spleen focus forming virus promoter; WPRE, wood chuck post transcriptional regulatory element; Ub, ubiquitin promoter; ΔU3, deletion in U3 region (SIN vector).

5.4 Transgene expression *in vitro*

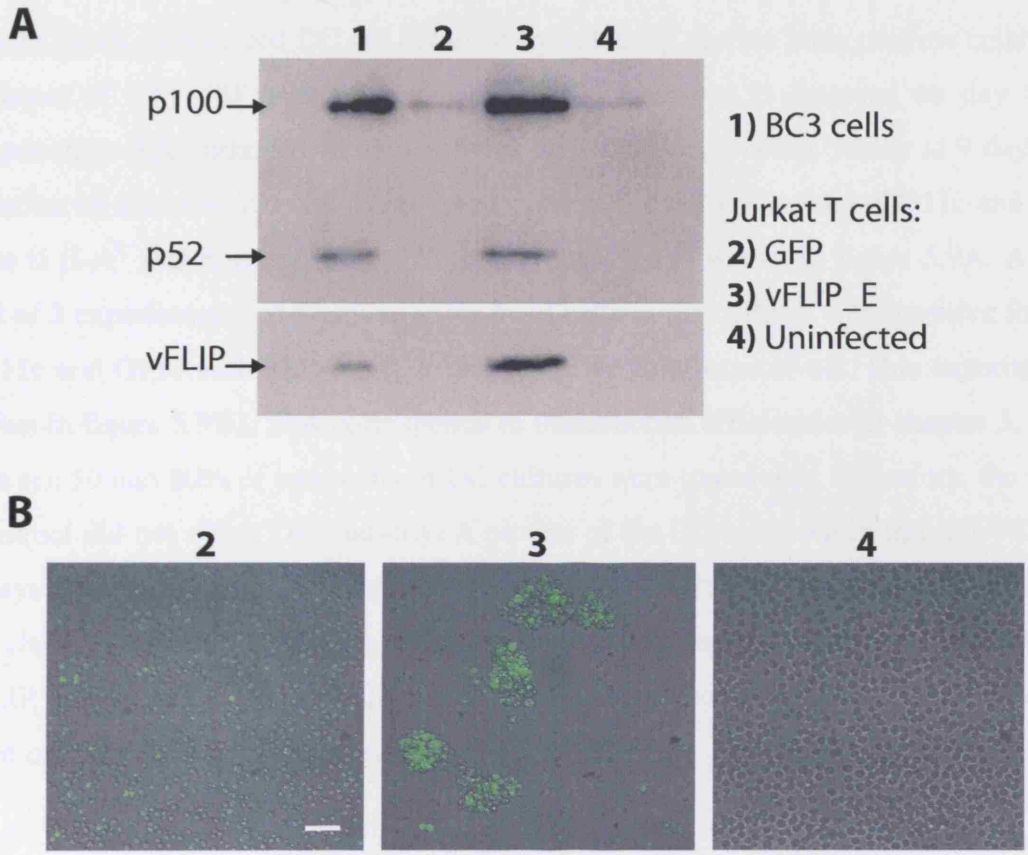
5.4.1 The vFLIP_E vector expresses vFLIP and GFP in Jurkat T cells and activates NF-κB through both the classical and alternative pathway

Firstly, we decided to test the expression of the constructs in the T cell line, Jurkat T cells, since these cells are easy to grow and more easily transduced than dendritic cells. Jurkat T cells were transduced at an MOI of 10 (instead of an MOI of 20, used for DC) and the cells were cultured until day 5 post transduction before detecting vFLIP, to ensure its expression. Figure 5.8A shows the results of a Western blot using Jurkat T cell lysates. vFLIP is strongly expressed in the vFLIP_E LV transduced cells as shown in the bottom panel. vFLIP was not detected in the control groups of Jurkat T cells that were uninfected or transduced with the GFP vector, but it was detected in the positive control (BC3) cells, which are a KSHV infected primary effusion lymphoma (PEL) line, expressing the latent proteins vFLIP, vcyclin and LANA.

It was also determined from the Western that both p100 and p52 were up-regulated in vFLIP_E LV transduced cells (see upper panel) and in BC3 cells, but not in the control samples. This data suggests that vFLIP was activating NF-κB in these cells, through both classical and alternative pathways, since p100 is induced by the classical pathway and processed to release p52 in the alternative pathway. However, it can't be ruled out that excessive accumulation of p100 might automatically lead to its processing and that vFLIP might only be driving the classical pathway.

Pictures of the cells that were taken using a confocal microscope are shown in figure 5.8B. The vFLIP_E LV transduced Jurkat T cells (identified by GFP expression), were clumped, suggesting that they were activated; NF-κB is known to activate adhesion molecules such as ICAM-1 (van de Stolpe et al., 1994). The control Jurkat T cells that were uninfected or transduced with the GFP vector were not clumped.

Figure 5.8

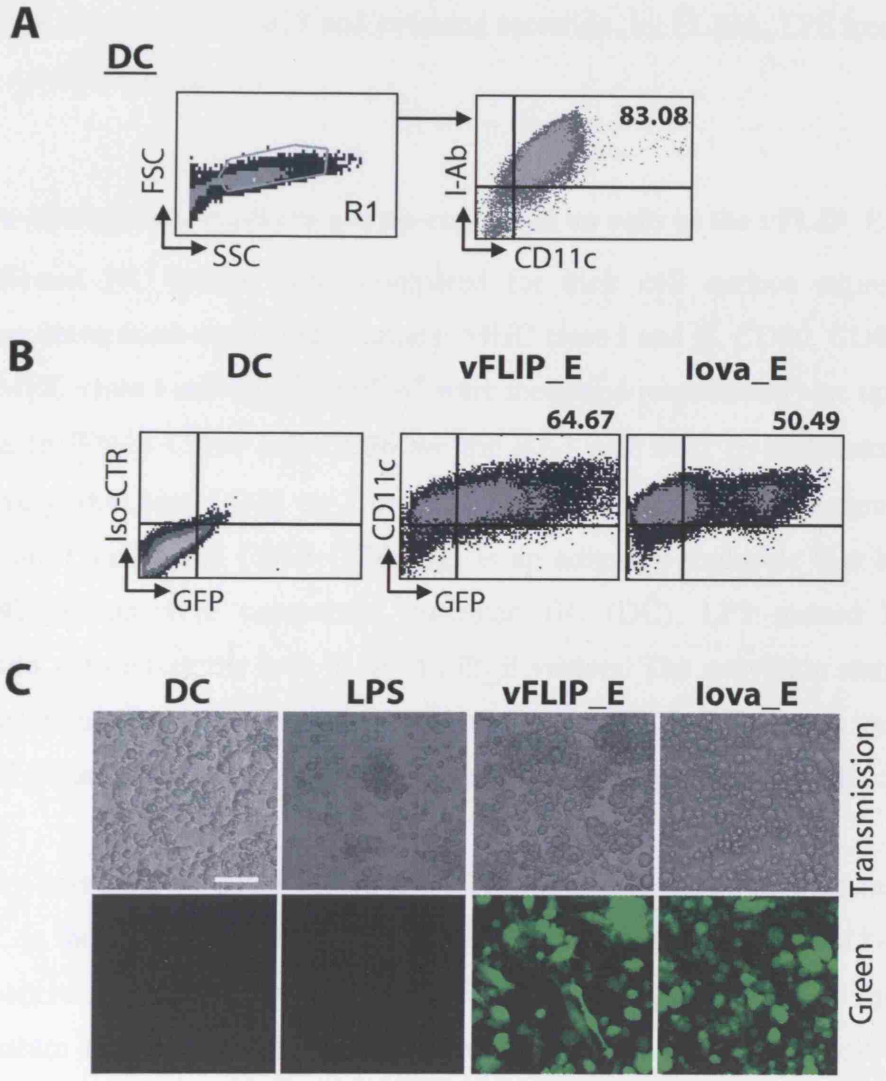


The vFLIP_E LV activates NF-κB in Jurkat T cells through both the classical and alternative pathways. Jurkat T cells were infected with either the GFP or vFLIP_E vectors at an MOI (multiplicity of infection) of 10, or left uninfected. **A)** At day 5 post infection, $2x10^6$ cells from each group were lysed in 500µl RIPA buffer and lysates (after boiling with sample buffer) were separated by SDS-PAGE. BC3 cell lysate (KSHV infected primary effusion lymphoma, PEL) was run as a positive control. Samples were duplicated so that one gel could be probed with an αp100/p52 antibody (Ab) (upper western) and the other probed with an αvFLIP Ab (lower western).vFLIP, 23 kDa. **B)** The same jurkat T cell cultures were analysed for GFP expression at day 6 post infection, using a confocal microscope. The scale bar represents 100µm.

5.4.2 BM-derived DC are efficiently transduced with the vFLIP_E vector.

A high purity of myeloid DC are obtained by culturing murine bone marrow cells in the presence of GM-CSF (see methods). The DC phenotype is assessed on day 5 post transduction (also referred to as day 5 for non-transduced cells), which is 9 days post isolation of the bone marrow cells. Most cells in the culture express CD11c and MHC class II (I-A^b was measured) by this time point. This is shown in figure 5.9A. A mean (out of 3 experiments) of 52.89 or 45.45 % of cells in the cultures were positive for both CD11c and GFP, with the vFLIP_E or Iova_E vectors respectively. One experiment is shown in figure 5.9B). This corresponds to transduction efficiencies in chapter 3, where between 50 and 80% of total cells in DC cultures were transduced. Therefore, the vFLIP construct did not affect DC viability. A picture of the DC is shown in figure 5.9C taken 6 days post transduction. DC treated overnight with LPS (this group is called, "LPS") are clumped, due to activation, but untreated DC (referred to as "DC") are not. The vFLIP_E expressing DC look slightly clumped, compared to the Iova_E expressing DC; most cells are GFP+ in both transduced DC cultures.

Figure 5.9



Bone marrow (BM)-derived DC are transduced *in vitro* with similar efficiencies, using either the vFLIP_E or Iova_E vectors. Transduction of BMDC was at MOI - 20 (see methods). **A)** DC phenotype was assessed by FACS, after 5 days. Live cells (uninfected) were gated (left-hand panel) and DC purity assessed (right-hand panel). The % of cells in the culture that were double+ for MHC II (I-Ab) and CD11c is shown. (50,000 events recorded per sample). **B)** GFP expression, 5 days post transduction. Live cells were gated (not shown) and the left panel shows uninfected DC, stained with an isotype control Ab (Iso-CTR) and the right-hand panels show transduced DC stained for CD11c. Numbers show the % of cells that are double+ after background from the Iso-CTR is subtracted. (80,000 events recorded per sample). The mean % of transduced DC was 52.89 for vFLIP_E (standard deviation, S.D. = 10.06) and 45.45 for Iova_E (S.D.= 4.44), out of 3 experiments. **C)** DC phenotype and GFP expression were also measured, using a confocal microscope, 6 days post transduction (one day post LPS treatment). The scale bar represents 50μm.

5.5 vFLIP activates BM-derived DC

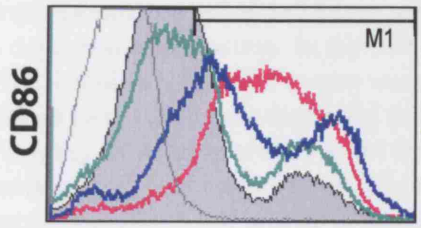
The effect of vFLIP on DC maturation was assessed by measuring up-regulation of co-stimulatory markers, by FACS and cytokine secretion, by ELISA. LPS treated DC were used as a positive control.

5.5.1 Co-stimulatory markers are up-regulated on cells in the vFLIP_E DC group

The different DC groups were compared for their cell surface expression of the following maturation-associated markers: MHC class I and II, CD80, CD86, CD40 and CD54. MHC class I and II (K^b and I-A^b were measured respectively) are up-regulated in response to IFN-γ; CD80 and CD86 are the B7-1 and B7-2 co-stimulatory molecules respectively, that bind CD28 on T cells; CD40 delivers its activation signal by binding CD40L on T cells, and CD54 (ICAM-1) is an adhesion molecule that binds LFA-1. Four DC groups were compared: Untreated DC (DC), LPS treated DC and DC transduced with either the Iova_E or vFLIP_E vectors. The activation status of the DC was assessed on day 5 post transduction (because the vFLIP_E DC were found to be less activated at earlier time points) and the results are displayed in figure 5.10.

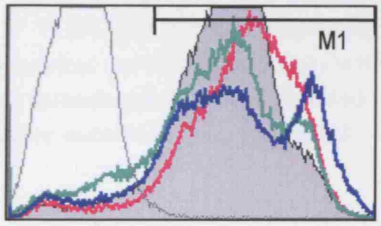
For every maturation marker assessed, DC in the vFLIP_E group were more activated than DC in the untreated and Iova_E groups, as shown by a shift in the % of cells that were positive for the marker, or in the MFI. The LPS treated DC often appeared to be more mature than the vFLIP_E DC e.g. a higher % of cells were positive for CD86 (88.15% vs. 71.88%), although the MFI was higher in the vFLIP_E group (729.72 vs. 502.28). It appeared that for some markers (such as CD86), there was a subpopulation of cells in the vFLIP_E group that were highly expressing the marker; the LPS DC in comparison were uniformly matured. It was therefore of interest to ascertain whether this subpopulation of activated cells was the vFLIP expressing ones in the culture. Therefore, we decided to analyse all the data by density plots and summarise 3-4 experiments per marker, to calculate whether differences between DC groups and between the transduced and non-transduced populations within groups were significant or not.

Figure 5.10



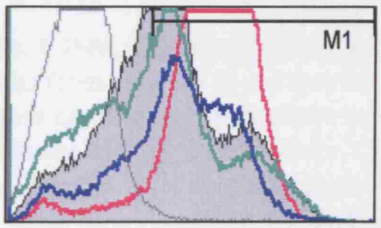
CD86

Marker	% Gated	Mean	Marker	% Gated	Mean
M1 DC	28.89	380.06	M1 Iova_E	42.18	440.36
M1 LPS	88.15	502.28	M1 vFLIP_E	71.88	729.72



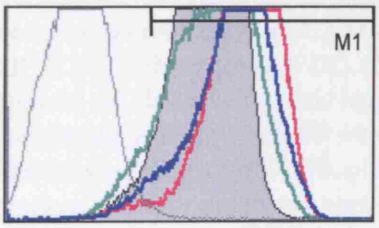
MHC II

Marker	% Gated	Mean	Marker	% Gated	Mean
M1 DC	92.01	471.43	M1 Iova_E	82.05	556.38
M1 LPS	93.00	666.47	M1 vFLIP_E	87.37	954.70



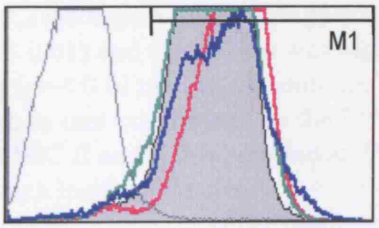
CD40

Marker	% Gated	Mean	Marker	% Gated	Mean
M1 DC	58.09	245.26	M1 Iova_E	50.99	267.44
M1 LPS	92.03	260.83	M1 vFLIP_E	69.69	200.00



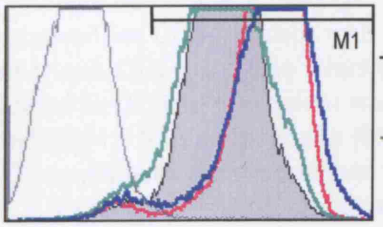
MHC I

Marker	% Gated	Mean	Marker	% Gated	Mean
M1 DC	94.30	210.08	M1 Iova_E	90.10	282.66
M1 LPS	97.33	489.58	M1 vFLIP_E	94.88	427.71



CD80

Marker	% Gated	Mean	Marker	% Gated	Mean
M1 DC	95.00	234.89	M1 Iova_E	90.85	259.81
M1 LPS	96.77	347.39	M1 vFLIP_E	91.67	350.94



CD54

Marker	% Gated	Mean	Marker	% Gated	Mean
M1 DC	97.19	334.76	M1 Iova_E	93.04	388.49
M1 LPS	96.84	852.26	M1 vFLIP_E	95.56	1077.30

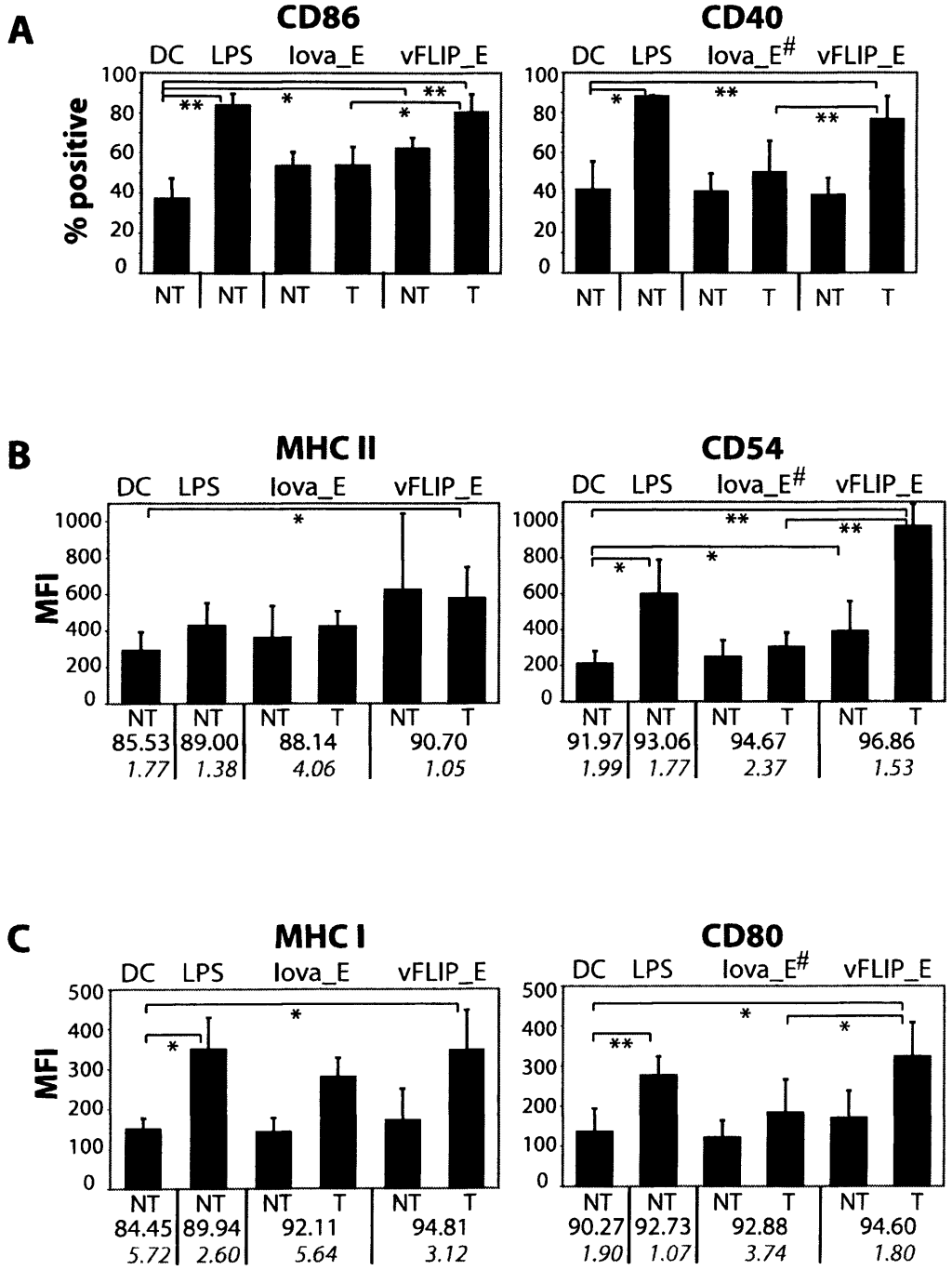
Figure 5.10

vFLIP_E LV activates DC by up-regulating co-stimulatory molecules. BMDC were transduced at MOI- 20 (see methods) and DC maturation was assessed by FACS, 5 days post transduction. In the LPS group, LPS (50ng/ml) was added one day before FACS analysis. 100,000 events were recorded per sample. Live cells were gated (not shown) and results are displayed as histograms. A sample stained with an isotype CTR Ab (grey outline) was used to set the gate of the % of cells + for each co-stimulatory marker (M1, % gated). The mean shows the mean fluorescence intensity within the gated population (all results to 2 decimal places). Untreated DC (DC) are shown in grey filled histograms, LPS treated DC are in pink, Iova_E DC are in green and vFLIP_E DC are in blue. One experiment shown. Results were repeated 3-4 times for each marker (at this time point) with similar results. FACS was also done at day 3 and 4 post transduction, which showed that vFLIP_E DC were less mature than on day 5 but more activated than untreated DC.

Figure 5.11

CD86, CD40, CD54 and CD80 are upregulated on vFLIP expressing BMDC Results from figure 4 (3-4 experiments) were analysed using density plots to find out whether co-stimulatory markers were up-regulated on transduced cells only, or on all cells in the vFLIP_E DC culture. Quadrant statistics (gates were set, using the isotype controls) were used to plot a summary graph for each marker showing the mean and S.D. of all experiments done. The vFLIP_E and Iova_E groups were subdivided into nontransduced (NT) and transduced (T) cells. If significant differences were found between the untreated DC (DC) and the LPS or vFLIP_E DC, and between the vFLIP_E DC and the Iova_E DC, they are shown on each graph. Any other significant differences are described in this legend. Differences were calculated using paired student *t* tests, where $p = < 0.05$ was considered significant. * $p = < 0.05$, ** $p = < 0.01$. **A)** Results for CD86 and CD40, plotted as the % of cells in each group that are + for the marker. 4 experiments are summarised for CD86, which was also slightly up-regulated on both Iova_E NT ($p = < 0.05$) and Iova_E T ($p = <0.01$) cells, compared to DC. CD86 expression was higher in the LPS group, than on vFLIP_E NT cells ($p = < 0.01$) and expression was higher on the vFLIP_E T cells, than the vFLIP_E NT cells ($p =< 0.01$). 4 experiments are summarised for CD40, although there was no LPS group in one experiment, so the LPS data is a summary of 3 experiments. **B)** Results for MHC II and CD54, plotted as MFI means, since most cells in all groups were + for each marker (the mean % + , in black, and the S.D., in grey italics, are shown under each graph). 3 experiments are summarised for MHC II, which was also slightly up-regulated on Iova_E T cells ($p = < 0.05$), compared to DC. 4 experiments are summarised for CD54, except with the LPS group, where 3 experiments are summarised. CD54 was also slightly up-regulated on Iova_E T cells ($p = < 0.05$), compared to DC and expression was higher on the vFLIP_E T cells than the vFLIP_ NT cells ($p = < 0.01$). **C)** Results for MHC I and CD80, plotted as MFI means (as in B)). 3 experiments are summarised for MHC I, which was also up-regulated on Iova_E T cells ($p = < 0.05$), compared to DC. 4 experiments (or 3 experiments for the LPS group) are summarised for CD80. # Here, 2 out of 4 experiments summarised were using the GFP instead of the Iova_E vector.

Figure 5.11


A
CD86
DC LPS Iova_E vFLIP_E
% positive
100
80
60
40
20
0
NT NT NT T NT T
CD40
DC LPS Iova_E# vFLIP_E
B
MHC II
DC LPS Iova_E vFLIP_E
MFI
1000
800
600
400
200
0
NT NT NT T NT T
85.53 89.00 88.14 90.70
1.77 1.38 4.06 1.05
CD54
DC LPS Iova_E# vFLIP_E
91.97 93.06 94.67 96.86
1.99 1.77 2.37 1.53
C
MHC I
DC LPS Iova_E vFLIP_E
MFI
500
400
300
200
100
0
NT NT NT T NT T
84.45 89.94 92.11 94.81
5.72 2.60 5.64 3.12
CD80
DC LPS Iova_E# vFLIP_E
90.27 92.73 92.88 94.60
1.90 1.07 3.74 1.80

5.5.2 CD80, CD86, CD40 and CD54 are significantly up-regulated on vFLIP expressing cells, compared to both non-transduced cells in the vFLIP_E group and transduced cells in the Iova_E group.

CD86 was found to be expressed on a subpopulation of untreated DC at the time point (day 5) analysed; it is likely that some DC start to mature due to culturing and pipetting (figure 5.10). However, the mean % of untreated DC expressing CD86 was only 37.17, compared to 84.01% after LPS maturation (see figure 5.11, significant differences were calculated using paired *t* tests and the p values are stated in the legend). Within the control vector group (Iova_E), significantly more cells in both the non-transduced and transduced populations were expressing CD86 than in the untreated DC group (53.74 and 53.93% respectively). 62.32% of non-transduced cells in the vFLIP_E group were CD86+ (which was not significantly higher than cells in the control vector group), compared to 80.53% of transduced cells in the vFLIP_E group; the latter result was significantly higher than both non-transduced, and transduced cells in the control vector group and non-transduced cells in the vFLIP_E group, while this result was not significantly different from the LPS group.

A subpopulation (41.33%) of untreated DC also expressed CD40 (see figure 5.10 and 5.11), compared to 49.97% of transduced cells in the control vector group, although this difference was not significant. In fact, CD40 was only up-regulated on transduced cells (76.58%) in the vFLIP_E group, but to a significantly lesser extent than after LPS treatment (88.03%).

Since MHC class II is expressed on DC anyway (it was present on 85 – 91 % of cells in all DC groups), up-regulation of expression was assessed by comparing the MFI values. Most differences in the MFI between groups were found not to be significant, mainly due to the variation between experiments. However, it was determined that MHC class II was up-regulated on both control vector transduced (MFI, 423.73) and vFLIP_E vector transduced cells (578.11), compared to untreated DC (290.98).

92 – 97% of cells in all DC groups were CD54+, but the MFI rose significantly after LPS treatment (from 211.29 to 599.4). CD54 was only slightly up-regulated on control vector transduced cells (303.65) and on non-transduced cells in the vFLIP_E group (388.61); both these results were significant. In contrast, CD54 was strikingly up-regulated on transduced cells in the vFLIP_E group (975.60); this result was significantly higher than untreated DC, control vector transduced cells and non-transduced cells in the vFLIP_E group, but not LPS treated DC.

MHC class I was expressed on DC in all groups (84 - 95% of cells), although the MFI significantly increased after LPS treatment (from 149.46 to 349.67). Other increases in the MFI were observed for both the control vector transduced cells (279.86) and for the vFLIP_E vector transduced cells (346.49); these two groups were not significantly different from each other. Also, the results for the LPS group were not significantly different to those for the vFLIP_E transduced population.

CD80 was expressed on 90-95% of cells in all DC groups; the MFI increased significantly (from 136.32 to 276.94) after LPS treatment. CD80 was only significantly up-regulated on transduced cells within the vFLIP_E group (323.69) and expression in this group was significantly higher than on control vector transduced cells (183.31) but not LPS treated DC.

In summary, these data suggest that vFLIP activates BM-derived DC by up-regulating the maturation-associated markers: CD86, CD40, CD54 and CD80; CD86, CD54 and CD80 expression levels were not significantly different to those on LPS treated DC. MHC class I and II were also up-regulated on transduced cells in the vFLIP_E group, but this was also the case on transduced cells in the control vector group and differences between these groups were not significant. These results suggest that the vector itself also plays a role in DC activation. CD86 was up-regulated on non-transduced cells in the control vector group, possibly owing to effects of vector particles / proteins or through a cytokine released. MHC II and CD54 (as well as CD86) were up-regulated on

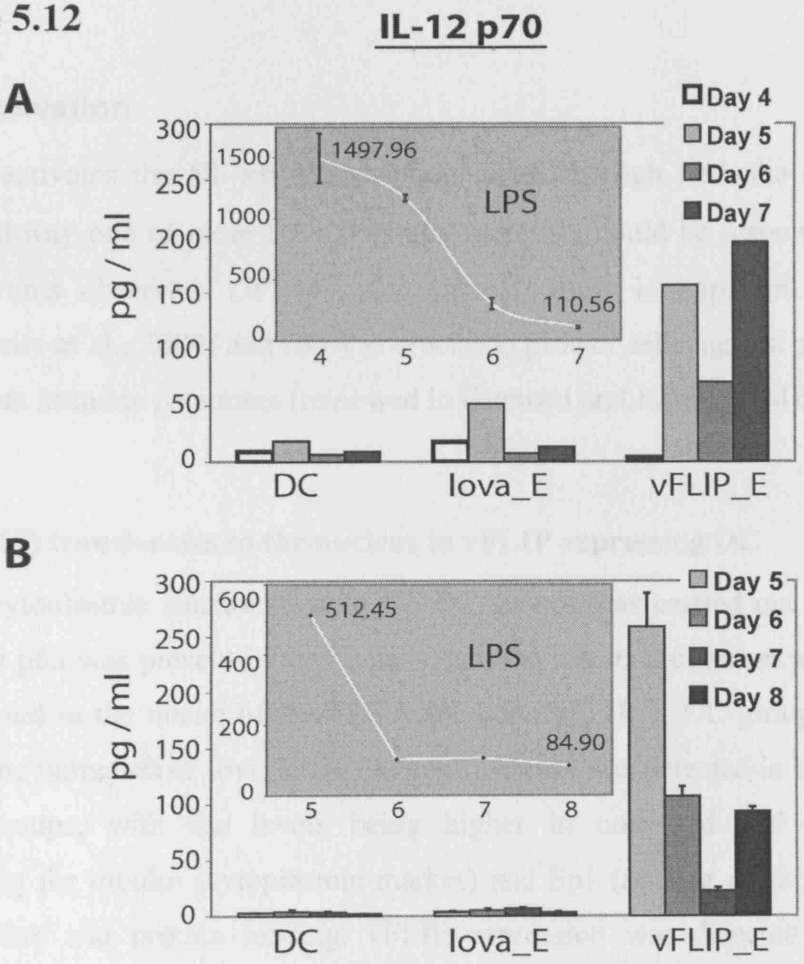
non-transduced cells in the vFLIP_E group, although differences between this group and the control vector groups were not always significant between experiments; effects could have been directed by cytokines, amounts of which could vary between experiments.

5.5.3 DC in the vFLIP_E vector culture secrete IL-12p70

It was important to look at cytokine secretion because upon pathogen sensing *in vivo,* mature DC signal to the adaptive immune system to direct differential responses: IL-12 drives differentiation of CD4+ T cells into Th1 effectors, whereas if enough IL-4 is present, Th2 development will ensue (Murphy and Reiner, 2002; O'Garra, 1998). We were interested in detecting IL-12p70 because Th1 responses are crucial to cell-mediated anti-tumour immunity (Winter et al., 2003). IL-12p70 is a heterodimeric cytokine composed of p35 and p40 chains, encoded by different genes, both of which are up-regulated by LPS (Morelli et al., 2001).

In order to measure IL-12p70, DC supernatants from the four DC groups were analysed at different time-points by sandwich ELISA (see methods) and the results (of 2 independent experiments, showing 4 time-points each) are displayed in figure 5.12. IL-12p70 was undetectable in the vFLIP_E DC supernatant on days 3 and 4 post transduction, but it was present in supernatants taken on days 5 - 9; the amount detected varied from 22 – 259 pg / ml. In comparison, the LPS treated DC secreted substantially more IL-12p70 (~500 – 1500 pg / ml, detected one day post LPS treatment). The control groups of untreated or Iova_E DC did not secrete IL-12p70, except for one supernatant sample taken (on day 5) from Iova_E DC, which contained 52 pg / ml, although this result was not repeated.

Figure 5.12



vFLIP_E DC secrete IL-12 p70 from day 5 post transduction. BMDC were transduced (see methods), or left untreated (DC) and supernatants were harvested from the cultures at day 3 - day 9 post transduction. At each time point, a separate well of DC was used because the DC did not recover, after supernatant was harvested, even if the volume was replaced with fresh medium + or - GMCSF. Supernatants were used to measure the concentration of IL-12 p70 by sandwich ELISA, using a kit (see methods). At days 3 and 4 post transduction, no IL-12 p70 was detected in any of the groups in 3 experiments done (data not shown, and see A). A and B are 2 independent experiments showing IL-12 secretion. **A)** Results for day 4 - day 7 post transduction. Bars show the mean of duplicate wells. The LPS positive CTR group is shown on a separate graph due to the difference in scale (supernatants were harvested at the same time-points as the other groups, as shown on the *x* axis). LPS was added on day 3 and IL-12 was measured in triplicate wells (mean and error bars shown, plus data values for day 4 and 7). **B)** Results for day 5 - 8. Bars show the mean and error bars of triplicate wells. The LPS CTR is displayed as in A), except that data points show the mean of duplicate wells and LPS was added on day 4.

5.6 NF-κB activation

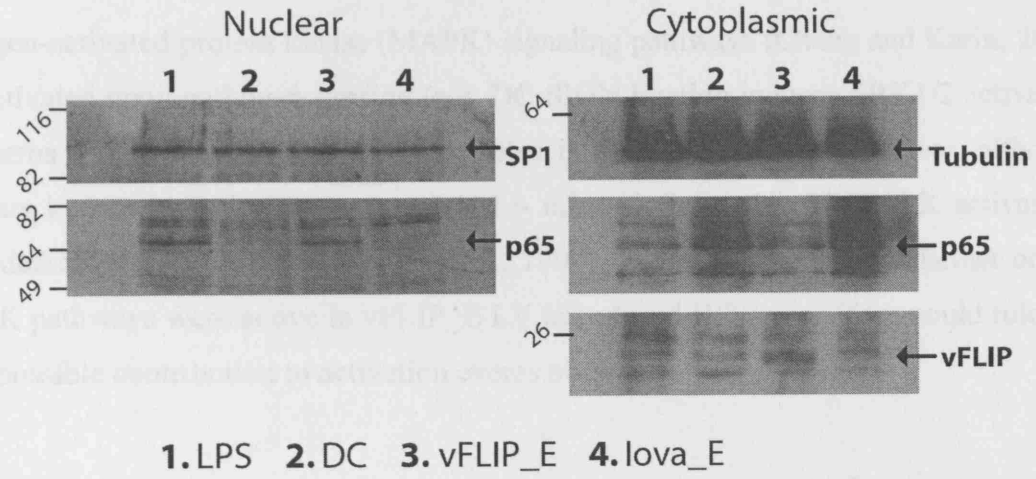
Since vFLIP activates the NF-κB transcription factor through both the classical and alternative pathway one or more NF-κB family members could be responsible for DC maturation events observed. Of particular interest, RelB is important in adaptive immunity (Speirs et al., 2004) and RelA is crucial to protect cells against apoptosis and to initiate innate immune responses (reviewed in (Bonizzi and Karin, 2004)).

5.6.1 RelA (p65) translocates to the nucleus in vFLIP expressing DC

Nuclear and cytoplasmic fractionation of the DC groups was carried out to determine whether or not p65 was present in the nucleus, and the results are shown in figure 5.13. p65 was detected in the nuclei of the LPS treated and vFLIP_E DC groups, but not in the nuclei of the untreated or Iova_E DC. In contrast, p65 was detected in the cytoplasm of all DC groups, with the levels being higher in untreated and Iova_E DC. Immunoblotting for tubulin (cytoplasmic marker) and Sp1 (nuclear marker) confirmed cell fractionation and protein loading. vFLIP expression was detected only in the vFLIP_E DC group; the αvFLIP Ab also reacts with background bands.

In summary, the data suggest that maturation events observed with vFLIP expressing DC are mediated through NF-κB p65 activation. One way to further illustrate this would be to co-express the IκB super-repressor to specifically block the NF-κB classical pathway and confirm a subsequent inhibition of DC activation. It would also be interesting to look for activation of the alternative pathway by detecting nuclear translocation of p52 / RelB by Western or immunostaining; the effect of this pathway on DC activation could be assessed by specifically blocking it e.g. by expressing short interfering (si) RNA hairpins against p100.

Figure 5.13



NF-κB p65 is present in the nucleus of vFLIP_E LV transduced DC. BMDC were transduced at MOI -20 (see methods) and $6x10^6$ cells / group were harvested on day 4 post transduction. Then, cytoplasmic and nuclear extracts were made by cell fractionation (see methods), and protein concentration was determined using the Bradford assay (see methods). Duplicate nuclear samples were loaded on one gel (13μg was loaded per lane, out of ~ 30μg obtained), in order to probe with an αSP1 Ab and an αp65 Ab (left-hand panels). Triplicate cytoplasmic samples were loaded on a second gel (26μg / lane, out of ~ 700μg obtained), in order to probe with the αp65 Ab, plus an αTubulin Ab and an αvFLIP Ab. Tubulin was not detected in the nuclear fraction, nor SP1 in the cytoplasmic fraction (not shown), as measured by re-probing blots. Protein sizes: SP1, 95-105kD; p65, 65kD; Tubulin, 55kD; vFLIP, 23kD. A repeat experiment (also on day 4 post transduction) showed similar results, with p65 only detected in the nuclei of the LPS and vFLIP_E DC groups.

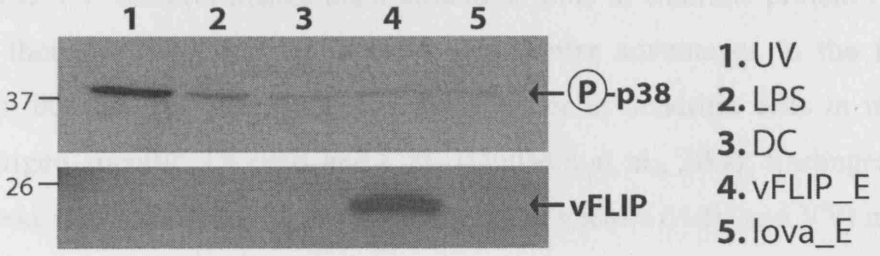
5.7 MAPK pathways

Mitogen-activated protein kinase (MAPK) signaling pathways (Chang and Karin, 2001) are activated upon pathogen sensing (e.g. DC-SIGN ligation induces ERK1/2 activation (Caparros et al., 2006)). Their possible roles in DC maturation are unclear, although they are known to be important e.g. *Mkk3*-/- mice (deficient in p38 MAPK activation) have defective IL-12 production (Lu et al., 1999). We wanted to know whether or not MAPK pathways were active in vFLIP_E LV transduced DC, to see if we could rule out their possible contribution to activation events observed.

5.7.1 p38 MAPK is not activated in vFLIP expressing DC

Activation of MAPK pathways can be demonstrated by Western, due to the availability of Abs specific for the phosphorylated MAPKs. The Western in figure 5.14 shows that the p38 MAPK pathway is not activated in vFLIP_E DC, despite the strong expression of vFLIP in these cells. In contrast p38 is activated in LPS treated DC, although to a lesser extent than in ultraviolet (UV) treated DC, which were used as an additional positive control. It can't be ruled out that p38 could be activated at later time points in the vFLIP expressing DC (either by vFLIP directly or through NF-κB target genes), since samples were only taken at days 2-5 post transduction. It is still unknown whether or not ERK and JNK MAPKs are activated in the vFLIP_E DC.

Figure 5.14



p38 MAPK is not activated in vFLIP_E LV transduced DC. BMDC were transduced at MOI -20 (see methods) and $3x10^6$ cells / group were harvested on day 3 post transduction. For the UV positive control, DC were subjected to 2 rounds of UV activation and then incubated for 30 minutes before harvesting. Total protein extracts were prepared using RIPA lysis buffer, plus protease inhibitors (see methods), and protein concentration was determined using the Bradford assay (see methods). Duplicate samples were loaded on the gel (20µg / well), in order to probe with an αphospho-p38 (Ⓟ-p38) Ab and an αvFLIP Ab. The phosho-p38 western was repeated on day 2 and day 4 post transduction, using the same batch of DC, with similar results (not shown). A further phospho-p38 western was done, with a different batch of DC, at day 5 post tranduction that showed similar results (not shown).

5.8 Discussion

The finding that lentiviral vectors stably express genes but do not notably activate cells (unlike AdV and VV vectors) makes them attractive tools to examine protein function and for gene therapy. However, lentivectors also confer advantages in the field of vaccine design, because they can express antigen genes in dendritic cells *in vivo* and prime both antigen specific Th cells and CTL (Dullaers et al., 2006; Esslinger et al., 2003; Palmowski et al., 2004; Rowe et al., 2006). Other vectors (AdV and VV) manifest the problem of stimulating a powerful immune response towards the vector itself.

The disadvantage of lentivectors as vaccines is their inability to mature DC (Breckpot et al., 2003; Esslinger et al., 2002; Veron et al., 2006), (although they must exert some activation effects *in vivo* in order to explain the immunisation data). The aim of this project, therefore, was to express a DC activator in the vector to improve vaccine efficacy. The advantage of this approach (as opposed to co-injecting an adjuvant) is that the DC activator relies on transgene expression; any initial presentation of viral proteins (Gag / VSV-G) during vector entry should be while the DC are immature. After integration, no viral genes are encoded; expression of the activator and the antigen in parallel should result in mature DC presenting the antigen of interest. In this respect, a lentivector co-expressing both activator and antigen might be a more potent cancer vaccine than those available to date.

We decided to use vFLIP from KSHV as a potential DC activator because of its known ability to activate NF-κB. We co-expressed vFLIP with GFP in a dual promoter lentivector and examined its effect on DC maturation *in vitro*, using murine bone marrow-derived DC. Here, ~~we~~ preliminary data have shown that vFLIP expressing DC exert many of the characteristics of LPS treated DC. For instance, their expression of CD80, CD86, MHC class I and II and CD54 is not significantly different to LPS DC. CD40 (although also up-regulated on vFLIP expressing DC) is significantly lower than on LPS DC. Interestingly, the maximum IL-12 p70 secretion by vFLIP expressing DC is still ~ 6

times lower than the maximum secreted by LPS DC (259 pg/ml compared to 1498 pg/ml respectively).

By using a control lentiviral vector expressing an ovalbumin construct with GFP (Iova_E), instead of vFLIP and GFP (vFLIP_E), we were able to establish which activation events were significantly enhanced with the vFLIP_E group, compared to the control vector group. These were the up-regulation of CD80, CD86, CD40 and CD54 and the secretion of IL-12p70. Additionally, some bystander effects were seen on the non-transduced cells in the vFLIP culture (up-regulation of MHC II, CD54 and CD86).

The mechanism of these effects is presumed to result from NF-κB activation, (at least by the classical pathway), since p65 (RelA) was detected in the nuclei of these cells (and not in the nuclei of the control vector transduced cells). It is possible that the alternative NF-κB pathway is also contributing to maturation effects seen, since vFLIP is reported to activate this pathway, and RelB is important in maturation of and antigen presentation by bone marrow-derived DC (Castiglioni et al., 2003). The genes, CD80 and CD54 contain κB binding sites (interestingly, it has been reported that ICAM-1 κB sites preferentially bind RelA homodimers (Ledebur and Parks, 1995)). MHC I H-2K^b also contains a κB binding site, (although this marker was up-regulated on control vector transduced cells as well). It is likely that CD86 and CD40 were indirectly up-regulated on vFLIP expressing cells through NF-κB target genes. IL-12 p40 also has a κB binding site, although an additional signal must have been supplied to lead to IL-12 p70 secretion. Effects on bystander cells in the vFLIP_E culture (MHC II up-regulation) could have been mediated by IL-12 or other cytokines secreted by the vFLIP expressing cells; some potential cytokines with κB binding sites are IFNβ, IFNγ, GMCSF and TNFα/β.

Rather than elucidating which cytokines are secreted and mediating which effects, the most interesting next step would be to examine the vFLIP vectors vaccine potential. To this end, we want to co-express Iova in the vFLIP vector and compare it to the vector expressing Iova alone. We plan to do this by modifying BMDC with the vectors and co-

culturing them with OT-I or OT-II cells in an ELISpot. Immunisation of mice with these vector vaccines could be assessed in a therapeutic setting, (using an ovalbumin expressing tumour); the vector expressing Iova alone is able to protect mice in a prophylactic setting (see chapter 3). It would also be interesting to see if the Iova_vFLIP vector alters the balance of the Th1/ Th2 adaptive response, compared to the Iova vector.*1.

(or a component of the preparation)

Our data (using the control lentiviral vector) also confirm that the vector itself does exert some activation effects on DC, although it doesn't induce maturation or IL-12 secretion. Effects seen (most distinctly MHC I up-regulation) could have resulted after DC innate recognition of virus. TLR7 and TLR9, located in endosomes, recognise viral ssRNA (e.g. the vector genome) and CpG DNA (possibly present due to plasmid contamination) respectively; receptor ligation results in IFN-α secretion (see figure 5.5).

Other possible activators include VSV-G and HIV-Gag, (which is cross-presented upon vector entry (Marsac et al., 2002)), the GFP transgene expressed or additional signals in the culture, such as GMCSF used to feed DC and known to mature them over time (Shi et al., 2006b).*2. This means that synergistic effects of the lentivector plus vFLIP could have contributed to maturation events seen with the vFLIP_E vector.

Our results showed that the only marked difference between the LPS treated DC and the vFLIP expressing DC was the amount of IL-12 p70 secreted. This suggests that activation of NF-κB is sufficient to induce some maturation but that one or more signaling pathways are also missing. MAPK pathways are known to be involved in DC maturation (Luft et al., 2006). One candidate MAPK is p38, reported to be involved in IL-12 p35 production (Lu et al., 1999). We found that p38 is not activated in the vFLIP_E DC cultures but it is activated in LPS treated DC.*3. Importantly, vFLIP can be used in this way to define the minimal pathways required for maturation of DC, due to its selective activation of NF-κB. Additional pathways could be activated in parallel by using constructs that constitutively activate different MAPK pathways for instance.

*1. We could analyse this by adoptively transferring OT-II cells into immunised mice and measuring expansion and cytokine secretion.

*2. The use of a heat killed vector or a non-enveloped vector would provide more information about the cause of this modest activation.

*3. However, this is only preliminary data and we now intend to measure the total amounts of each MAPK as well as the phosphorylated amounts of each MAPK.

Another reported effect of vFLIP is to protect cells against apoptosis through activation of NF-κB (Sun et al., 2003); anti-apoptotic genes containing κB sites are: Bfl1, Bcl-x_L, Nr13 and IAPs, although NF-κB can also induce the proapoptotic genes Fas and Fas ligand. There remains the possibility that vFLIP can directly inhibit apoptosis by blocking caspase-8 recruitment to the death inducing signaling complex (DISC), in the same way as its homolog cFLIP (Irmler et al., 1997), but this has not been proven. Either way, preliminary data (not shown) suggested that the vFLIP expressing DC had a survival advantage. After one month in culture, the vFLIP expressing DC retained their mature DC morphology, exhibiting extensive dendrites; 20% of them still expressed CD86. Iova expressing DC and uninfected DC also survived after one month, but the cells were round, not as adherent and did not express CD86. It would be interesting to explore the effect of vFLIP on DC survival. It could be attractive to extend the life-span of activated transduced DC *in vivo* as another strategy to improve vaccine efficacy (see chapter 4 for discussion on DC life-span). However, this brings us to the question of safety.

While NF-κB signaling is important to tumour surveillance and rejection because it drives efficient antigen presentation, release of proinflammatory cytokines and Th1 responses, it is also involved in tumour growth, because it activates growth factors (TNF and IL-1) and inhibits apoptosis (Karin and Greten, 2005). It would therefore be crucial to target the vFLIP vector to DC (by surface targeting for example (Carter et al., 2006)) and perhaps to use an inducible or DC specific promoter (Nopora and Brocker, 2002).

These data have implications for KSHV biology as it remains a possibility that the virus infects DC *in vivo*. KSHV was reported to be present in BM-derived DC from multiple myeloma patients (Raje et al., 1999; Rettig et al., 1997). However, several other studies did not confirm these findings (Bellos et al., 1999; Tarte et al., 1999; Yi et al., 1998). Experimental infection of human cord blood-derived stem cells with KSHV results in enhanced immunostimulatory properties of the progeny DC (Larcher et al., 2005), which may be due to vFLIP. HHV-8 has also been reported to infect myeloid DC and

macrophage cultures using DC-SIGN as a receptor (Rappocciolo et al., 2006). In these cultures, antigen stimulation of CD8 T cells was inhibited.

In conclusion then, vFLIP from KSHV is a potential DC activator, due to its ability to activate NF-κB through both classical and alternative pathways. Here, we have shown that vFLIP can be used as a DC activator because it is able to mature DC; co-stimulatory markers are up-regulated and DC start to secrete IL-12 p70. Detection of p65 (RelA) in the nuclei of these DC supports the role of NF-κB in maturation events observed. LPS treated DC, which are deemed fully mature, secrete substantially more IL-12 p70 than vFLIP expressing DC, suggesting that NF-κB activation alone is not sufficient for optimal IL-12 secretion. These results demonstrate that vFLIP can be used as a tool to determine the minimal signaling pathways required for DC maturation. Most importantly, the data suggest that a lentiviral vector co-expressing both a tumour antigen and vFLIP could be a more potent cancer vaccine than those available to date.

Chapter 6: Epilogue and future directions for cancer vaccines

This thesis has covered research on lentivirus-based vaccines that has ranged from improving T helper responses in mice to analysing dendritic cell signaling pathways. The contrasting projects presented here, whilst being different enough to have maintained my enthusiasm throughout my PhD, are similar enough to allow me to tie the results together in this concluding chapter. By summarising my findings and those of others in the field, I will go on to discuss future directions of lentivirus-based vaccine research. Finally, I will refer back to general advances in cancer immunology (mentioned in chapter 1) and suggest possible routes towards the development of a vaccine for cancer.

6.1 The significance of this thesis

My first aim (in chapter 3) was to find out if lentivectors could stimulate CD4+ T cell responses to their antigen transgene (it was already known that they could induce CTL responses in mice). Despite some evidence for overlapping pathways of processing and presentation of antigens on both MHC class I and II, regardless of their location (Groothuis and Neefjes, 2005; Schmid and Munz, 2005), we assumed that lentivector encoded antigens would not access the endocytic pathway (unless they possessed a signal sequence). By constructing a lentivector expressing a cytoplasmic ovalbumin antigen, I confirmed this assumption to be true *in vitro* and *in vivo*.

Lentivectors encoding ovalbumin fusions designed to traffic the antigen into the endocytic pathway were also engineered. I found that both CD8+ and CD4+ T cell responses were improved after injecting a vector expressing ovalbumin fused to the invariant chain (Ii). These results are meaningful in the design of lentivirus-based cancer vaccines, since most tumour antigens are cytoplasmic and depend on uptake (by DC) to be processed through the MHC class II pathway. Interestingly, patients who do harbour TILs (tumour infiltrating lymphocytes) are often found to respond to tumour antigens

that are naturally presented on MHC class II e.g. MAGE-3 and NY-ESO-1. T cell responses to these antigens in particular are associated with good prognosis (Jager et al., 2000; Manici et al., 1999). Therefore, cancer vaccines that enhance presentation of such antigens on MHC class II by DC, as well as on MHC I, should boost these natural anti-tumour responses. We propose that processing and presentation of tumour antigens and in turn anti-tumour immunity could be improved by fusing antigens to Ii chain and expressing them from a lentivector (see chapter 3).

We also found that lentiviral vectors are relatively immunogenic vaccines compared to vaccinia vector and peptide emulsified in complete Freund's adjuvant (pepCFA) (see chapter 3). This can be partly explained by our results from chapter 5, which show that the vector can intrinsically modulate some maturation-associated DC markers. As yet, we have not determined the mechanism of these observed effects; one possibility is that the removal of the HIV accessory proteins has restored susceptibility to innate viral recognition pathways. Another possibility is that such effects are independent of HIV (and caused by VSV-G or DNA plasmid contamination, for instance). It is important to add that the activation effects observed *in vitro* are modest; our data conclusively show that the vector does not mature DC (see chapter 5), although it may exert more of an effect *in vivo*.

This brings us to the vFLIP results: another goal was to enhance vaccine efficacy by the addition of a candidate DC activator. We found that insertion of vFLIP from KSHV into the vector rendered it capable of inducing maturation effects (up-regulation of co-stimulatory molecules) on DC that were similar to those caused by LPS (see chapter 5). The only noticeable difference was the relatively low secretion of IL-12p70 by the vFLIP expressing DC. Considering these promising results *in vitro*, I am eager to know how the vFLIP vector (co-expressing ovalbumin fused to invariant chain) will perform as a vaccine *in vivo*. Even if a vFLIP vector itself is too radical to use as a vaccine, it is a valuable tool both to prove the principle that lentivectors can be engineered to express DC activators, and to assess the contribution of the NF-κB pathway to DC maturation.

During my PhD, I also wanted to analyse transduction *in vivo*: chapter 4 is, therefore, devoted to tracking *in vivo* modified cells in order to collect data on antigen expression and presentation, and its effects on immunity. Our preliminary results show that DC in particular stably express and present the lentivector encoded antigen. This finding has exciting implications for vaccine design, especially in light of recent data on DC subsets and their functions, and DC precursors; it may be possible to engineer lentivectors to stably present antigens in particular subpopulations of DC. This may allow the repeat mobilisation of DC effectors after injection of adjuvants. This approach would be valuable because it is unlikely that one injection of a lentivector will be sufficient to tackle an established tumour; we found that strong T cell memory was only maintained after boosting with vaccinia vector (see chapter 3).

In brief, I have found that lentivirus-based vaccines have the potential to activate DC, to stimulate cell-mediated immunity, and to modify DC to effect long-lived antigen presentation. Therefore, lentiviral vectors present themselves as an unexpected solution to common problems faced by current cancer vaccines e.g. short-lived antigen presentation and the lack of cognate T cell help. By manipulating the tropism, the ability to activate DC (or not), and the length of antigen presentation on MHC class I and/or class II, lentivectors could be tailored to be either immune stimulating or tolerance inducing vaccines. These benefits are, however, a trade-off for the problems accompanied with lentivector vaccines, namely the need to target the vector for safety and efficacy reasons and the risk of insertional mutagenesis and/or the emergence of replication competent virus. The task of overcoming these problems is underway (see chapter 1), which will hopefully allow us to design a sophisticated vector vaccine that comprises novel immune activation properties, as well as enhanced safety features.

6.2 Parallel advances in the field

Research in the field of lentivirus-based vaccines, prior to 2004 is discussed in chapter 1. Since then, the following advances have been made, with regards to *in vivo* administration of lentivector vaccines: Kris Thielemans lab in Brussels published a

paper (Dullaers et al., 2006) at the same time as I did on the ability of lentivectors (encoding ovalbumin) to stimulate CD4+ T cell responses in mice. While my approach was to optimise cognate T cell help, they aimed to prove the importance of CD4+ T cells in helping the CD8+ T cell response. By depleting CD4+ T cells prior to immunisation, they clearly showed that these cells are important for lentivector induced CD8+ T cell responses. This study also showed that injected lentivectors (as well as transferred DC, transduced with lentivectors) can slow tumour growth in a therapeutic setting. A separate group also demonstrated the value of lentivector immunisation in a therapeutic tumour setting, using a tyrosinase antigen (Kim et al., 2005).

Frederic Levy's lab in Lausanne subsequently showed that lentivectors induce superior (more long-lasting) CD8+ T cell responses than peptide plus adjuvant, when using the tumour antigen, Melan-A (Chapatte et al., 2006). Finally, a recent study in Louis Falo's lab (He et al., 2006) demonstrated that subcutaneously injected lentivectors (expressing ovalbumin) transduce skin-derived DC, which then migrate to lymph nodes and are responsible for the induction of T cell immunity. Overall, these studies support the development of lentivirus-based cancer vaccines.

6.3 Lentivirus-based vaccines

Considering that I have been working with a model antigen and normal (not transgenic or diseased) mice, it is important to stress that this thesis is concerned with creating a vaccine model rather than a disease specific vaccine. My future plans: to test the vFLIP vector vaccine *in vivo*, to confirm that DC stably present antigen, and to investigate the effect of this, will allow me to add to this model. The model consists of a lentivector expressing both an antigen fused to Ii, as well as an activator (e.g. vFLIP). Although the vector appears to naturally select DC for expression of antigens to some extent, the vaccine model should contain a DC specific promoter (at least for vFLIP expression); CD11c would be a good choice because of its strong expression in cDC. The model also proposes that the vector should be injected subcutaneously if transient antigen expression is desirable, or intravenously if stable antigen expression by DC is required.

This model is a base from which to design a vaccine to suit a particular disease setting. In the case of cancer, the model could be developed to consist of a vector co-expressing NY-ESO-1 and vFLIP under DC specific promoters, for example. It would also be important to include other tumour antigens in the vaccine design to avoid tumour escape. The best way to achieve this, with the lentivector approach would be to express each antigen from a separate vector (to avoid immunodominance) and inject multiple vectors.

The first clinical trial involving lentiviral vectors (initiated by our lab) has just been approved. The vaccine protocol involves priming with a lentivector expressing NY-ESO-1 driven by the SFFV promoter, followed by boosting with an NY-ESO-1 expressing fowlpox vector. Previous results from our lab (Palmowski et al., 2004) and my results in chapter 3 have shown this vaccine regimen (lentivector + poxvirus vector) to be extremely potent.

6.4 The next cancer vaccine

The active maintenance of tolerance is still an obstacle even to the most potent cancer vaccine. Since there is now an exciting body of data on this topic, it is possible to generate new cancer therapies aimed at combating tolerance. Also, there is a broadening understanding of how the immune system recognises patterns of danger (such as dsRNA from viruses or LPS from bacterial cell walls), which are necessary to trigger immunity. The task now is to combine cancer vaccines with danger signals and inhibitors of tolerance in a triple vaccine approach (see figure 1.1). Dual vaccine approaches have already reached clinical trials (see figures 1.2 and 1.4); it is reassuring to see that progress made in basic research is quickly translated into new medicines. Lentivirus-based vaccines co-expressing antigens with activators may not require additional danger signals, but they could be more effective if combined with an anti-CTLA-4 antibody, for instance, especially in prime-boost protocols with poxvectors. It is hoped that such vaccines will induce better clinical responses in patients than those available to date.

It is important to stress that with cancer vaccines there is always the risk of an auto-immune response and vaccines should be carefully tested before entering the clinic.

However, we are still a long way from developing an effective vaccine for cancer. It may be impossible even to develop one vaccine for each type of cancer (e.g. breast cancer, melanoma), considering that tumour associated antigens differ greatly among patients. The challenge to develop a cancer vaccine can perhaps be compared to the task to make a HIV vaccine (although we have been using components of HIV to make a cancer vaccine, our vaccine could also be redesigned to combat HIV). With cancer vaccines, the difficulty is that we are trying to break tolerance, whereas at least HIV is recognised as a pathogen by the IS. On the other hand, HIV is more expert at evading immunity than our cancer cells; in this sense it is only our lack of knowledge (of cell biology as well as cancer immunology) that is preventing us from combating cancer. In conclusion, I believe that a cancer vaccine is in sight because our knowledge is evolving faster than our cancers are.

In terms of how the research discussed in this thesis should continue, I think that the most sensible idea would be to focus on the DC maturation project. Although tracking vector modified cells in vivo is important and my preliminary data has yielded some intriguing results, it is a difficult project that involves many more questions than answers. Therefore, future experiments should be to finish dissecting the signaling pathways activated by vFLIP in DC and to construct a vector expressing both ovalbumin and vFLIP. The vaccine efficacy could then be compared to the vector expressing ovalbumin alone. This could easily be done by adoptively transferring CFSE labelled OT-I and OT-II cells into immunised mice and measuring expansion and cytokine secretion. A more long-term aim would be to target the vFLIP vector to DC and to express a tumour antigen such as NY-ESO-1, in order to make a more potent cancer vaccine than those available.

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