

Efficacy of B16OVA Tumour Cell Lysate Conjugated to *Rabbit Haemorrhagic Disease Virus* Virus-Like Particles as an Anti-Tumour Vaccine

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A thesis submitted in partial fulfillment of the requirements of
Bachelor of BioMedical Science with Honours majoring in Infection and Immunity
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November 2011

Abstract

By presenting antigen to T cells dendritic cells (DC) carry out a central role in the activation of T cell mediated immunity to cancer. Tumour cell lysate (TL) as a source of tumour antigens offers the advantage over single, defined tumour-associated antigens (TAA) of being able to stimulate polyclonal T cell responses to heterogeneous tumours containing both known and unknown antigens. However TL alone does not generate a robust, long-lasting anti-tumour response. Virus-like particles (VLP) coupled to defined TAA have been shown to stimulate strong anti-tumour responses but the majority of cancer antigens remain undefined. This project aimed to develop VLP-antigen conjugates by coupling unidentified TAAs in TL to *Rabbit Haemorrhagic Disease Virus* (RHDV) VLP. TL was generated from the melanoma cell line B16OVA that secretes the model antigen, ovalbumin (OVA). Bone-marrow derived DCs (BMDC) were pulsed with VLP-TL and the BMDC maturation response evaluated by assessing upregulation of the key DC surface markers, CD40, CD80, CD86 and MHC-II by flow cytometry. Subsequently antigen-pulsed DC were co-cultured with OVA MHC-I and MHC-II peptide-specific OT-I and OT-II splenocytes and the T cell proliferative and cytotoxic response measured. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-I splenocytes proliferated in response to VLP-TL indicating T cell activation; OT-II splenocytes on the other hand showed no such response. IFN- γ was detected in the supernatant of both OT-I and OT-II co-cultures, indicating a cytotoxic response. Inhibition of T cell proliferation and cytotoxicity was seen in the presence of VLP or TL alone and VLP-TL was sometimes able to overcome this inhibition. *In vivo* CTL-mediated cytotoxicity was also examined with VLP-TL vaccinated mice showing a significant TL-specific cytotoxic response, demonstrating proof of principle for future *in vivo* assays of VLP-TL. These results indicate that VLP-TL may have a beneficial effect on the ability of DC to stimulate T cell proliferation and anti-tumour cytotoxicity. Further investigations with increased dosages are warranted to ascertain whether or not the effect seen is dose-dependent.

Acknowledgements

I have thoroughly enjoyed learning how the process of scientific research works this year. It was a privilege to be one of Professor Margaret Baird's last Honours students and to study under this very classy lady who also happens to be a great scientist. I am deeply grateful to Dr Sarah Young for her help, insights and the humanity she brings to this corner of the scientific world. Vernon: I will never be a virologist, but I have learned much from you this year and for that I thank you.

I am immensely grateful to Michelle Wilson, my technician extraordinaire, Flow Cytometric Magician, Mouse Wrangler and guardian angel. You made this year so much easier, so enjoyable and I could not have done it without you.

Thank you to Vivienne Young of the Ward Lab for putting up with my requests for more VLP and VLP conjugates.

I would like to acknowledge Sally McCormick who has been a wonderful academic head of the BioMed Sci Hons programme and Sue Butt who provided excellent administrative support.

Enormous thanks must go to my ever-patient and immensely supportive husband, without whom my sanity may have been lost forever.

Thanks also to my family and friends who have endured four years of relative neglect and cheered me on from the sidelines.

And importantly thanks to the University of Otago Microbiology and Immunology Department for having me as a student and providing the funding which made this learning experience possible.

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Abbreviations

AB	antibody	PBS	Phosphate Buffered Saline
ACT	adoptive cell transfer	PE	phycoerythrin
APC	allophycocyanin	pg	pico-gram
CD	Cluster of differentiation	RBC	red blood cell
CFSE	carboxyfluorescein diacetate succinimidyl ester	RHDV	<i>Rabbit Haemorrhagic Disease Virus</i>
CTL	cytotoxic T lymphocyte	RNA	ribonucleic acid
DC	dendritic cell	RPMI	Roswell Park Memorial Institute (medium)
dH₂O	distilled water	SATA	N- Succinidimyl S-Acetylthioacetate
DMEM	Dulbecco's Modified Eagle Medium	ss	single stranded
DNA	deoxyribonucleic acid	SSc	side scatter
DPBS	Dulbecco's Phosphate Buffered Saline	Sulfo-SMCC	sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate
ds	double stranded	TAA	tumour-associated antigen
FACS	Fluorescence Activated Cell Sorter	TB	Trypan Blue
FBS	Foetal Bovine Serum	TCF	Tissue Culture Flask
FCS	Foetal Calf Serum	TCR	T Cell Receptor
FITC	Fluorescein isothiocyanate	TL	tumor lysate
FSc	forward scatter	VLP	virus-like particle
GM-CSF	granulocyte macrophage-colony stimulating factor		
HLA	human leukocyte antigen		
HRP	horseradish peroxidase		
IMDM	Iscove's Modified Dulbecco's Medium		
IFN-γ	interferon-gamma		
kDa	kilodalton		
mg	milli-gram		
ml	milli-litre		
μg	micro-gram		
μl	micro-litre		
MQ	milli-Q water		
OT	ovalbumin transgenic		

Chapter 1. Introduction

1.1 Vaccination and Cancer

The first recorded instances of vaccination appeared in the literature in the late 1700s after it was noted that weakened versions of disease-causing pathogens were capable of protecting people from illness ¹. Advances in the understanding of how our immune systems function has allowed us to use vaccines in a targeted way to protect millions worldwide from devastating infectious diseases and, in more recent years, from certain types of cancers. One success story is the prophylactic vaccine Gardasil formed from *Human papillomavirus* virus-like particle (HPV VLP) which gives protection against cervical cancer ². Cancer can be immunologically difficult to detect as cancer cells are self cells which have taken a deviated developmental route, via point mutation(s), post-translational modification, or possibly a chromosomal translocation which creates one or more fusion proteins ³. Discoveries and improvements in molecular technologies and cell culture techniques have allowed the identification of tumour-associated antigens (TAA), for example in melanomas and B cell lymphomas, however for the vast majority of cancers specific TAA(s) remain undefined and thus inaccessible to antigen-targeted vaccines or therapies. Presenting an additional problem is the fact that most human tumours are unable to be grown as cell lines. This limits their use as immune activators in DC or T cell adoptive cell therapy (ACT) which seeks to help the patient's immune system recognise its own TAA(s) via *ex vivo* activation of DC or T cells with specific antigen. It is for this reason that several groups have turned their attention to tumour lysate (TL), a source of undefined antigen, as a potential immune stimulator. DCs and T cells are two of the key players which potentially have the capacity to use TL to the immune system's advantage.

1.2 Dendritic Cell Debut

In October 2011 Ralph Steinman won a Nobel Prize for the 1973 discovery (with colleagues Cohn *et al*) of dendritic cells (DCs) in the mouse spleen ⁴. A few years subsequent to this discovery another Steinman collaboration showed that DCs were the necessary antigen-presenting cells (APCs) for inducing the T cell response ⁵. A critical contribution to our understanding of DC function came with the discovery that DCs can serve two broad functions: antigen presentation and T cell sensitization; and that these may be independently regulated. In 1989 members of a Steinman group showed that freshly isolated Langerhans cells (skin-resident DCs) are better at capturing antigen than at stimulating T cell proliferation ⁶ and by 1990 they were able to demonstrate that DCs as APCs can consistently prime antigen-specific mouse T cells *in vivo* ⁷.

It was also shown in the 1980s that the T cell-mediated immune response is directed against antigens presented by the Major Histocompatibility Complex (MHC) on the surface of APCs ^{8,9}. In 1990 the ability of antigen-pulsed DCs to activate the T cells of naïve mice was analysed. It was found that T cell activation was specific to the antigen with which the DCs were pulsed and that the T cells could only recognize (were restricted to) the antigen when presented on the MHC molecule on the surface of the DC. In other words, DCs could present exogenous antigens on their surface MHC molecules directly to naïve T cells *in vivo*. This was the first hint at the MHC- presentation pathway in DCs with visualization of small amounts of processed antigen in intra-DC vacuoles ¹⁰.

Bone marrow-derived dendritic cells (BMDCs) are the key APCs ¹¹ in both the pathogen and cancerous cell elimination pathways and as such are pivotal players in cancer immunotherapy. Immature DCs are highly efficient, phagocytic, antigen capturing cells ¹² which continuously monitor the internal environment, transporting both benign and immunogenic antigens from the periphery to lymphoid organs for exposure to cognate helper and killer T cells *in vivo*, stimulating either tolerance ¹³ or immunogenicity as

appropriate. Immature DCs can be stimulated to mature by bacterial and viral antigens as well as by inflammatory cytokines ^{14,15,16,17}. In addition to high levels of immunostimulatory Major Histocompatibility classes I and II (MHC-I,-II)-peptide complexes, activated mature DCs up-regulate the co-stimulatory molecules CD80, CD86 as well as adhesion molecules ICAM-1, ICAM-3 and LFA-3 ¹⁸, which, along with the CD40-CD40L interaction ¹⁹ between the DC and T cells, provides potent stimulation for T cell proliferation and activation ²⁰. Charles Janeway made an important contribution to the field of immunology with his suggestion that non-antigen-specific DC activity could be initiated through interactions between Pattern Recognition Receptors (PRRs) which bind specifically to various categories of extraneous antigen such as bacteria and viruses ²¹ (Pg 53). However it was Polly Matzinger who recognised the paradox inherent in autoimmune disease and foetal tolerance, and proposed the Danger Theory ^{22,23} of immune initiation which puts forward the idea that the immune system also reacts when cells or tissues are damaged, rather than simply distinguishing 'self' from 'non-self'. Thus, 'danger signals' released from, or upregulated by, cells which are dying or in distress are also able to activate APCs and provide the co-stimulation necessary to activate T cells. With the identification of various 'danger signals' and the discovery of a range of human tumour associated antigens (TAAs) came the prospect of being able to create vaccines and therapies which could overcome the peripheral tolerance which allows the unhindered growth of many tumours and provoke an effectual immune response. The modern era of immunotherapy ostensibly began with Ralph Steinman and colleagues' discovery of the critical role of dendritic cells as antigen-presenting cells and their importance in the interface between adaptive and acquired immunity ⁵.

The finding that granulocyte macrophage-colony stimulating factor (GM-CSF) could stimulate the generation of immature DCs from mouse and human blood and bone marrow ^{24,25,26} greatly facilitated study of these cells as it meant that larger quantities of DCs could be easily generated *in vitro*. From 1992 on studies were generally carried out with BMDCs since the femurs and tibiae of one mouse gave the same amount of DCs

previously derived from 10-20 mouse spleens which meant that enough DCs were available to be employed for inducing immunity *in vivo* and for carrying out cell fractionation and molecular studies.

Huge improvements in molecular technology in the second half of the 1990s enabled more detailed study of the physiology and function of DCs. Sallusto and colleagues were the first to show that immature DCs have high phagocytic (endocytic) ability which is down-regulated upon activation (maturation). Upregulation of T cell co-stimulatory molecules (MHC, CD40, CD80 and CD86) on the surface of mature DC^{27,12} enhances their ability to activate their cognate T cell via the T cell receptor (TCR). Mayordomo *et al* noted, for example, that DCs cultured with GM-CSF and IL-4 had higher levels of MHC class II and costimulatory molecules CD80 and CD86 compared with DCs cultured with GM-CSF alone or GM-CSF and TNF- α , and that these DCs were more potent at stimulating the mixed lymphocyte reaction (MLR)²⁸. This was an important study that demonstrated the effectiveness of tumour peptide-pulsed DCs as both vaccines (having the ability to resist lethal tumour challenge and/or prevent or delay tumour growth) and as therapeutics (having the ability to cause regression of established tumours in two tumour models)²⁸

It should be noted that while this study focused on the activating interaction between DCs & T cells, DCs are also able to stimulate other immune effector cells including B cells, natural killer T cells and natural killer cells²⁹ which are all then able to bring their unique arsenals to bear on the specific tumour antigens via AB capture & tumour cell killing.

1.3 Capturing T cell Cytotoxic Capability

By the early 1990s the molecular basis of antigen recognition by T cells had been solved and many of the diverse pathways that control T cell activation and functional occupation had been mapped out. As a consequence the possibility arose of outlining numerous approaches for intervening with the immune system to design protecting

vaccines, in order to bring about a successful reaction against tumour antigens ³⁰. Scientists started devising ways they could intercept, hijack and manipulate these newly understood immune mechanisms to potentially realize better patient outcomes in infections, cancer, autoimmunity and transplant rejection. The realisation that some cancers expressed TAAs ³¹, which in spite of T cell recognition by the host, failed to elicit an eliminatory immune response, spawned the concept of vaccination against autologous cancer cell antigen(s). There were two main approaches: (1) Identify a TAA to use in a vaccine and (2) enhance the immune stimulatory nature of the tumour cells and 'let the immune system decide which antigen to attack' ³⁰. Concepts such as adoptive cell therapy (ACT) entered the literature (although this idea had been trialled since the late 1980s). Instead of stimulating the immune system ACT provides ex vivo activated T cells (or antibodies) to the cancer patient.

By the mid-1990s it was known that immune responses to tumours varied between immunogenicity and tolerance and some data pointed to the identification of newly changed cells by the immune system as being significant with regards to tumour growth ³². There was a growing awareness that defective antigen presentation by DCs may be responsible for generation of tolerance by stimulation of tumour-specific suppressor T cells, thus strategies for enhancing antigen presentation to prevent induction of tolerance to tumours were being examined ³². There was also a realisation of the central importance of DCs as APCs and that the type of antigen along with expression of the appropriate co-stimulatory molecule as well as the functional state of the DC that is presenting the antigen all work together. A DC presenting antigen plus co-stimulation is not sufficient to induce a robust immunogenic T cell response - DCs also secrete immune response-enhancing cytokines (eg chemokines that cause the trafficking of tumour killing cells into the tumour area) and other essential components for stimulation of naïve T cells.

1.4 Clinical Trials

With increasing knowledge of DCs and their interactions with T cells, new types of potential therapeutics were able to be visualised, and work on the variables which needed to be refined for the success of these newly imagined therapies was underway ²⁰. By this time many studies were demonstrating that specific peptides, pulsed onto DCs, could elicit tumour-specific immunity ^{33,34,35,36,37,38,39,28,40,41,10,42}. The knowledge that specific T cell responses could be generated by DCs presenting specific antigen sparked studies into both the identification of TAAs and the generation of specific immune responses to these antigens. Melanoma was particularly antigenic with known spontaneous regressions observed in patients and so various melanoma antigens were fairly rapidly identified, leading the way for antigen-based vaccines ^{43,44,36,45,46}. By the mid-90s a growing body of evidence in mouse models was providing a rationale for clinical trials evaluating various aspects of DC immunotherapy to commence or continue in cancer patients. Following these promising results with both defined and undefined TAAs, the first publications of clinical trials with various approaches to tumour immunotherapy began appearing in the literature in the late 1990s ^{46,45,47}. In 1998 the Nestle group were first to report on a vaccination trial in melanoma patients ⁴⁸. Early immunotherapy efforts included the use of immune-enhancing adjuvants to boost immunogenicity to the TAAs ⁴⁹. However, in spite of the promise and hope, sadly, objective clinical responses were limited. The Steinman group noted in 1997 that cytotoxic T lymphocyte (CTL) effector function was not sustained by tumours ⁵⁰, and this had important implications for immunotherapy. They found that *'repetitive vaccination or adoptive transfer of activated tumour-specific CTL [was] necessary to sustain antitumour activity in cancer patients'* ⁵⁰.

1.5 Tumour Lysate as Vaccine?

Building on the knowledge gained from previous studies with peptide-pulsed DCs Schnurr *et al* published in 2001 the first study showing that, *in vitro*, tumour lysate-pulsed DCs could generate T cells specific for pancreatic carcinoma ⁵¹. The use of tumour lysate attempted to evade the disadvantages of vaccination against single tumour antigens such as: immune escape due to mutation of that single antigen; the question of whether that identified tumour antigen was actually capable of stimulating an immune response even if it was vaccinated against; and the fact that vaccination against a single TAA was restricted to those patients with a specific HLA-type thus reducing the broad-reaching effect of this immunotherapy. The benefit of un-fractionated tumour-derived antigens from tumour cell lysates or whole tumour cells is that they contain multiple known as well as unknown antigens which can be presented to T cells by both MHC class I and class II pathways. An obvious drawback of the use of tumour lysate with its inherent normal self antigens mixed in with abnormal TAAs was the spectre of raising an autoimmune response to tissues that share lysate epitopes ⁵². However, Schnurr states that '*in clinical trials using lysate or whole tumour cells as the source of antigen, no clinically relevant autoimmune responses were detected*' ⁵¹. This is biologically plausible since normal peripheral tolerance mechanisms should ensure that the 'different' tumour cells would be targeted, not the normal tissue cells. That being said, some melanoma patients treated with TAA vaccination have been reported to develop vitiligo, an autoimmune response to normal melanocytes ⁵³ which causes lightening of skin pigmentation. A more immediate problem with this study lay in the fact that pulsing the DCs with lysate from pancreatic carcinoma cell lines did not cause DC maturation, as gauged by the absence of the DC maturation marker CD83. However, the response induced varied between different tumour cell types. Pulsing the DCs with both lysate and the adjuvant Keyhole Limpet Haemocyanin (KLH) boosted the T cell response against the TAAs demonstrating that KLH can be employed as a 'helper antigen' to enhance tumour-specific immune responses. But critically this study

also demonstrated that tumour lysate alone is not sufficient to stimulate a strong anti-tumour immune response – an adjuvant was clearly also required to ensure strong DC stimulation and therefore proper T cell activation. Lack of stimulation of T cells by lysate alone may be due to the tumour cells secreting immune inhibitory substances which 'turn off' the T cells, alternatively the lysate may not contain any DC stimulatory molecules, such as pro-inflammatory cytokines, which are absolutely essential for DC maturation and thus induction of a T cell response ⁵⁴.

1.6 Virus-Like Particle Nanotechnology

The 1990s saw the publication of successful attempts to create virus-like particles (VLP) - viral protein(s) generated in insect or yeast systems, which spontaneously self-assemble into stable protein 'cages', of nanometer dimensions, which lack genomic material ⁵⁵. The VLP were indistinguishable in size, morphology and antigenicity from their native virus. Researchers were hopeful that this new technology could be used as a type of Trojan horse vaccine to deliver a targeted immune-stimulating particle along with the antigen(s) of choice ^{56, 57}. Indeed, Banchereau's 2000 review of DCs included what may prove to be a prophetic paragraph with regards to the future role of VLPs: *'Ultimately we predict that DCs will be targeted in vivo by **'intelligent missiles'**, **man-made viruses** ... expressing specific ligands that can bind to either all DCs or to a specific subset. The missile may be loaded with (a) DC modulators (activators or inhibitors) to induce or suppress a given immune response or (b) **antigens** together with DC modulators for vaccination'* ⁵⁸.

Recombinant VLP can be created by generating empty VLP from different, but related, viruses (eg VP1 from polyomaviruses from humans, monkeys, hamsters and birds), dissociating the VLP into pentamers and reassociating the pentamers back into recombined VLP by defined ion and pH conditions ⁵⁹. Alternatively they can be created by inserting genes for viral capsids into the genome of a viral vector which can be

triggered to express the VLP. The VLP expressed may be either a single protein VLP, or further genetic modification can allow the inclusion of other VLP surface proteins, such as specific antigens. There are problems inherent in the latter method such as size limitations on peptides included in the VLP, along with changes in conformation and inaccessibility of introduced peptides. The Ward lab at Otago University, which supplied the VLP for this study, used chemical conjugation of antigen onto VLP via a hetero-bifunctional linker ⁶⁰ to overcome these limitations. Resolution of viral molecular structures allowed the identification and modification of viral capsid amino acids which facilitated conjugation of various proteins. Genetic manipulation can easily produce mutant VLP which display lysines or cysteines in available regions of the viral capsid, and molecules can be chemically conjugated to these amino acids either inside or outside the VLP ⁶¹. Our model tumour antigen, OVA, has a lot of available primary amines which are well suited to conjugation onto the RHDV VLP lysines using the hetero-bifunctional linker Sulfo-SMCC.

VLP from the human viruses Hepatitis B (HBV) and *Humanpapillomavirus* (HPV) have been successfully used in the formulation of two commercially available vaccines: Enderix (an HBV vaccine) and Gardasil (a cervical cancer vaccine). Vaccines made from human VLP can run into the problem of pre-existing antibodies in individuals who have been exposed to the virus from which the VLP is made. These anti-bodies will have a neutralising effect on the desired VLP-induced immune response. Using a non-human VLP such as RHDV VLP to generate a vaccine avoids this issue since humans will not have been previously exposed to this rabbit disease-causing virus. However, subsequent 'boosts' with the same VLP-based vaccine, if required, may also encounter a previously non-existent antibody response against the non-human VLP. This too can potentially be circumvented by using one VLP type for the priming vaccine and a different VLP type for the booster vaccine.

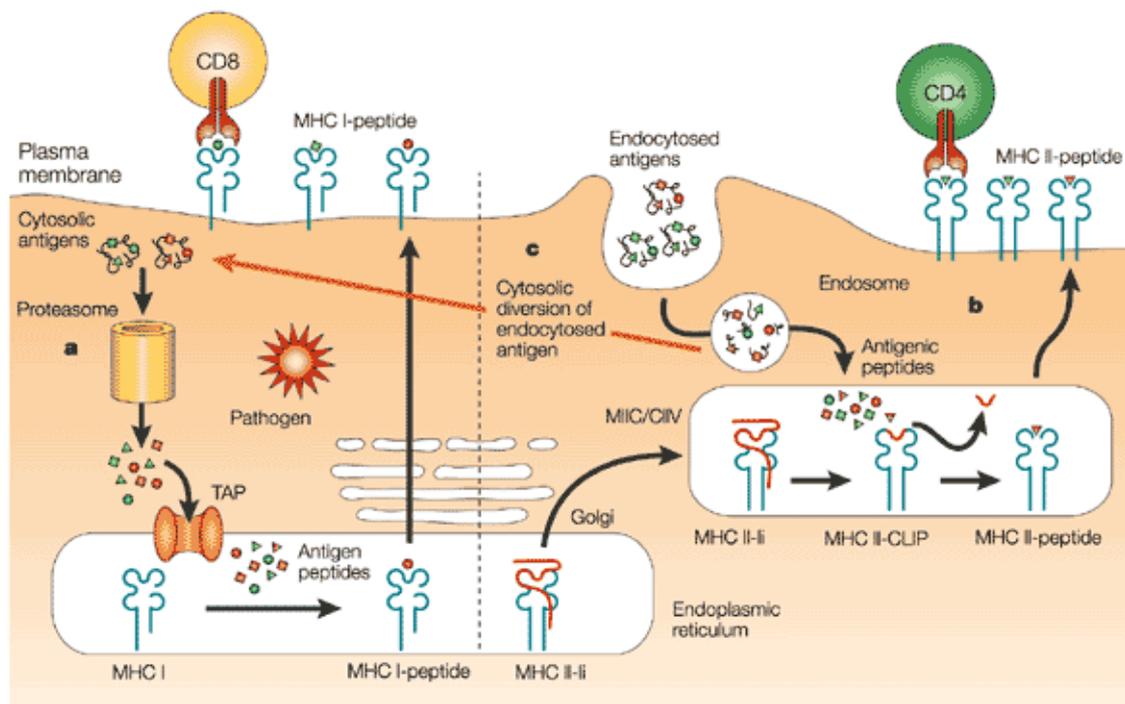
The knowledge that different proteins are displayed on the surface of normal versus tumour cells has opened the way for using viruses as targeted therapeutics by conjugating

specific antigens to viruses, or the more benign virus-like particles, for delivery to specific cells (via virus-specific receptors) and/or generation of an immune response against that antigen (by antigen-specific T cell or antibody responses). An enormous benefit of VLPs is that some are able to induce strong T cell and antibody responses, even in the absence of adjuvants ^{62,63,64,65,66,67,68}, thus stimulating both cytotoxic and humoral arms of the adaptive immune response. Lenz's 2001 paper provided proof of principle that TAAs coupled to VLPs could be used to induce both a cell-mediated (CTL) and humoral (antibody) response against specific antigens ⁶³. Lenz and colleagues showed that it was the regular array of viral protein(s) interacting with specific receptors on DCs which caused uptake of the VLP by the DC and subsequent DC maturation. This occurred in the absence of any other viral gene products such as dsRNA or glycosylated viral envelope proteins since these are generally absent from VLPs.

1.7 Cross-Presentation and CD40 Ligation are Crucial

During the early 2000s much was becoming clearer with regards to the endogenous and exogenous ⁷⁰ pathways of antigen presentation by DCs on their MHC class I and II molecules respectively, but the ability of DCs to cross-present antigen on both MHCI and II (and thus activate both cell-mediated and humoral arms of the adaptive response) was only just beginning to be appreciated and the mechanisms and circumstances worked out. Figure 1.1 outlines our current understanding of cross-presentation pathways. An important paper by Nguyen *et al* in 2002 provided the first analysis of a triple transgenic mouse model (P14/RIP(GP x Tag2)) in which immunity to TAAs could be followed during the endogenous transformation from normal cells to tumours. This study differed from initial experiments with transplantable tumours which would have had confounding variables such as inflammation and cell stress induced by mechanical damage during subcutaneous (s.c.) or intradermal (i.d.) injection of the tumour cells and as such were not 'naturally/spontaneously' arising tumours ⁷¹. With this model they were able to show that tumors did not lead to T cell tolerance *in vivo*, but, crucially, '*despite the overwhelmingly*

tumor-specific T cell repertoire in P14/RIP(GP x Tag2) animals and evidence for their activation by TAAs, immunosurveillance (was) inefficient in preventing tumor development'. They hypothesized that this was likely due to the limited degree of cross-presentation in the regional LNs, which would result in insufficient numbers of activated tumor-specific T cell to protect the host from tumor growth.



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FIGURE 1.1 Antigen processing pathways of the MHC-I and MHC-II molecules showing cross-presentation to both CD4 and CD8 T cells. a | MHC class I molecules present peptides that are primarily derived from endogenously synthesized proteins of either self or pathogen origin. These proteins are degraded into peptides by the proteasome and then transported through the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum for loading on MHC class I molecules. b | By contrast, MHC class II molecules present proteins that enter the cell through the endocytic route. During maturation of MHC class II molecules, they are prevented from binding to endogenous antigens in the endoplasmic reticulum by association with the invariant chain (Ii). Invariant chain–MHC class II complexes (MHC II–Ii) move through the Golgi to the MIIC/CIIV compartment where the invariant chain is degraded to CLIP (for class II-associated invariant-chain peptide). CLIP is then removed from the CLIP–MHC class II (MHC–CLIP) complexes and exchanged for antigenic peptide. c | Dendritic cells can endocytose antigens from other cells and cross-present them to CD8⁺ cytotoxic T lymphocytes. The TAP-dependence of such cross-presentation indicates that it involves diversion of the cellular antigens into the conventional MHC class I pathway, although the mechanism(s) for this diversion are as yet undefined. In most cases, these antigens will also be processed into the MHC class II presentation pathway for recognition by CD4⁺ helper T cells. (MIIC, MHC II loading compartment; CIIV, MHC II vesicles.) Figure from Heath and Carbone, 2001
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Since it was known that cross-presentation can lead to the stimulation of both CD4+ and CD8+ T cells, and that VLP can activate the immune response, a study by Ruedl *et al* looked at cross-presentation of VLP by DC ⁷³. They found that certain DC subtypes are better at cross-presentation than others, and that the primary APC involved in VLP-cross-presentation are CD8- DC. This paper also showed that the particulate nature of VLP ensured processing of VLP (and therefore VLP-conjugated antigen) by DCs and not by other cell types. In other words it makes sense to target DCs for VLP uptake in tumour immunotherapy. Work by Morón in 2003 further confirmed VLP processing and cross-presentation by DCs ⁷⁴. However, further study by the Ruedl Bachmann group and others showed that the *in vivo* immune response to VLP depends on the type of VLP (compare *Papillomavirus* VLPs with *Polyomavirus* VLP ⁶³) and the type of cancer (compare pancreatic cell carcinoma cell lines ⁵¹). Just like tumour cell lysate-pulsed DCs in Schnurr's work ⁵¹, VLP alone were inefficient at inducing CTL responses but they become very powerful vaccines if applied together with substances that activated the DCs ^{64,75}. This is further confirmed by Bachmann and Jennings in their chapter of the book 'Novel Vaccination Strategies' when they write: '*Despite efficient presentation of VLP-derived peptides on MHCI molecules VLPs induce poor T cell responses in vivo in absence of additional stimuli, especially if highly purified antigen preps are used for immunization*'... *However if DCs ... are stimulated while simultaneously vaccinating with VLPs, then very strong CTL responses may be induced.* ⁶⁴ *In essence, co-administration of VLPs with stimuli of the innate immune system closely mimics the course of events during a viral infection*' (P417-418) ⁶². Thus VLP and VLP conjugates which are able to activate DC represent the best strategy for immunotherapeutic immune activation.

The final critical step in strong T cell response activation was confirmed by a study which showed the necessity for CD40 ligation along with antigen presentation and co-stimulation by CD80 and CD86 ⁵⁴. Maturation of the DC with its concomitant upregulation of co-stimulatory molecules on its surface switches the DC function from tolerance-inducing to immunity-inducing interactions with T cells. Immature DCs can present antigen to T cells,

but without CD28/CD80,86 or the CD40-CD40L interaction the T cell will not be activated. If tumour antigen(s) are presented to T cells by immature DCs the T cells won't be activated against that antigen(s) and as a result the tumour will not be eliminated. For this reason it is crucial that the DCs are matured before presenting tumour antigen to T cells. From a tumour immunotherapy perspective VLP has been shown in some cases to help ensure this maturation, and VLP combined with an adjuvant ensures even better maturation ⁷⁵. The overarching aim of this study was to ascertain whether or not TL coupled to VLP can provide this adjuvant effect and overcome the sometimes immune inhibitory responses seen by VLP or TL alone.

1.8 Aims

Despite all the promise clinical trials to date have yielded disappointing results, with only occasional significant tumour regressions and no improved survival ⁷⁶. Despite the identification of some tumour-associated antigens, the vast majority remain undefined and as such cannot be targeted by vaccines or therapies. Few human cancers are able to be cultured as cell lines therefore cells from the tumour itself are the only possible source of tumour antigen for these patients. The use of tumour lysate aims to remove the necessity for isolating a patient's TAA(s) by creating a method for using undefined antigens from the patient's cancerous cells. Lysate-pulsed DCs would be expected to induce a polyclonal expansion of T cells, including MHC-II-restricted T helper cells which carry out a vital role in the activation of CTLs. CTLs are almost certainly the most important cells in executing the antitumor immune response ⁷⁷ and generating CTL clones with manifold antigenic definitions would appear to be an advantage in heterogenous tumours, potentially lowering the threat of tumour escape variants.

Previous work has shown that VLP coupled to defined TAA is capable of eliciting a good immune response ⁷⁸. This study sought to move these investigations a step closer to clinical application by assessing the possibility of coupling undefined TAA in the form of tumour

lysate to VLP, thus removing the necessity for isolating a patient's TAA(s) by creating a method for using the mass of undefined antigens from the patient's cancerous cells. Given that tumour lysate alone can be insufficient to stimulate DC⁵¹ and certain VLP have been shown to generate strong DC and T cell responses^{64,63,67} this study aimed to ascertain whether the combination of RHDV VLP and B16OVA TL could overcome the immune deficient response to tumour lysate without the requirement for a DC-stimulating adjuvant.

The specific aims of this study were as follows:

- Prepare tumour lysate from B16OVA tumour cells for coupling to RHDV VLP and assess the level of the model tumour antigen, OVA, in the tumour lysate
- Assess BMDC maturation phenotype following exposure to VLP-TL *in vitro*
- Assess the capacity of VLP-TL-pulsed DC to stimulate proliferation and cytotoxic cytokine production by OT-I and OT-II T cells *in vitro*
- Assess the ability of VLP-TL vaccination to generate cytotoxic T cells specific for our model tumour antigen (OVA) *in vivo* using a murine model

Chapter 2: Materials and Methods

2.1 Animals – Source, Characteristics, AEC Permission

Specific pathogen-free male and female C57BL/6 mice sourced from the Department of Animal Laboratory Sciences (University of Otago, Dunedin, NZ) or the Animal Resources Centre (Canning Vale DC, Western Australia) were used as donors for bone marrow-derived dendritic cells (BMDCs) and splenic DCs. OVA Transgenic I (OT-I) and OVA Transgenic II (OT-II) mice whose T cell receptors specifically recognize the OVA peptides OVA₂₅₇₋₂₆₄ (known as SIINFEKL) and OVA₃₂₃₋₃₃₉ respectively, were sourced from the HTRU (Hercus Taieri Resource Unit, Mosgiel, NZ). All experimental protocols were approved by the Animal Ethics committee, University of Otago (AEC number ET39/10).

2.2 Genotyping T Cell Receptors from OT-I and OT-II Transgenic Mice

OT-I and OT-II transgenic mice were tail tipped and blood collected into Alsevi's solution. RBC lysis buffer was added to lyse RBCs prior to staining residual cells with primary antibodies:

- 1 μ L per 1×10^6 cells (0.2 μ g) APC-conjugated anti-CD8 or anti-CD4 respectively (BD Pharmingen, BD Biosciences)
- 1 μ L per 1×10^6 cells (0.2 μ g) PE-conjugated V α 2 (PE Rat anti-mouse V α 2 TCR (B20.1), BD Biosciences Pharmingen)
- 1 μ L per 1×10^6 cells (0.5 μ g) biotinylated V β 5.1 (Biotin mouse anti-mouse V β 5.1, 5.2 T-cell receptor (MR9-4); BD Biosciences Pharmingen)

Cells were incubated for 30 minutes on ice before addition of 0.1 μ g (0.5 μ L per 1×10^6 cells) of streptavidin-conjugated PerCP (PerCP Strep; BD Biosciences Pharmingen) for a further 20 minutes. Cells were washed of unbound antibody in FACS buffer, centrifuged

(250 x g, 5 minutes, 4°C) and resuspended in 200 µL FACS buffer after which transgenic TCRs were detected via Flow Cytometry.

2.3 Generation of Murine Bone Marrow-Derived Dendritic Cells (BMDC)

The tibiae and femurs of C57/BL6 mice were isolated aseptically and placed in Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, Paisley, Scotland) enriched with 5% heat inactivated foetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria). The prepared bones were washed in cold 70% ethanol for two minutes and rinsed twice in cold DPBS containing 5% FBS (DPBS5). The ends of the bones were removed and the bone marrow flushed through onto a sieve with a 25 gauge needle and 2 mL syringe filled with cold DPBS5. The cell suspension was collected in a 50 mL Falcon tube (BD BioSciences) and centrifuged for 7 minutes at 250 x g. The supernatant disposed of and 3-10 mL of warm ACK RBC lysis buffer added for 5 minutes to lyse RBCs. Residual cells were washed three times in DPBS5 and resuspended in 1 mL complete Dulbecco's Modified Eagle Medium (cDMEM; Gibco) supplemented with 5% FBS and 20 ng/mL Granulocyte Macrophage Colony Stimulating Factor (GM-CSF; R&D Systems, Minneapolis, MN, USA) (cDMEM5+GM) for counting. The cells were re-suspended at 1×10^6 cells/mL in cDMEM5+GM, plated into six well plates at around 5 mL per well and incubated at 37°C plus 10% CO₂ for four days. On day four approximately 75% of the culture media was removed and replaced with a corresponding volume of fresh media. On day six cells were harvested, washed with DPBS5 and resuspended at the correct concentration for pulsing with antigen.

2.4 Pulsing of BMDC with Antigen

Day 6 cultured BMDC were resuspended at 1×10^6 cells/mL and the treatments added at the concentrations listed below. DC were incubated with protein or adjuvants overnight,

except for the OVA peptides which were only added 2-3 hours prior to harvest due to the fact that no processing is necessary for these antigens to be presented on MHC class I or II molecules and thus less time is required.

Proteins or adjuvants used to stimulate DCs are listed below with the concentrations:

- Lipopolysaccharide (LPS): 5 µg/ml
- Unmethylated cysteine-phosphate-guanine (CpG; Gene Works): 5 µg/ml
- RHDV VLP (VP60): 50 µg/ml
- Tumour lysate (TL): 50 µg/ml
- Supernatant (undiluted) (SNU): 50 µg/ml
- Supernatant (concentrated) (SNC): 50 µg/ml
- VLP coupled to tumour lysate (VLP-TL): 50 µg/ml
- VLP coupled to SNU (VLP-SNU): 50 µg/ml
- VLP coupled to SNC (VLP-SNC): 50 µg/ml
- VLP coupled to SNU spiked with OVA (VLP-SN+OVA): 50 µg/ml
- OVA protein: 20 µL/ml
- OT-I OVA peptide (OVA₂₅₇₋₂₆₄/SIINFEKL): 100 µg/ml
- OT-II peptide (OVA₃₂₃₋₃₃₉): 100 µg/ml

2.5 CFSE Labeling of Splenocytes and Co-Culture with Murine Dendritic Cells

Background

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a highly fluorescent cellular dye. Spontaneous and permanent binding of CFSE to lysine and other available amines means that it can be used to covalently label stable intracellular and cell surface molecules. Upon cell division the fluorescent molecules are divided equally between each daughter cell and a halving of fluorescent intensity can be observed with each cycle of cell proliferation. Up to eight cell divisions can be seen before CFSE fluorescence can no longer be distinguished among background autofluorescence of unlabelled cells⁸⁰.

Day seven pulsed DC were harvested in warm DPBS5, counted and resuspended at 1×10^5 cells/ml.

Spleens were isolated from OT-I or OT-II mice, as appropriate, and single cell suspensions created by gently pressing the spleens through 70 μm cell strainers (BD Biosciences) with the plunger of a sterile 2 mL syringe. The cell suspensions were treated with ACK RBC lysis buffer and segregated for use in proliferation assays using carboxy fluorescein diacetate N-succinimidyl ester (CFSE; Sigma) or ELISA assays. The amount of cells required to generate supernatant for the ELISA assay was determined and these cells were removed and plated with DCs in 96 well plates at a DC:splenocyte ratio of 1:10. The remaining splenocytes were resuspended at 2×10^7 cells/mL in DPBS to be labeled with CFSE. DPBS with no FBS added is used for re-suspension at this step since FBS will quench the CFSE. Splenocytes were added to CFSE diluted 1 in 4000 in DPBS was added to splenocytes and these cells were incubated at room temperature for eight minutes. The tube was swirled gently every 2 minutes to ensure even labeling of the cells. Cells were washed three times in DPBS5, resuspended in cDMEM5, counted and plated with DCs in 24 well plates at a DC:splenocyte ratio of 1:10. Cells were incubated at $37^\circ\text{C} + 10\% \text{CO}_2$ for 72 hours. At this time point 100 μL per well of supernatant was removed from the ELISA plate for IFN- γ detection and cells from the CFSE plate analysed for proliferation by Flow Cytometry.

2.6 Thymidine Incorporation

In Experiments 13 and 14, proliferation at 72 hours was measured by H^3 -methyl thymidine (Amersham Biosciences, UK) incorporation. After 72 hours of co-culture (as above) in 96 well round-bottom plates (Nunc) 50 μCi of H^3 -methyl thymidine (Perkin Elmer, Boston, MA, USA) was diluted in a 50 μL volume of cDMEM, added to each well and the plate incubated a further 18 hours at 37°C in 10% CO_2 . Cells were harvested by TomTec HarvesterTM onto grid-printed filter mats. The filter mats were dried and placed in sample

bags along with 5 mL BetaPlate Scint™ (Wallac Oy, Turku, Finland). The sealed bags were slotted into plastic cassettes and proliferation was determined by incorporation of H³ thymidine into cells' DNA as measured by counting β-scintillation in a MicroBeta Plus Liquid Scintillation Counter™ (Wallac Oy). This reading was measured in counts per minute (cpm).

2.7 Fluorescent Labeling of Cell Surface Proteins for Flow Cytometric

Analysis

Background

Immunofluorescence is an indispensable means of detecting proteins of interest. Cell surface proteins can be labelled with specific antibodies which have been covalently bound to highly fluorescent markers known as fluorophores such as phycoerythrin (PE), a red fluorescent protein which emits orange-yellow light, or allophycocyanin (APC), which emits red light. Multiple proteins can be labelled in one cell preparation. Fluorophore-labelled antibodies recognise their cognate antigen, bind durably, and unattached antibodies are eliminated by washing. Passing cells through the flow cytometer in a stream of fluid allows identification of the cell based on its size (forward scatter), granularity (side-scatter) and fluorescent qualities. Attached fluorochromes are excited by lasers allowing their recognition by detection equipment.

For DC activation experiments DCs were pulsed as described in section 2.4 and harvested at 24 hours with ice cold DPBS into 15 mL falcon tubes (BD Biosciences). The cells were then centrifuged at 250 x g for 5 minutes at 4°C, supernatants were decanted and cells resuspended by vortex in the residual supernatant. Fluorophore-coupled anti-mouse antibodies and their equivalent isotype controls were added to the cells at 0.5 µg/mL (CD11c and the CD11c isotype control) or 1 µg/mL (all other antibodies). Cells were incubated with antibodies on ice in the dark for 30 minutes at the end of which 1 mL of FACS buffer was added to wash off unbound antibodies and the cells centrifuged (250 x g

for 5 minutes at 4°C). Cells were resuspended in 200 µL FACS buffer if cells were to be run through the FACSCalibur flow cytometer within a few hours. Alternatively, if cells were to undergo FACS analysis within a week cells were 'fixed' by resuspension in 200 µL 2% paraformaldehyde (PFA), wrapped in tin foil and stored at 4°C.

Antibodies used for labeling of cell surface proteins, and their isotype controls, are listed in Table 2.1.

Table 2.1. Antibodies (and the fluorophores to which they were conjugated) and isotype controls used in Flow Cytometry to identify DC activation markers CD40, CD80, CD86 and MHC-II

Fluorophore	Antibody	Catalogue Number	Supplier
APC	Anti-mouse CD11c, Clone: N418	117310	BioLegend
APC	Armenian Hamster IgG1, λ2 isotype control, Clone: HTK888	400911	BioLegend
PE	anti-mouse CD40 (3/23)	553791	BD Biosciences Pharmingen
PE	Rat IgG2a, κ Isotype control	553930	BD Biosciences Pharmingen
PE	Hamster anti-mouse CD80 (16-10A1)	553769	BD Biosciences Pharmingen
PE	Hamster IgG2, κ Isotype control	550085	BD Biosciences
PE	Rat anti-mouse CD86 (GL1)	553692	BD Biosciences Pharmingen
PE	Rat IgG2a, κ Isotype control	553930	BD Biosciences Pharmingen
PE	Rat anti-mouse I-A/I-E (M5/114.15.2)	557000	BD Biosciences Pharmingen
PE	Rat IgG2b, κ Isotype control	553989	BD Biosciences Pharmingen

For T cell proliferation assays co-cultured cells were harvested with ice-cold DPBS, centrifuged (250 x g, 5 minutes, 4°C), the supernatant discarded and the cells resuspended in the residual supernatant by vortex. The antibody listed in Table 2.2 was added to OT-I T cell co-cultures (0.5 µL).

Table 2.2. Details of antibody used to identify CD8+ splenocytes in OT-I CFSE proliferation assays

Fluorophore	Antibody	Catalogue Number	Supplier
APC	Rat anti-mouse CD8a, Clone: 53-6.7	553035	BD Pharmingen, BD Biosciences

The antibody listed in Table 2.3 was added to OT-II T cell co-cultures (0.5 μ L).

Table 2.3. Details of antibody used to identify CD4⁺ splenocytes in OT-II CFSE proliferation assays

Fluorochrome	Antibody	Catalogue Number	Supplier
APC	Rat anti-mouse CD4, Clone: RM4-5	553051	BD Pharmingen, BD Biosciences

Cells were incubated with antibodies on ice in the dark for 30 minutes. Unbound antibody was washed off by the addition of 1 mL FACS buffer, the cells centrifuged (250 x g, 5 minutes, 4°C), supernatant discarded and the cells resuspended in 200 μ L FACS buffer for FACS analysis. If cells were not to be run through the FACSCalibur within a few hours they were resuspended in 2% Paraformaldehyde (PFA) for FACS analysis within one week. (PFA at 8% was diluted 1 in 4 in FACS Buffer to create 2% PFA solution). At least 10,000 events were acquired in all flow cytometric analyses.

2.8 Culture of B16OVA Tumour Cells

Vials of B16OVA cells were removed from liquid nitrogen storage and thawed quickly in a 37°C waterbath. In a laminar flow hood DPBS5 was immediately added to the thawed cells and the cells centrifuged at 300 x g for 5 minutes at 4°C. (This removes the freezing mix which contains the cryoprotectant dimethyl sulfoxide (DMSO) added to prevent cell death during freezing). The supernatant was discarded and the cells resuspended in 10 mL Iscove's Modified Dulbecco's Medium (IMDM). A further 20 mL IMDM was aliquotted into a BD Falcon tissue culture flask (TCF; BD Biosciences) and the resuspended cells pipetted into the TCF. Cells were incubated at 37°C with 5% CO₂. Cells were grown to approximately 80% confluence prior to being split into two (or more) tissue culture flasks for further expansion of cell numbers.

Cells were split by pouring and pipetting off non-adherent cells into a falcon tube and adding enough warm TrypLE Express (Invitrogen) to cover the surface of the TCF. (TrypLE is

a recombinant trypsin-like enzyme used for the dissociation of attachment-dependent cell lines from plasticware). The TCF was incubated at 37°C with 5% CO₂ for 3-5 minutes until adherent cells lifted off into suspension. An equal volume of FBS was added to quench the trypsin reaction, the TCF surface washed with DPBS and the adherent cell suspension added to the non-adherent cells. The falcon tube was topped up with DPBS and centrifuged at 250 x g for 7 minutes at 20°C. The supernatant was discarded and the cells resuspended in 10 mL IMDM5+Geneticin. The cells were divided equally between two fresh TCFs and media added up to 25 mL. The cells were incubated at 37°C with 5% CO₂ until they reached approximately 80% confluence.

Once the desired volume of cells was reached (up to 10 x 175cm² TCFs) cells in active growth (log) phase (approximately 80% confluent) were treated with 10 µg/mL Brefeldin-A (Sigma) for 16 hours prior to harvest to allow OVA protein to accumulate inside the cells.

2.9 Preparation of Tumour Lysate

Tumour lysate (TL) preparation was carried out under sterile conditions, on ice, in a laminar flow hood, 16 hours after the addition of Brefeldin-A to the B16OVA tumour cells. Cells were harvested using Hanks Buffered Salt Solution (HBSS) Complete mini-EDTA-free protease inhibitor (PI) tablets (Roche Diagnostics Ltd, Mt Wellington, Auckland, NZ) were dissolved in coupling PBS. Brefeldin-A-treated B16OVA cells were resuspended in the protease inhibitor cocktail before being subjected to four repeat cycles of freeze-thaw lysis at -80°C and 37°C respectively. The protease inhibitor solution was important for minimizing protein degradation by proteases released during lysis. The resultant lysate was sheared by slowly aspirating and decanting the TL with a 20 gauge needle and 5 mL syringe. Approximately half the volume of the TL was aspirated and released to avoid creating bubbles which denature proteins. Shearing is necessary to break up the viscosity of the crude TL as viscous TL will not centrifuge into a soluble fraction and an insoluble pellet. The total protein

concentration was ascertained by spectrophotometer (Nanodrop, Thermo Fisher) where 1 A280 = 1 mg/mL. SDS-PAGE and immunoblotting (Western Blot) using mouse anti-OVA antibodies were employed to ascertain the presence of OVA protein in the lysate. (See Section 2.14))

2.10 Concentration of B16OVA tumour cell supernatant

Two milliliters (2000 μ L) of B16OVA tumour cell supernatant was concentrated down to 20 μ L, a 100 fold concentration by Trichloroacetic Acid (TCA) protein precipitation in order to increase the protein concentration including that of OVA, our model antigen. The supernatant (500 μ L) was incubated with equal volumes of 10% TCA, on ice, for 20 minutes and then centrifuged at full speed for 15 minutes at 4°C. No pellet was visible so the incubation and centrifugation steps were repeated. Again no pellet was visible so a further 35 minute incubation in 100% TCA was carried out followed by 15 minute centrifugation at 4°C. Once again no pellet was seen but we proceeded to the ethanol wash step at this stage. The supernatant was pipetted off and the 'pellet' washed in 500 μ L of ice-cold ethanol. The ependorf tube was re-spun in an effort to keep what little pellet there was stuck to the tube and the ethanol was pipetted off. The lid of the ependorf tube was left open so the pellet could dry for 15 minutes at room temperature. The concentrated supernatant was diluted one in ten for a final 10 x concentration. SDS-PAGE sample buffer was added (20 μ L) and the pellet resuspended by slowly pipetting up and down 10-20 times. The pH was tested by placing a 10 μ L drop onto pH paper (the pH should be 6.8). The concentrated supernatant was stored overnight at -20°C and the presence of OVA detected by Western blot the next day.

2.11 Preparation of Rabbit Haemorrhagic Disease Virus-like Particles

Preparation of RHDV VLP was carried out by the Ward Lab (Department of Microbiology and Immunology, University of Otago, Dunedin, NZ), according to methods previously described ⁸¹. Briefly, sf21 insect cell cultures were inoculated with recombinant baculovirus which expressed the VP60 gene. Released VLP were purified and separated on a cesium chloride (CsCl) step gradient. The VLP band was removed and checked for purity by resolving the VLP on 10% denaturing acrylamide gels and staining with Coomassie Brilliant Blue G250. The resultant VLP were stored in 50% glycerol to be used as VLP alone, or in chemical conjugation reactions.

2.12 Coupling Tumour Lysate, Supernatant and OVA to RHDV VLP

Conjugation of RHDV VLP to tumour lysate, supernatant, and concentrated supernatant, was also carried out by the Ward Lab (Department of Microbiology and Immunology, University of Otago, Dunedin, NZ), according to methods previously described ⁸¹². VLP-conjugates were stored in 50% glycerol at -20°C and used on ice during DC pulsing.

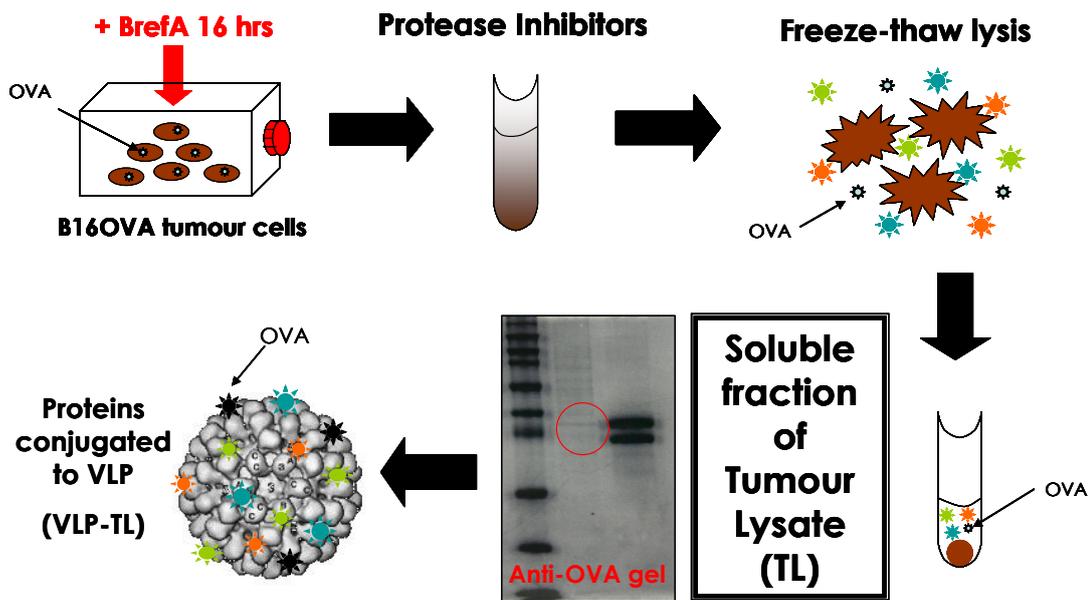


FIGURE 2.1 **Flow Diagram overview of TL conjugation to VLP.** Brefeldin-A-treated cells are resuspended in a solution of protease inhibitors prior to freeze-thaw lysis. Centrifugation yields a pelleted insoluble fraction which is discarded. The soluble fraction is analysed for OVA content and undergoes conjugation to VLP.

2.13 Gel Electrophoresis

VLP-conjugates and tumour lysates were boiled for 5 minutes in sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2-ME; Gibco, Paisley, Scotland) and evaluated on 10% and 8% SDS-polyacrylamide gels, with 0.1% SDS, using the Laemmli buffer system (Laemmli, 1970). Broad Range (New England Biosciences, Beverly, MA, USA) and Prestained Novex (Invitrogen, Carlsbad, CA, USA) molecular weight markers were loaded alongside samples. Polypeptide bands were visualized using Coomassie Brilliant Blue-G250 (BDH Chemicals, Poole, UK).

2.14 Immunoblotting (Western Blot)

Protein immunoblotting uses protein-specific antibodies to identify proteins of interest and was used to detect OVA in the tumour lysate and supernatant. Tumour lysate, concentrated supernatant and VLP conjugates were run through 10% or 8% SDS-polyacrylamide gel electrophoresis and transferred onto methanol rinsed polyvinylidene difluoride (PVDF) membranes (Millipore, Carlsbad, CA, USA). The membrane, along with Whatman 3MM filter paper (Maidstone, UK) was soaked in anode or cathode buffer for ten minutes before being assembled on a Trans-Blot Semi-Dry Blotter (BioRad, Hercules, CA, USA). The transfer ran for 30 minutes at 22 volts and 264 mA. The membrane was then rinsed in methanol and left to dry overnight on filter paper.

2.14.1 Detection of OVA protein

Anti-OVA (A6075, Sigma) (2 μ L) was diluted in 20 mL of sodium casein alanate solution. All antibody dilutions were previously determined by Ward lab members. The dry membrane was placed in the solution and incubated for 1 hour at room temperature. Three washes were carried out where the membrane was placed on the rocker for five minutes in PBS with 0.02% Tween. The particular bands were detected using the SuperSignal® West Pico chemiluminescence system (Thermo Scientific) and the image acquired by ChemiDoc (BioRad).

2.14.2 Detection of RHDV VLP

The anti-OVA-treated membrane was stripped of anti-OVA antibody. Stripping buffer was heated to 50°C and the membrane incubated in the hot buffer for 30 minutes with rocking. This was followed by four 10 minute washes in 1% Tris buffered saline. Methanol

was added until the membrane turned opaque; the membrane was then washed in milliQ water and incubated in 1% BSA in PBS Tween overnight at room temperature with rocking. RHDV detection proceeded with the membrane being incubated with 30 μ L of anti-SH3 (SH3 is a peptide on RHDV VLP) (anti-SH3 generated by Ward lab; concentration unknown) diluted in 20 mL sodium casein alanate solution, for 1 hour at room temperature with rocking. Three 10 minute washes in PBS Tween were carried out and the membrane further incubated with anti-rabbit HRP (6 μ L diluted in 20 mL sodium casein alanate solution) for 1 hour at room temperature with rocking. The membrane was washed a further three times (10 minutes each) in PBS Tween and the antibody-specific bands detected using the SuperSignal® West Pico chemiluminescence system as above.

2.15 Enzyme Linked Immunosorbent Assay (ELISA)

Background

ELISA is a common biochemical method used to detect antibodies or antigens in a sample. Briefly, an unspecified quantity of antigen is non-specifically adsorbed onto a micro-titre plate and then a specific antibody for that antigen is added. The antibody is bound to an enzyme which changes colour when the enzyme's substrate is added, allowing detection of the antibody-antigen binding reaction.

Alternatively a 'sandwich ELISA' can be carried out where a capture antibody is first adsorbed onto the plastic well plate and the antigen of interest added, which binds to this antibody. A detection antibody is then added, which forms a complex with the antigen. Finally, an enzyme-linked secondary antibody is added which binds to the detection antibody, and again, once the enzyme's substrate is applied, the initial antibody-antigen binding reaction can be identified by a colour change which is measured by spectrophotometry. All ELISA assays involve the establishment of a standard curve with known quantities of the antigen or antibody of interest. The spectrophotometric read-outs of your sample are then measured against the standard curve.

2.15.1 Direct Anti-OVA ELISA

To create a standard curve OVA protein (Sigma) was diluted to 20 ng/mL in coating buffer, 100 µL dispensed into wells A1, B1, A2 and B2 and doubling dilutions carried out to wells A11, B11. Wells A12, B12 were left blank. Serum-free supernatant from B16OVA tumour cells was added (100 µL per well) to Rows B1-12 and C1-12, the plates wrapped in cling film and incubated overnight at 4°C. The plate was washed six times in wash buffer and blotted on absorbent paper to remove excess liquid. Non-specific binding was blocked with 200 µL/ well blocking buffer per well and the plate incubated for 1 hour at 37°C. The primary antibody (rabbit anti-OVA (courtesy of S. Hook, Pharmacy Dept)) was diluted to 4 µg/mL in coating buffer and 100 µL dispensed into each well. The plate was washed six times, blotted and 100 µL/well of Goat anti-mouse horseradish peroxidase conjugate (HRP; BD Pharmingen) was added (diluted 1 in 5000 in blocking buffer) and the plate incubated for 20 minutes at 37°C. A further six washes and blotting were carried out before the addition of 100 µL/well of cold tetramethylbenzidine (TMB) substrate (TMB Single Solution (Ready to use), Invitrogen Corporation, Camarillo, CA, USA, Cat #: 00-2023). This reaction was stopped with 100 µL/well of 1N H₂SO₄ and the absorbance read at 450 nm within 30 minutes on a BioRad Microplate Reader.

2.15.2 Indirect Anti-OVA ELISA

Rabbit anti-OVA (courtesy of S. Hook, Pharmacy Dept) was diluted to 4 µg/mL in coating buffer and 100 µL dispensed into BD Falcon Microtest 96 well ELISA plates (BD Biosciences) which were wrapped in cling film and incubated overnight at 4°C. The plates was washed six times in wash buffer and blotted on absorbent paper to remove excess liquid. Non-specific binding was blocked with 200 µL/ well blocking buffer per well and the plate incubated for 1 hour at 37°C. The plate was washed again, six times, and blotted. Blocking

buffer (100 μ L) was dispensed into wells A1, B1 through A12, B12. The OVA standard was diluted in blocking buffer to 20 ng/mL and 100 μ L dispensed into wells A1, B1, A2 and B2. Doubling dilutions were carried out to wells A11, B11 and Wells A12, B12 left blank. The OVA-containing tumour cell supernatant was added (100 μ L per well) and the plates incubated for two hours at 37°C. Plates were again washed six times, blotted and 100 μ L of mouse anti-OVA (BD Pharmingen, BD Biosciences, Cat: 554410) added to each well (2 μ g/mL). The plates were incubated for 30 minutes at 37°C and again washed six times and blotted. Goat anti-mouse horseradish peroxidase conjugate (Strep-HRP; BD Pharmingen) was added (100 μ L /well, diluted 1 in 3000 in blocking buffer) and the plate incubated for 20 minutes at 37°C. A further six washes and blotting were carried out before the addition of 100 μ L/well of cold tetramethylbenzidine (TMB) substrate (TMB Single Solution (Ready to use), Invitrogen Corporation, Camarillo, CA, USA, Cat #: 00-2023). This reaction was stopped with 100 μ L/well of 1N H₂SO₄ and the absorbance read at 450 nm within 30 minutes on a BioRad Microplate Reader.

2.15.3 Anti-IFN- γ ELISA

Purified rat anti-mouse interferon-gamma (IFN- γ) (BD Pharmingen, BD Biosciences, Cat 551216) was diluted to 4 μ g/mL in coating buffer and 50 μ L dispensed into BD Falcon Microtest 96 well ELISA plates (BD Biosciences) which were wrapped in cling film and incubated overnight at 4°C. Plates were washed six times in wash buffer and blotted on absorbent paper to remove excess liquid. Non-specific binding was blocked with 200 μ L/well blocking buffer per well and the plate incubated for 1 hour at 37°C. Plates were washed again, six times, and blotted. Blocking buffer (100 μ L) was dispensed into wells A1, B1 through A12, B12. The interferon- γ standard was diluted in blocking buffer to 10 ng/mL and 100 μ L dispensed into wells A1, B1, A2 and B2. Doubling dilutions were carried out to wells A11, B11 and Wells A12, B12 left blank. Samples were added (100 μ L per well) and the plates incubated for two hours at 37°C. Plates were again washed six times, blotted and

100 μ L of biotinylated rat anti-mouse IFN- γ (BD Pharmingen, BD Biosciences, Cat 554410) added to each well (2 μ g/mL). The plates were incubated for 30 minutes at 37°C and again washed six times and blotted. Streptavidin-horseradish peroxidase conjugate (Strep-HRP; BD Pharmingen) was added (100 μ L /well, diluted 1 in 3000 in blocking buffer) and the plates incubated for 20 minutes at 37°C. A further six washes and blotting were carried out before the addition of 100 μ L/well of cold tetramethylbenzidine (TMB) substrate (TMB Single Solution (Ready to use), Invitrogen Corporation, Camarillo, CA, USA, Cat #: 00-2023). This reaction was stopped with 100 μ L/well of 1N H₂SO₄ and the absorbance read at 450 nm within 30 minutes on a BioRad Microplate Reader.

2.16 Fluorescence Assisted Cell Sorting (FACS)

DC and T cell phenotype was analysed by flow cytometric analysis. Cultured cells were harvested with ice cold DPBS into 15 mL Falcon tubes (BD), centrifuged for 5 minutes at 250 x g (4°C) and the supernatant discarded. The cells were resuspended in 800 μ L ice cold FACS buffer transferred to FACS tubes and centrifuged again. The supernatant was discarded, the cells resuspended by vortex in the remaining liquid and antibodies added to the appropriate tubes. The cells were incubated on ice for 30 minutes with their respective fluorochrome-conjugated anti-CD11c (0.5 μ L), anti-CD40 (1 μ L), anti-CD80 (1 μ L), anti-CD86 (1 μ L) or anti-I-A (mouse MHC-II) (1 μ L). After incubation the tubes were topped up with 1 mL of FACS buffer and centrifuged (250 x g, 5 minutes, 4°C). The supernatant was discarded and the cells were resuspended in FACS buffer and processed via flow cytometry within a few hours. Alternatively cells were fixed in 2% paraformaldehyde (PFA) for flow cytometric analysis within a week. Fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson) and analysed with FlowJo software version 9.3.2 (Treestar)

2.17 In vivo Cytotoxicity

Four groups of four C57/BL6 mice received intra-tail vein (i.v.) vaccinations of RHDV VLP, TL, VLP-TL or VLP conjugated to OVA. Each treatment was diluted in 100 μ L PBS so that each mouse received 50 μ g of the treatment in an equal volume of PBS. One week later 11 donor C57/BL6 mice were sacrificed (1 donor per 1.5 recipients) and cell suspensions of splenocytes prepared as described previously (See Section 2.5). Half of the splenocytes were pulsed for 2-3 hours with 1 μ g/mL SIINFEKL (the target peptide of the OVA protein) and labeled with a high concentration (1/2500 dilution) of CFSE (as described previously, see 2.5 Co-Culture of Murine DC with Splenocytes). The remaining splenocytes were incubated for 2-3 hours without SIINFEKL peptide and labeled with a low concentration (1/25000 dilution) of CFSE (as described previously, see Section 2.5). The CFSE^{HI} and CFSE^{LO} splenocytes were mixed together at a 1:1 ratio and delivered i.v. into the tail veins of the vaccinated mice. Each mouse received a total of 2×10^7 CFSE-labelled splenocytes (1×10^7 CFSE^{HI} cells and 1×10^7 CFSE^{LO} cells). Three days later the vaccinated mice were sacrificed and single cell suspensions made of their splenocytes (see Section 2.5). A 10% dilution of the splenocytes was prepared for flow cytometric analysis and run through the FACSCalibur on a low flow rate.

2.18 Calculation of Percentage of Specific Cell Lysis

The following calculation was used to ascertain the specific lytic activity of cytotoxic T lymphocytes (CTLs) in the *in vivo* cytotoxicity assay:

$$(1 - \text{ratio unprimed} / \text{ratio primed}) \times 100$$

Unprimed denotes the negative control treatment (VP60 in our experiment) and primed signifies each treatment (TL, VLP-TL) or the positive control (VLP-OVA).

The ratio of the unprimed is calculated as the percentage of CFSE^{LO} divided by the percentage of CFSE^{HI}.

2.19 Software

Quantity One® 1-D analysis software version 4.6.6 (BioRad) was used for image acquisition of chemiluminescent Western Blot membranes.

FACSCalibur™ (BD) flow cytometric acquisition data was generated in CellQuest™Pro version 5.2.1 (BD Biosciences) and exported to FlowJo 9.3.2 (TreeStar) for analysis.

IFN- γ production data by T cells was generated by Microplate Manager version 5.2.1 (BioRad).

Thymidine incorporation results were produced by MicroBeta Scint.

2.20 Statistical Analyses

Where statistics are shown Students two-tailed paired t-tests were performed using GraphPad Prism version 5.0 (La Jolla, CA, USA). P-values of 0.05 or less were deemed to be significant. Bonferroni correction post-tests were applied.

Chapter 3: Results

3.1 DC Exposed to Antigen Display Typical Maturation Phenotype

Dendritic cells were generated from bone marrow precursors. Using this culture system we showed that 70% of cells expressed CD11c, a marker which is specific to DC (Fig 3.1). Functional activity of DCs is denoted by the elevated expression of both major histocompatibility complex class II (MHC-II; also known as I-A) and the co-stimulatory molecules CD40, CD80 and CD86. We examined the effect of the known DC activators bacterial lipopolysaccharide (LPS; Sigma) and unmethylated cysteine-phosphate-guanine (CpG; Gene Works) on dendritic cell maturation which is characterised by upregulation of the cell surface markers CD40, CD80, CD86 and MHC-II. Bone marrow-derived dendritic cells (BMDC) were pulsed with (exposed to) LPS or CpG overnight and analysed via flow cytometry (Fig 3.1). An increase was seen in the percentage of cells positive for CD40, CD80 and CD86 and MHC-II. An increase in the mean fluorescence intensity (MFI) for MHC-II was seen in DC exposed to LPS and CpG compared to un-pulsed DC. (Fig. 3.2) Approximately 10% of untreated DC displayed CD40 (Fig 3.2). This figure increased dramatically to around 70% of DC upon exposure to LPS and around 60% upon exposure to CpG (Fig. 3.2). Around 20% of immature DC were positive for CD80 and this figure jumped to around 50% on exposure to LPS or CpG (Fig. 3.2a). The percentage of un-activated DC displaying CD86 was between 30 to 55% and this percentage hiked to around 70% post stimulation (Fig. 3.2). MHC-II was present on 50-80% of untreated DC and this number increased only slightly after treatment (Fig. 3.2a). In addition an increase in the mean fluorescence intensity (MFI) for MHC-II was seen in DC exposed to LPS and CpG compared to un-pulsed DC. (Fig. 3.3)

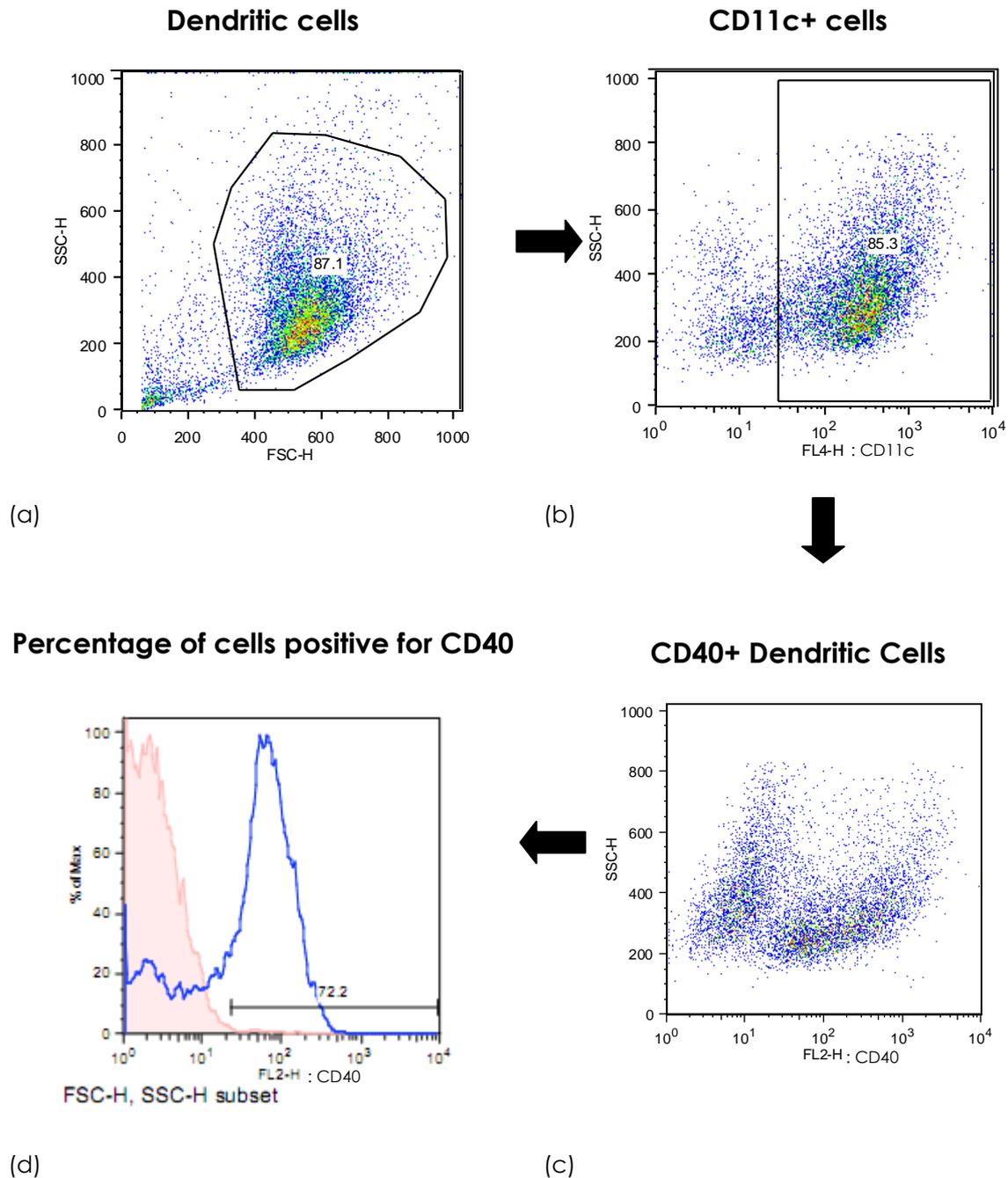


FIGURE 3.1. **Dendritic cell gating strategy.** Representative images showing (a) gating of dendritic cells based on where the cells sit in the FSC SSC dot plot (b) selection of CD11c positive cells based on cells positive for the fluorochrome 4 (FL4: APC) (c) selection of cells positive for specific markers (CD40, CD80, CD86 or MHC-II) based on fluorochrome 2 (FL2: PE) (d) Histogram of PE-positive cells showing 'true negative' isotype control (shaded) versus DC pulsed with LPS (solid line). The marker figure indicates the percentage of DCs treated with LPS positive for the activation marker CD40.

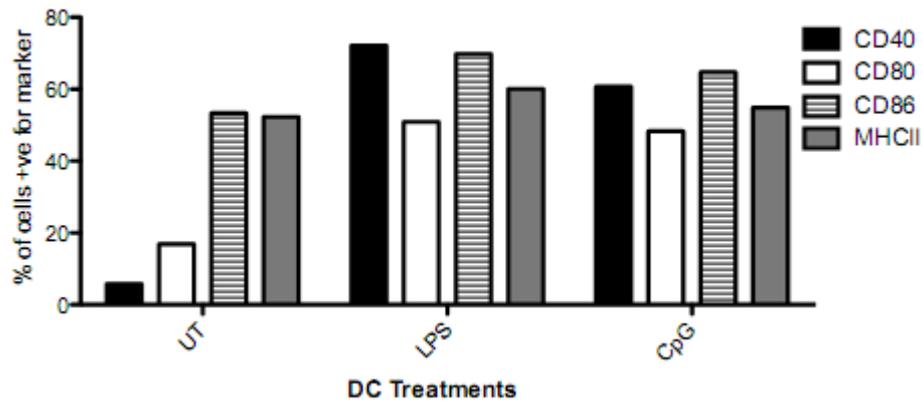


FIGURE 3.2. **LPS and CpG induce increased expression of CD40, CD80, CD86 and MHC-II on GM-CSF differentiated DCs.** Bone marrow precursor cells cultured for 6 days with GM-CSF (20 ng/mL) were pulsed with LPS (5 μ g/mL) or CpG (5 μ g/mL) and harvested on Day 7. Differentiated, pulsed dendritic cells (BMDC) were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the percentage of cells positive for the markers analysed by Flow Cytometry. Representative quantitative example of flow cytometric analysis of eight samples.

Markers CD40 and CD80 showed subtle increases in MFI (Fig. 3.3) while the MFI of CD86 approximately doubled. As expected the MFI of MHC-II started higher and increased greatly in response to bacterial stimulation.

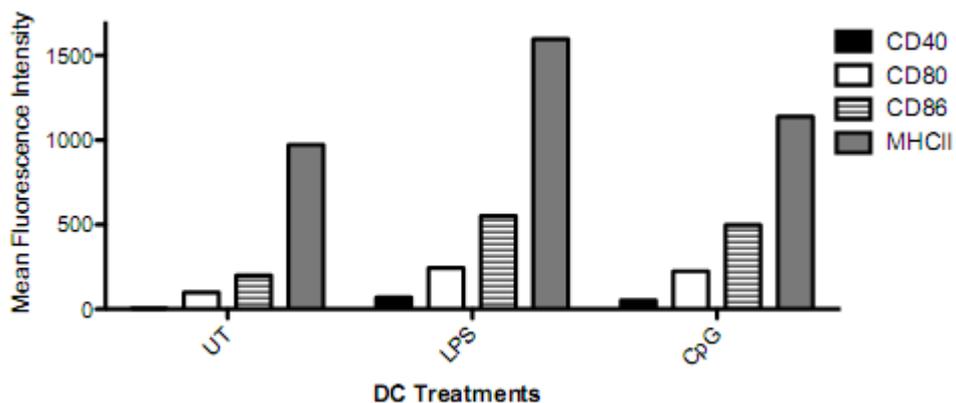


Figure 3.3. **LPS and CpG induce increased mean fluorescence intensity (MFI) of CD40, CD80, CD86 and MHC-II on GM-CSF differentiated DCs.** Bone marrow precursor cells cultured for 6 days with GM-CSF (20 ng/mL) were pulsed with LPS (5 μ g/mL) or CpG (5 μ g/mL) and harvested on Day 7. Differentiated, pulsed dendritic cells (BMDC) were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the MFI of the markers analysed by Flow Cytometry. Representative quantitative example of eight flow cytometric analyses.

3.2 Detection of OVA Protein in Tumour Lysate, B16OVA Tumour Cell Supernatant and VLP Conjugates

In order to detect the presence of our model antigen in the tumour lysate immunoblotting (Western Blotting - WB) was initially carried out on B16OVA tumour cell lysate. This assay detected no OVA protein in this lysate. In order to increase the level of OVA protein B16OVA cell supernatant was concentrated and a further SDS-PAGE gel detection of OVA was carried out (Fig. 3.4). Two faint bands were visible on the gel at the level of the OVA positive control bands.

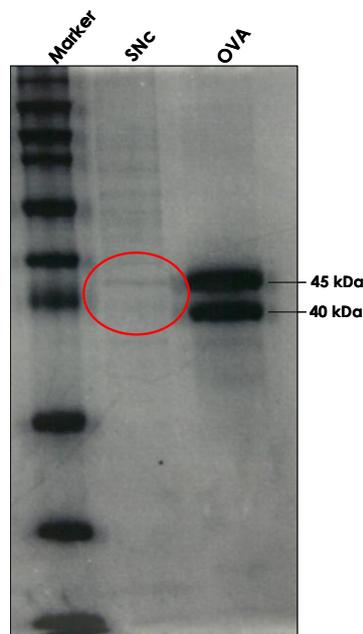


FIGURE 3.4 OVA protein can be detected in concentrated B16OVA tumour cell supernatant. Ten times concentrated B16OVA supernatant (obtained by TCA precipitation) was subjected to SDS-PAGE. OVA protein was run as a positive control. The masses (in kilo Daltons) of the two key OVA proteins are indicated. Lanes: 1, Broad Range protein molecular weight marker; 2, concentrated supernatant (SNc); 3, OVA positive control

In order to ascertain whether or not OVA could be detected in actual tumour lysate Brefeldin-A-treated B16OVA cells, in which OVA had accumulated prior to lysis, were assayed for OVA by WB, along with VLP coupled to TL (VLP-TL), VLP coupled to OVA (VLP-OVA) and VLP coupled to TL or SN which had been spiked with OVA (Fig. 3.5). Faint but clear OVA bands were seen in the VLP-OVA and VLP-TL+OVA but not in the VLP-SN+OVA,

indicative of free OVA in these first two preparations. No OVA was observed in VLP-TL or either of the two tumour lysate batches assayed (TL2 and TL3).

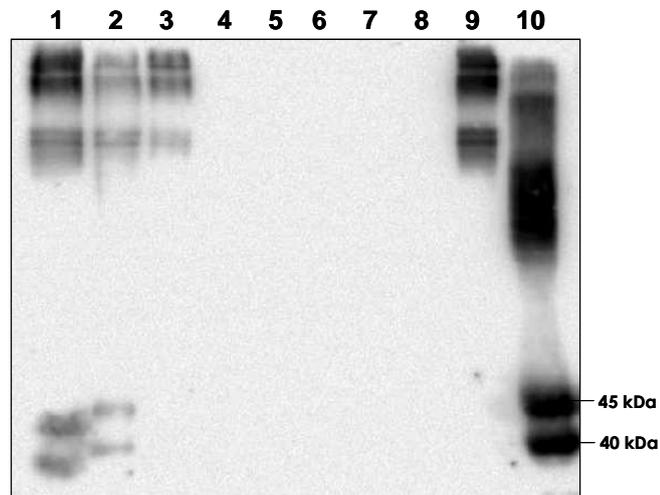


FIGURE 3.5 OVA protein can be detected in VLP conjugates containing OVA but not in VLP-SN+OVA, VLP-TL or B16OVA tumour cell lysate. B16OVA tumour cells were treated with Brefeldin-A for 16 hours prior to lysis. The lysate and VLP-conjugates were subjected to SDS-PAGE followed by Western Blot. Lanes: 1, VLP-OVA; 2, VLP-TL+OVA; 3, VLP-SN+OVA; 4, VLP-TL2; 5, TL2; 6, TL3; 7, VLP3 (VP60); 8, marker lane; 9, Dialysed VLP-OVA; 10, OVA.

To ascertain the presence of VLP in the VLP-conjugates an anti-RHDV VLP WB was also performed (Fig. 3.6). Clear bands at the 60 kDa molecular weight level show the presence of VLP, in the VLP-OVA, VLP-TL+OVA, VLP-SN+OVA and VLP3 lanes. Taken together, bands in the high molecular weight region (around 105 kDa) of both probes give an imprecise indication that conjugation between VLP and OVA has occurred in VLP-OVA, VLP-TL+OVA, VLP-SN+OVA. VLP and not OVA was seen in the VLP-TL, indicating that the level of OVA coupled to VLP, amidst the other TL proteins, was below the threshold of detection of this assay.

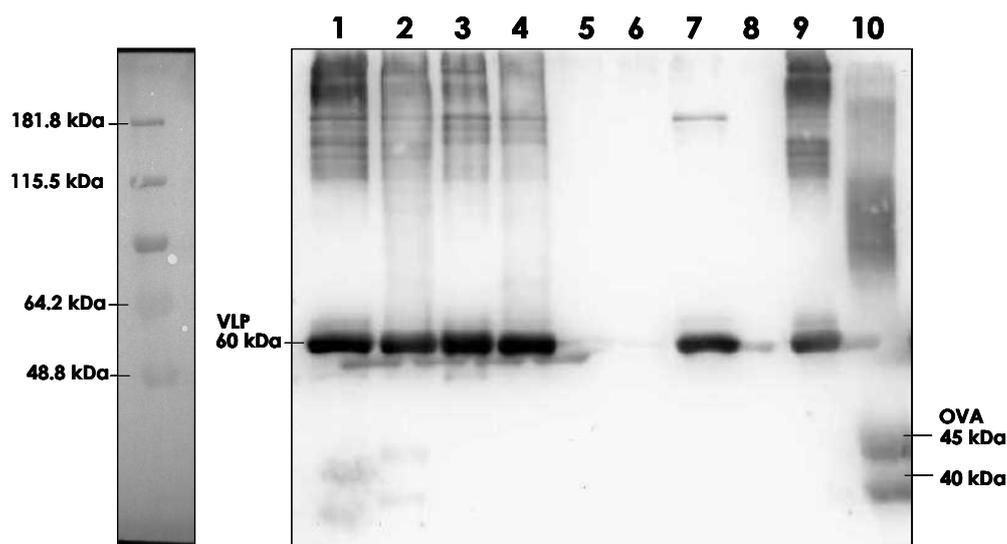


FIGURE 3.6 **RHDV VLP can be detected in VLP conjugates.** Following OVA detection the membrane was stripped of OVA protein, blocked and incubated with anti-SH3 and finally anti-rabbit HRP. Enhanced Chemiluminescence (ECL) detection by ChemiDoc was then carried out. Left panel: Novex molecular weight marker. Right panel: anti-SH3 Western Blot. Lanes: 1, VLP+OVA; 2, VLP-TL+OVA; 3, VLP-SN+OVA; 4, VLP-TL2; 5, TL2; 6, TL3; 7, VLP3 (VP60); 8, marker lane; 9, Dialysed VLP-OVA; 10, OVA.

Due to the negative result from the initial WB more sensitive anti-OVA ELISA assays were also undertaken. Coating the ELISA plate with OVA (direct ELISA) yielded 10.24 ng/mL OVA in the undiluted supernatant of B16OVA cells grown in serum-free media (See Appendix 2, Table S2.1). Coating the ELISA plate with anti-OVA antibodies (indirect ELISA) gave a reading of 0.128 ng/mL (See Appendix 2, Table S2.2). Both readings had very low optical densities (OD). The tumour lysate was not assayed by anti-OVA ELISA.

3.3. VLP-TL is Able to Induce Upregulation of Dendritic Cell Maturation Markers

Instead of generic activators DC were next pulsed with various treatments (Table 3.1) to assess their impact on DC phenotype. Supernatant (SNu) refers to the conditioned medium in which tumour cells were cultured.

Table 3.1: Abbreviations and definitions of treatments used in DC activation experiments

Abbreviation	Definition
UT	Untreated (DC negative control)
VLP	Virus-like particle alone
TL	Tumour Lysate alone
VLP-TL	VLP conjugated to TL
SNu	Supernatant undiluted
VLP-SNu	VLP conjugated to SNu
SNc	Supernatant concentrated
VLP-SNc	VLP conjugated to SNc
VLP-SN+OVA	VLP conjugated to SNu which had been spiked with OVA
LPS	Lipopolysaccharide (DC functional control)

In some experiments the treatments caused either little change or an inhibitory response when compared to untreated DC (see Appendix 2, Supplementary Data, Fig. S2.9). The activation marker CD40 was generally upregulated the most in response to VLP coupled to tumour lysate (VLP-TL) or concentrated supernatant (SNc) (Fig. 3.7). TL, SNc or VLP-TL were all able to cause upregulation of the co-stimulatory molecule CD80 to very similar degrees (Fig. 3.7). VLP-TL often caused the highest increase in the level of the co-stimulatory molecule CD86, though the difference was not great between VLP-TL and TL. Finally, in three out of four experiments the greatest increase in MHC-II MFI was seen in response to VLP-TL and this was mirrored in the percentage of cells positive for MHC-II. However, the difference between VLP-TL and VLP-SNu or VLP-SNc was minimal in terms of cells positive for MHC-II (Fig. 3.7 and Appendix 2, Supplementary Data, Fig. S2.7 and S2.8). The results for the various supernatant treatments (SNu, SNc, VLP-SNu and VLP-SNc) were much less consistent with VLP-SNu or VLP-SNc usually effecting either no difference or an inhibitory result (Fig. 3.7, Fig. 3.8 and Appendix 2, Supplementary Data, Fig. S2.7 and S2.8) as compared to VLP, TL or VLP-TL.

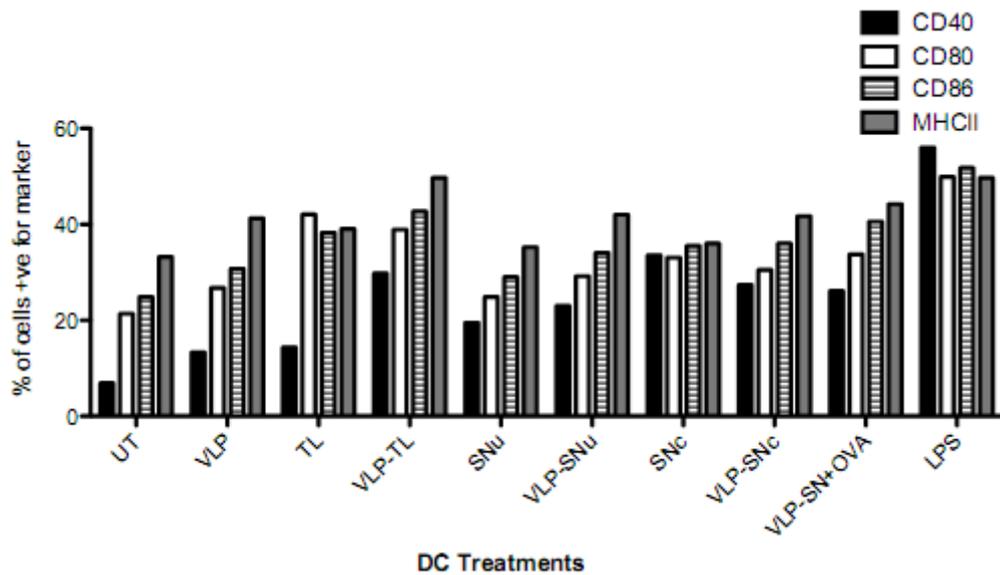


FIGURE 3.7 VLP-TL is able to induce upregulation of dendritic cell maturation markers. Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the percentage of cells positive for the markers analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNu: undiluted supernatant; VLP-SNu: VLP coupled to SNu; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

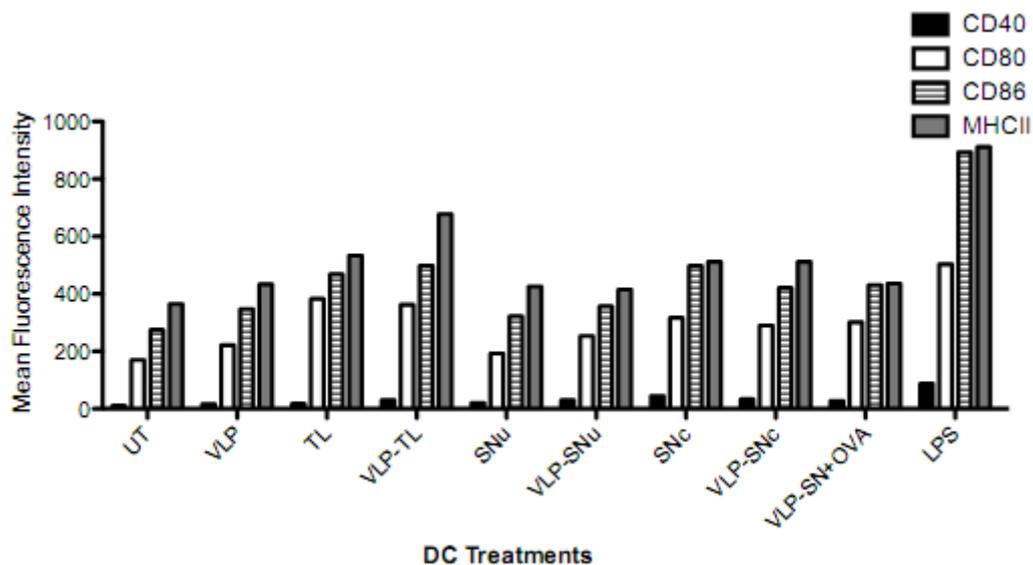


FIGURE 3.8. VLP-TL stimulates an improved upregulation of DC MFI. Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the MFI analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNu: undiluted supernatant; VLP-SNu: VLP coupled to SNu; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

3.4 T Cell Proliferation

Having assessed the effect on DC phenotype we now investigated whether the activated DC phenotype correlated with T cell proliferation indicative of the generation of cytotoxic T cells.

3.4.1 VLP-TL-Stimulated Proliferation as Assessed by Thymidine Uptake is Superior to That of VLP or TL Alone

T cell proliferation is one of the first indicators of T cell activation. Thymidine incorporation into the DNA of proliferating cells was used as a measure of T cell proliferation in two experiments and in both assays VLP-TL was found to be superior to all treatments at stimulating OT-I T cell proliferation (Fig. 3.9). 'Error bars' are only shown to visually compare the two different experiments. There were no replicates or multiple counts within each experiment.

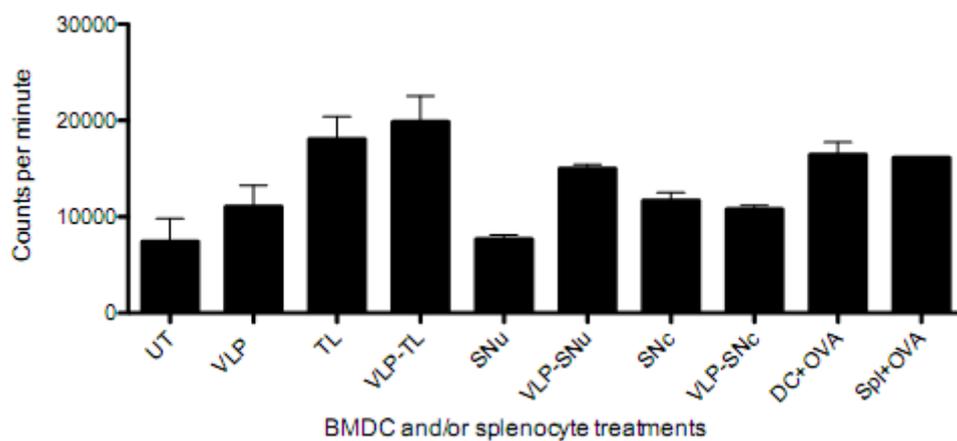


FIGURE 3.9. **VLP-TL-stimulated thymidine uptake is superior to that of VLP or TL alone.** 50 μ Ci of H³-methyl thymidine was added to splenocytes which had been cultured with antigen or protein-pulsed DC for 72 hours. After 18 hours of incubation thymidine incorporation was measured by counting β -scintillation (counts per minute). UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNU: undiluted supernatant; VLP-SNU: VLP coupled to SNU; SNC: concentrated supernatant; VLP-SNC: VLP coupled to SNC; DC+OVA: DC pulsed with whole OVA protein; Spl+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC. Results of the two experiments carried out are shown.

3.4.2 VLP-TL Can Induce Improved Proliferation of CD8+ Splenocytes

Labelling splenocytes with the dye carboxy fluorescein diacetate N-succinimidyl ester (CFSE) is an alternative method of determining cell proliferation. After 72 hours of co-culture with DCs pulsed with the various treatments (VLP, TL, VLP-TL, SNU, VLP-SNU, SNC, VLP-SNC) OT-I splenocytes (the bulk of which are T cells) were stained with anti-CD8+ and analysed by flow cytometry for proliferation (Fig. 3.10). DCs which had not been pulsed with antigen or protein (UT) were used as a negative control. DC that had been pulsed with VLP coupled to supernatant which was spiked with OVA (VLP-SN+OVA) was used as one functional control. Spiking the supernatant with OVA ensured that OVA, to which the OT-I T cells are exquisitely sensitive, was highly likely to be coupled to the VLP and would therefore generate a good proliferative response. This is exactly what was seen (Fig. 3.11). The OT-I OVA peptide SIINFEKL which requires no processing by DC was used as a secondary functional control. The first experiment showed that VLP-TL was superior to all other treatments at stimulating OT-I T cell proliferation (Fig. 3.11). One further test obtained the same finding but with a vastly diminished increase in actual proliferation numbers across all treatments (Appendix 2, Supplementary Data, Table S2.4). While only two of the four assays returned a result of VLP-TL being superior to all other treatments, in three of the four VLP-TL stimulated higher proliferation than either VLP alone or TL alone, although the difference between VLP and VLP-TL were often small.

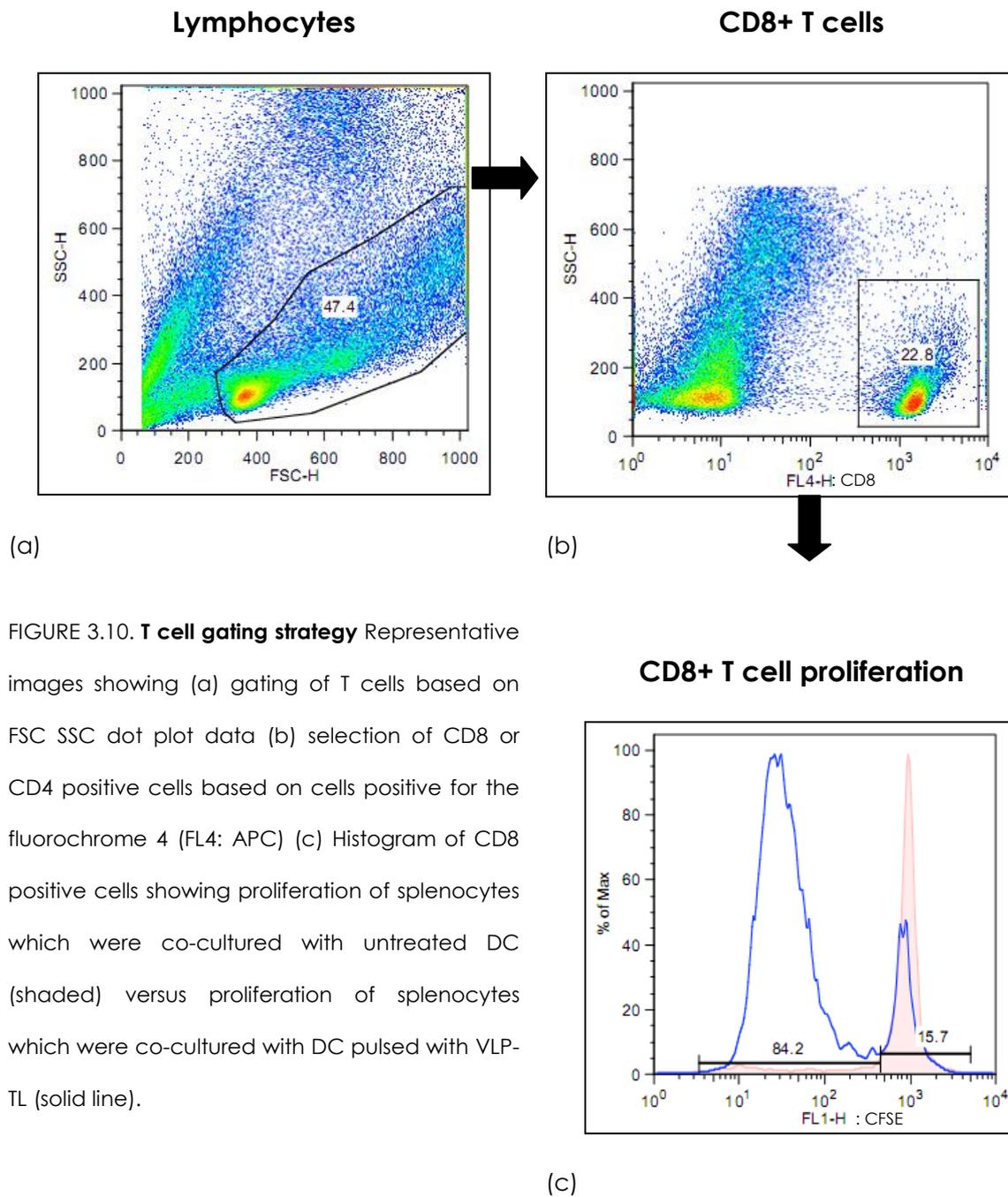


FIGURE 3.10. **T cell gating strategy** Representative images showing (a) gating of T cells based on FSC SSC dot plot data (b) selection of CD8 or CD4 positive cells based on cells positive for the fluorochrome 4 (FL4: APC) (c) Histogram of CD8 positive cells showing proliferation of splenocytes which were co-cultured with untreated DC (shaded) versus proliferation of splenocytes which were co-cultured with DC pulsed with VLP-TL (solid line).

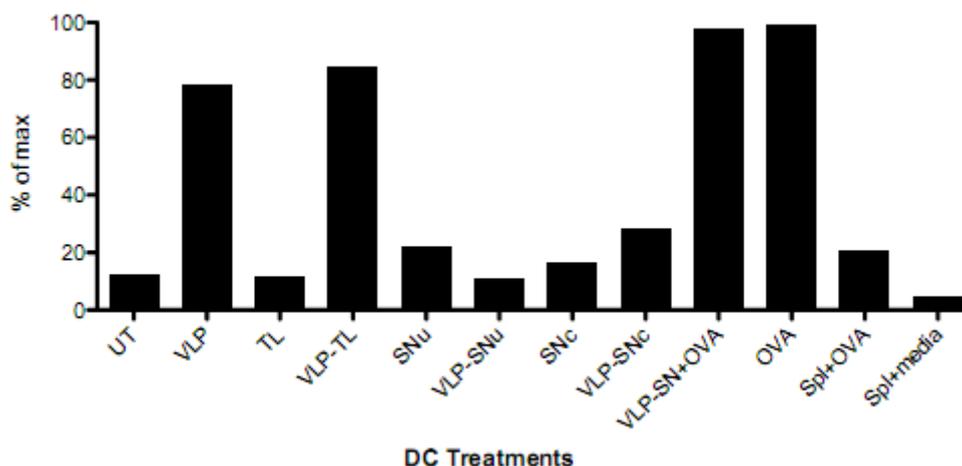


FIGURE 3.11. **VLP-TL can induce improved proliferation of CD8+ splenocytes.** Immature (UT) or mature (treatment-pulsed) DC were co-cultured with CFSE-labelled OT-I splenocytes for 72 hours and proliferation was analysed. Quantification of FACS Calibur flow cytometric results is shown. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNU: undiluted supernatant; VLP-SNU: VLP coupled to SNU; SNC: concentrated supernatant; VLP-SNC: VLP coupled to SNC; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; OVA: whole OVA protein; Spl+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC; Spl+media: splenocytes alone, no DCs. Representative data from one of six experiments. See Appendix 2, Supplementary Data, Table S2.4 for data from repeat experiments.

3.4.3 VLP, TL or VLP-Conjugates are Unable to Trigger CD4+ Splenocyte Proliferation

After addressing the issue of CD8+ splenocyte proliferation the next question to be answered was whether or not CD4+ splenocytes could also be induced to proliferate by any of our treatments. After 72 hours of co-culture with DCs pulsed with the various treatments (VLP, TL, VLP-TL, SNU, VLP-SNU, SNC, VLP-SNC) four repeated batches of OT-II splenocytes showed no proliferation in response to any of the treatments (Fig. 3.12). Once again untreated DCs (UT) were used as a negative control and VLP-SN+OVA was a successful positive control. The OT-I OVA peptide was replaced with the OT-II OVA peptide OVA₂₃₂₋₃₃₉ as a secondary functional control and this also demonstrated a good proliferative response. There was slightly more background proliferation in the UT controls than in the treated cells and the expected high levels of proliferation in the positive control wells. No proliferation was observed in the wells containing splenocytes alone.

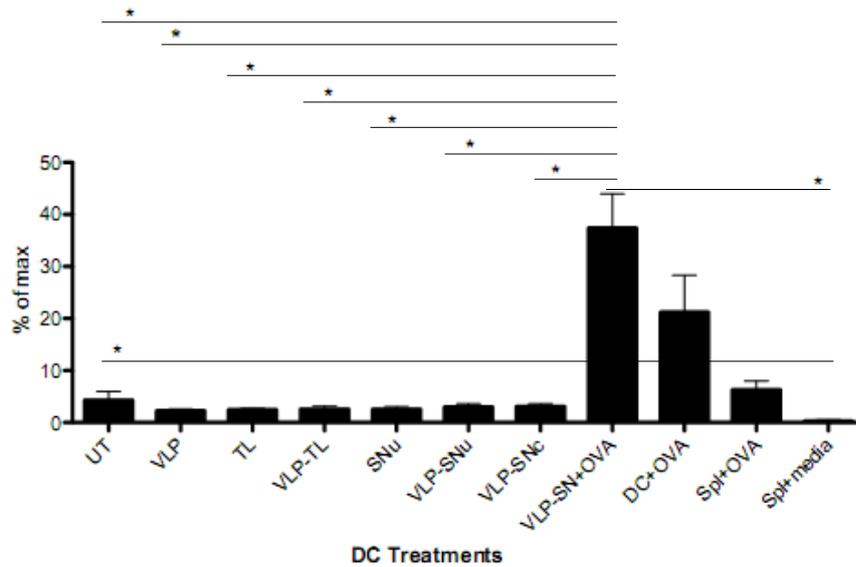


FIGURE 3.12. **VLP, TL or VLP-conjugates are unable to trigger CD4+ T cell proliferation.** Immature (UT) or mature (treatment-pulsed) DC were co-cultured with CFSE-labelled OT-II splenocytes for 72 hours and proliferation was analysed. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; SNU: undiluted supernatant; SNC: concentrated supernatant; VLP-TL: VLP coupled to tumour lysate; VLP-SNU: VLP coupled to SNU; VLP-SNC: VLP coupled to SNC; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; OVA: whole OVA protein; Splenocytes+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC; Splenocytes+media: splenocytes alone, no DCs. Quantification of FACSCalibur flow cytometric results from 4 experiments is shown. *, $p < 0.05$ using paired Student's t test before Bonferroni correction.

3.5 T Cell IFN- γ Production

In tandem with the CFSE proliferation studies we analysed whether cytotoxic T cell activation by antigen-pulsed mature DC was indicated by IFN- γ production, the second crucial gauge of T cell cytotoxic competence.

3.5.1 Production of IFN- γ by OT-I Splenocytes Can Be Improved By VLP-TL

After 72 hours of co-culture with DCs pulsed with the various treatments (VLP, TL, VLP-TL, SNU, VLP-SNU, SNC, VLP-SNC) the supernatant of the OT-I splenocytes' co-culture was evaluated by anti-IFN- γ ELISA. VLP-SN+OVA and the OT-I OVA peptide SIINFEKL were again used as positive controls with UT DC providing a negative control. The successful negative and positive controls indicated that the assays themselves were valid, however results

among the various treatments varied. Three assays were able to show a trend of improved IFN- γ output by splenocytes co-cultured with DC which had been pulsed with VLP-TL (Fig. 3.13).

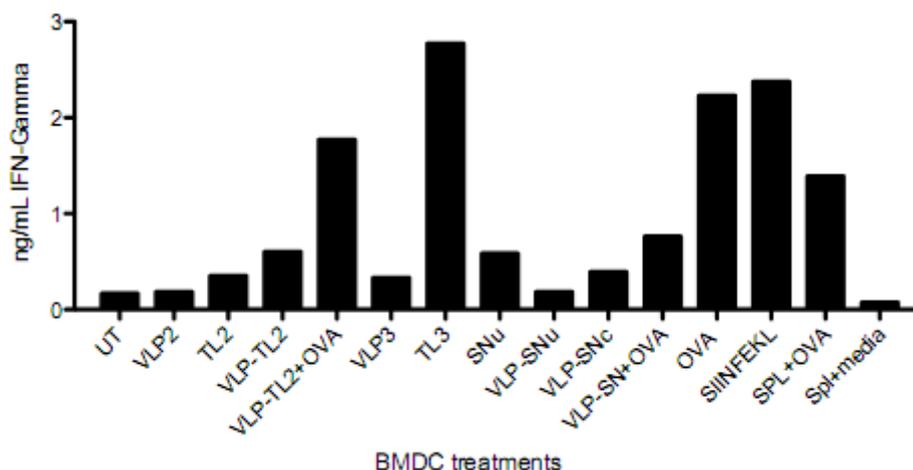


FIGURE 3.13 Production of IFN- γ by OT-I splenocytes can be improved by VLP-TL. Immature (UT) or mature (treatment-pulsed) DC were co-cultured with CFSE-labelled OT-I splenocytes for 72 hours and IFN- γ levels in the supernatant were analysed by direct anti-IFN- γ ELISA. UT: untreated; VLP2: unconjugated VP60 virus-like particles, 2nd batch; TL2: tumour lysate, 2nd batch; VLP-TL2: VLP coupled to 2nd batch of tumour lysate; VLP-TL2+OVA: VLP coupled to 2nd batch of TL spiked with OVA; VLP3: 3rd batch of VP60 VLP; TL3: 3rd batch of tumour lysate; SNU: undiluted supernatant; VLP-SNU: VLP coupled to SNU; SNC: concentrated supernatant; VLP-SNC: VLP coupled to SNC; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; OVA: whole OVA protein; SIINFEKL: OT-I-specific peptide of OVA protein; Spl+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC; Spl+media: splenocytes alone, no DCs. Representative graph showing quantification of one of four ELISA results. See Appendix 2, Supplementary Data, Fig. S2.6 for data from repeat experiment.

3.5.2 Stimulation of IFN- γ Production in OT-II Splenocytes by VLP-TL May be Dose Dependent

The final aspect to consider was IFN- γ production by CD4⁺ splenocytes. Once again, following 72 hours of co-culture with DCs pulsed with the various treatments (UT, VLP, TL, VLP-TL, SNU, VLP-SNU, SNC, VLP-SNC, VLP-SN+OVA or the OT-II OVA peptide OVA₂₃₂₋₃₃₉) the supernatant of the OT-II splenocytes co-culture was evaluated by anti-IFN- γ ELISA. Figure 3.14 shows the collated results of four experiments with error bars again used to visually show the range of results between experiments. Two of the three initial assays showed essentially no IFN- γ response to any treatments other than the positive controls. One assay

returned a result of IFN- γ being produced with the amount emitted as a result of stimulation by VLP-TL or VLP conjugated to either of the supernatants being less than that produced by cells stimulated by TL, SNU or SNC. The assay was repeated with the same treatment dose (50 $\mu\text{g}/\text{mL}$ of each treatment) and a very slight increase in IFN- γ production was stimulated by VLP-TL compared to VLP or TL alone. The assay was repeated again with double the treatment dose ((100 $\mu\text{g}/\text{mL}$ of each treatment) and again the IFN- γ produced by cells stimulated with VLP-TL was slightly greater than that produced by cells stimulated with VLP or TL alone. Figure 3.14 shows the collated results from four experiments in which the DCs were pulsed with 50 $\mu\text{g}/\text{mL}$ of each treatment.

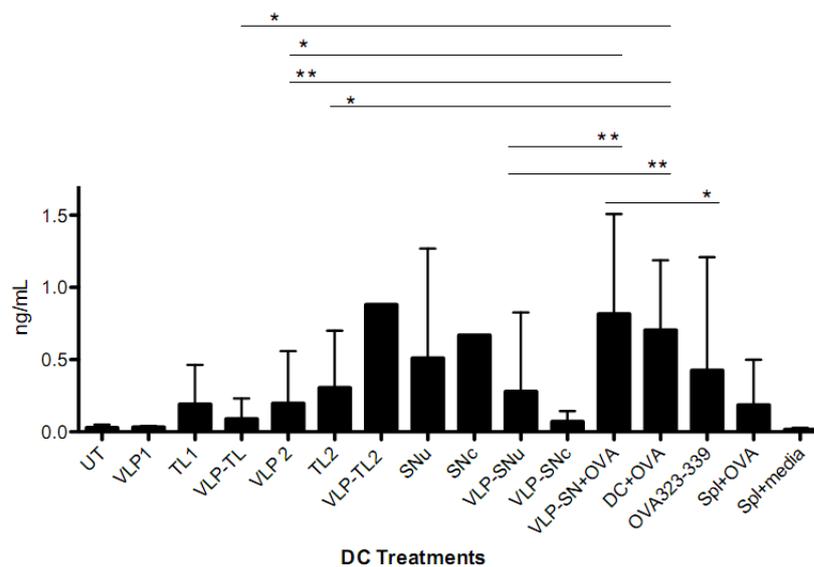


FIGURE 3.14 **Stimulation of IFN- γ production in OT-II splenocytes by VLP-TL may be dose dependent.** Immature (UT) or mature (treatment-pulsed) DC were co-cultured with CFSE-labelled OT-I splenocytes for 72 hours and IFN- γ levels in the supernatant were analysed by direct anti-IFN- γ ELISA. UT: untreated; VLP1: unconjugated VP60 virus-like particles, first batch; TL1: tumour lysate, first batch; VLP-TL: VLP coupled to tumour lysate; VLP2: unconjugated VP60 virus-like particles, second batch; TL2: tumour lysate, second batch; VLP-TL: VLP coupled to second batch of tumour lysate; SNU: undiluted supernatant; SNC: concentrated supernatant; VLP-SNU: VLP coupled to SNU; VLP-SNC: VLP coupled to SNC; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; DC+OVA: DC pulsed with OVA; OVA₃₂₃₋₃₃₉: DC pulsed with OT-II peptide; Spl+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC; Spl+media: splenocytes alone, no DCs. Collated data from 4 experiments. *, $p < 0.05$; **, $p < 0.001$ using paired Student's t test before Bonferroni correction.

3.6 Vaccination with VLP-TL Stimulates a Cytotoxic Response *In Vivo*

Evidence of CD8⁺ T cell proliferation and IFN- γ production implied the differentiation of CD8⁺ T cells into cytotoxic T lymphocytes (CTL). To investigate this further, four groups of four C57/BL6 mice were vaccinated with VLP or VLP-TL or VLP-OVA. One week later donor splenocytes were prepared, half of which were pulsed with the OVA peptide SIINFEKL and labelled with a high concentration of CFSE. The remaining half were left unpulsed and labelled with a low concentration of CFSE. The labelled splenocytes were mixed 1:1 and injected into the vaccinated mice (See Materials and Methods 2.17). Three days later the mice were sacrificed and their cytotoxic response to the OVA labelled splenocytes assessed. In mice vaccinated with VLP which was not conjugated to any peptide no *in vivo* cytotoxic response was seen (Fig. 3.15). The positive control of VLP conjugated to OVA peptide generated a cytotoxic reaction but this was not significant ($p = 0.0768$; Fig.3.15). Mice vaccinated with TL alone also demonstrated significant cytotoxicity ($p = 0.0186^*$) as did the mice which received vaccinations of VLP-TL, although the response of the latter was not numerically as great ($p = 0.0040^{**}$; Fig.3.15).

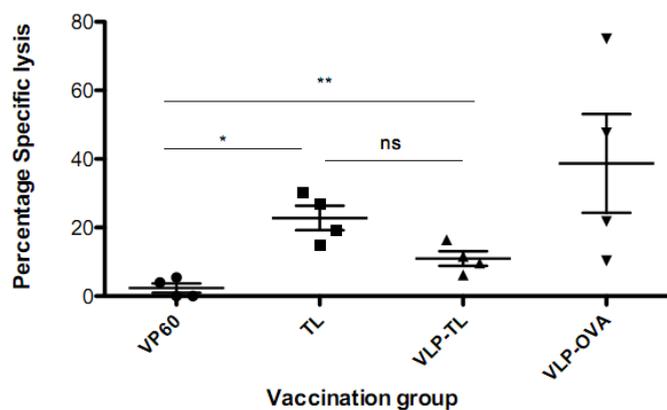


FIGURE 3.15. **Vaccination with VLP-TL stimulates a cytotoxic response *in vivo*.** Each dot represents one mouse which received 50 μ g of the vaccination treatment indicated in a volume of 100 μ L of PBS. One week later donor splenocytes labeled CFSE^{HI} (OVA pulsed) and CFSE^{LO} (unpulsed) were administered i.v. Three days later mice were sacrificed and their cytotoxic response to OVA peptide analysed by FACS Calibur Flow Cytometry. CTL lysis of OVA-pulsed, CFSE^{HI} cells was expressed as a percentage of the lysis obtained by the negative control (VP60). The value shown was calculated as $(1 - \text{ratio primed}/\text{ratio unprimed}) \times 100$. *, $p < 0.05$; **, $p < 0.001$ using paired Student's *t* test before Bonferroni's correction was applied.

While results have been variable and further refinement of experimental parameters is required these data do suggest that VLP-TL is capable of stimulating DC maturation, and subsequent CD4+ and CD8+ T cell proliferation and cytotoxic capacity, both *in vitro* and *in vivo*.

Chapter 4: Discussion

According to the World Health Organisation (WHO) cancer remains a principal cause of death globally with projected deaths from cancer rising to an estimated 12 million in the year 2030. The current oncological standard of care involves surgery to physically remove those tumours which can be accessed successfully. Surgery is usually accompanied by cytotoxic chemotherapy and/or radiation therapy in an effort to control the spread of the disease. However, there are many types of cancers for which surgical, chemotherapeutic or radiotherapeutic options are limited or unavailable. More importantly, even if surgery, chemotherapy or radiation are successful at eliminating the cancer, none of these approaches are able to prevent the return of that cancer as they do not stimulate an adaptive immune response which generates immunological memory against that tumour's antigens. Thus there is an urgent need for better approaches to cancer treatment, particularly ones which utilize and augment the immune response to cancers. This is precisely the goal of tumour immunotherapy, and this study sought to further the tumour immunotherapy cause by investigating whether the T cell response to tumour-associated antigen (TAA) could be strengthened by coupling TAA to VLP.

Much of our understanding of VLP-TL comes from previous work which showed that specific tumour antigens coupled to RHDV VLP induced a tumour-specific cytotoxic T cell response⁸¹. This study set out to move these investigations closer to clinical application by analyzing the response of T cells to RHDV VLP coupled to tumour lysate in which the antigens are undefined. This is important because many cancer antigens remain unknown. We hypothesized that RHDV VLP coupled to tumour lysate would generate a stronger T cell proliferative and cytotoxic response than either VLP or TL alone.

4.1 DC Exposed to Antigen Switch to Typical Maturation Phenotype

Dendritic cells (DC) which acquire immunostimulatory antigen are induced into maturation as they travel from the peripheral site of antigen acquisition to the lymph node, site of antigen presentation. It is these mature DC that are able to provoke the activation of antigen-specific T cells⁸⁷. Mature, activated DC, complete with high levels of major histocompatibility complex I or II (MHC I/II) molecules with which to present specific peptides, upregulated co-stimulatory molecules (CD40, CD80 and CD86), and secreting particular cytokines, such as interleukin-12 (IL-12), are able to bring about a strong, immunogenic T cell response to the cognate antigen. Immature DC, deficient in MHCII and co-stimulatory molecules, interacting with T cells will induce an immunotolerant T cell reaction and the particular antigen will be ignored. In the case of cancer this will lead to cancerous cells, which display altered self antigens, being ignored. Therefore immune therapies against cancer need to be able to present tumour antigens in an immunogenic way. We have utilised a virus-like particle as an antigen scaffold, able to present heterologous antigens to the immune system. This VLP has been shown to be able to deliver defined antigens⁶⁰, however most tumour antigens remain undefined. Therefore for translatable use in the clinic, we wished to determine whether tumour lysate (undefined antigens) loaded onto our VLP would be able to trigger a T cell response. That being the case, one of the first points to ascertain was whether or not VLP-TL could cause DC to display a typical mature phenotype which can be identified primarily by an increase in the cell surface molecules CD40, CD80, CD86 and MHCII.

Upregulation of these markers can be quantified by either an increase in the percentage of cells positive for the marker or an increase in the number of molecules on the cell surface, which is measured by their mean fluorescence intensity (MFI). On the journey from the periphery to the lymph node (LN) the function of the DC switches from that of primarily antigen-uptake to one of primarily antigen-presentation²⁰ in preparation for encountering T cells in the LN. DC differentiated from monocytes by the inclusion of GM-CSF in the

culture medium display the hallmarks of immature DC, having high endocytic function and a low ability to induce T cell proliferation²⁰. According to the literature a relatively low level of surface MHC-II is one of the characteristics of these cells as these molecules undergo a rapid internalization and recycling process by way of a constitutive intracellular pool^{27, 12}. As the DCs mature in response to antigen surface MHC-II levels increase up to four times within 24 hours due to an increase in manufacture of MHC-II coupled with a reduction in recycling which leaves more molecules on the cell surface²⁰. This allows DC to quickly stack many peptides for display on its surface, increasing its ability to present antigen to the appropriate T cell. While levels of MHC-II tend to be lower on unactivated DC, levels of this molecule are still higher than that of the co-stimulatory molecules and thus the MFI of MHC-II becomes another important indicator of the level of MHC-II activity. We found consistently higher levels of MHC-II in untreated DC than had been described in the literature and therefore did not observe as large an increase in the percentage of cells positive for MHC-II after treatment, however large increases in the MFI of MHC-II were reliably observed.

In line with the literature, approximately 10% of immature DC displayed CD40 (Fig 3.2). This figure increased dramatically to around 70% of DC upon exposure to LPS and around 60% upon exposure to CpG (Fig. 3.2). This is a crucial indicator of DC maturation. The interaction between CD40 on the DC and CD40 ligand (CD40L) on T cells triggers a chemical signal cascade inside the DC which activates production of the chemokine interleukin-12 (IL-12) by the DC^{19, 91}. IL-12 is critical for driving the immune response toward a cell-mediated response¹⁹, precisely what is required for a cancer vaccine and therefore increased CD40 on the surface of the DC is what we would want to see generated by the VLP-TL.

The surface markers CD80 and CD86 are present on around 30-60% of immature DC therefore their rise in numbers is not as dramatic as that of CD40, however this increase is

still important given their role as key co-stimulatory molecules of the DC-T cell interaction. They both bind CD28 (which helps activate the T cell response via intercellular signalling) and CTLA4 (part of the T cell attenuation system after the response is no longer required).

In accordance with the literature upregulation of all our monitored cell surface markers was seen in the presence of both LPS and CpG and this upregulation was also observed in the presence of VLP-TL (See Section 4.3). CpG is a more clinically relevant positive control⁸⁸ however we elected to continue with LPS as a functional control because the response by DC was more striking in the presence of LPS than CpG and enabled a clearer positive control. CpG was not as able to generate a high, clear positive signal.

4.2 Detection of OVA Protein in Tumour Lysate, Tumour Cell Supernatants and on VLP-Conjugates

Given that OVA is a secreted protein it is not unreasonable that no OVA was detected in the tumour cell lysate. It was for this reason that we elected to assess the presence of OVA secreted into the supernatant of B16OVA cells. In addition, we treated the cells with Brefeldin-A (Bref-A), a fungal metabolite which inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus, thus hindering protein secretion (10). Cells treated with Bref-A prior to lysis accumulated OVA intracellularly however OVA was still not identified in the lysate prepared from these cells (Fig. 3.5). Tumour lysates generated by freeze-thaw lysis aim to mimic the 'necrotic', stress-induced cell death present in the tumour environment which can elicit anti-tumour activity. Following the first unsuccessful attempt at OVA detection several improvements were made to the tumour lysate preparation method which will be discussed later (Section 4.3).

In any preparation of VLP coupled to protein there will be three elements: free VLP (60kDa), free protein (in this case OVA, at the 40 and 45 kDa levels) and VLP-which has

conjugated to the protein (which will be seen at the high molecular weight of around 105 kDa). After stripping the membrane of anti-OVA and re-incubating with anti-SH3 which binds to whole VLP a clear band was seen at the 60 kDa molecular weight level in the VLP-TL lane, indicative of the presence of VLP. The stripping process (See Materials and Methods Section 2.14.2) was not entirely successful and some OVA luminescence remained on the membrane. While clear bands are seen at the VLP level, it would have been preferable to have carried out the anti-RHDV detection on a separate membrane for better clarity of the correlation between VLP and OVA. Having said that, the clustering of bands in the high molecular weight (MW) levels after both probes, while not proving that OVA and VLP have conjugated, shows that they are at least aggregating. In an ideal world one OVA protein (45 kDa) would couple to one VLP creating a molecule of around 105 kDa. However, what actually happens is that one OVA molecule, having a lot of available primary amines, tends to link with the available NH groups of two or more VLPs, causing the high molecular weight aggregations that can be seen at the top of the Western blot membranes (Fig.s 3.5, 3.6). It is for this reason that the anti-OVA and anti-VLP antibodies are detected at the top of the membranes, while only free OVA and free VLP are detected at their MW level. This aggregation was taken as an insensitive indication that some conjugation has occurred.

Given the possibility that the level of OVA was beneath the threshold of detection for the WB we opted to carry out an anti-OVA ELISA, a more sensitive detection method. The first approach was to coat the ELISA plate with OVA (direct ELISA) and this yielded a figure of approximately 10 ng/mL of OVA in the undiluted supernatant of B16OVA cells which had been cultured for 3 days in serum-free medium. However, doubling dilutions resulted in increases in the amount of OVA present which didn't make sense. We tried again with an indirect ELISA approach of coating the plate with anti-OVA antibodies which gave a result of 0.128 ng/mL of OVA in the undiluted supernatant. This time the doubling dilutions led to reductions in the amount of OVA. However, both of these assays had very low optical

density readings (OD) which led us to question the validity of both assays. For example, the undiluted supernatant generated an OD of 0.0628, which, according to the standard curve should have equated to around 3 ng/mL of OVA, but instead resulted in a reading of 0.128 ng/mL of OVA. Nonetheless, in spite of the low OD, we reasoned that this result combined with the faint bands at OVA level in the SDS-PAGE gel (Fig. 3.4) gave us enough of a hint that OVA was present in our treatments for us to press on toward the exquisitely sensitive OT-I T cell read out system.

4.3 Dendritic Cell Activation in the Presence of VLP-TL

Given the importance of DC activation by VLP and VLP-conjugates for an immunotherapeutic immune response it was important to assess the impact of our VLP-TL on DC maturation. It has been shown that significant upregulation of the DC activation markers CD40, CD80, CD86 and MHC-II can be induced by RHDV VLP alone ⁶⁸ and by RHDV VLP coupled to green fluorescent protein (GFP) antigen (14). Thus, having established what an immature versus an activated DC phenotype looked like we could now see what effect VLP-TL would have on DC maturation and whether or not VLP coupled to TL would be enough to activate DC in the absence of an adjuvant. Similar to what has been reported previously ^{89,51} tumour lysate alone sometimes had an inhibitory effect (See Appendix 2, Supplementary Data, Fig.s S2.1, S2.2,) but in most experiments TL had a stimulatory effect, as has also been seen before⁴⁰. In one experiment, all treatments caused inhibition of all four DC maturation markers, and the VLP-TL while less inhibitory than TL alone was unable to bring the marker levels back to baseline (Data not shown). The findings of this study which showed a small stimulatory effect on DC activation are in contrast to previous work which found that RHDV VLP-TL did not stimulate DC maturation ⁸¹ without the addition of an adjuvant. It is possible that the presence of an adjuvant may aid VLP-TL to increase the slight maturation of DCs observed in this study, assisting VLP-TL to realise full DC stimulatory capacity. A study underway in Wellington, New Zealand is showing promising results with recombinant VLP expressing the adjuvant alpha-

galactosylceramide (α -GalCer) (unpublished data). Indeed Win *et al* showed that the addition of an adjuvant to DC pulsed with tumour lysates was superior to VLP-lysate alone at bringing about DC maturation and T cell cytotoxicity⁸¹.

Inconsistent results were continuously reported with the undiluted supernatant (SNU), concentrated supernatant (SNc), and VLP coupled to supernatants (VLP-SNU and VLP-SNc). SNU or SNc would sometimes activate the DC to a higher level than TL, but not consistently. No significant or apparently helpful patterns or trends were noticeable with these treatments. Indeed coupling VLP to SNU or SNc most often resulted in either no difference or an inhibitory effect (Fig. 3.7, Fig. 3.8 and Appendix 2, Supplementary Data, Fig.s S2.3, S2.4) when compared to SNU or SNc alone. Given the more pertinent application of TL than SN in the clinic, attention was then focused on VLP coupled to TL.

VLP-TL and SNc were the two prime candidates for best upregulation of CD40 on DC. It stands to reason that there would be a small amount of antigen coupled to VLP compared to a relatively high level of proteins found in the SNc thus the VLP-TL may be an efficient method for making that small amount of antigen immunostimulatory. TL, SNc or VLP-TL were all able to cause upregulation of CD80 to very similar degrees. While a trend of increases was seen in the levels of the markers CD40, CD86 and MHC-II between TL and VLP-TL-treated DCs, CD80 seemed to be stimulated to similar levels by both TL and VLP-TL. In three out of four experiments the best increase in MHC-II MFI was seen in response to VLP-TL.

The fact that there was minimal difference between the ability of VLP-TL, VLP-SNU or VLP-SNc to effect the best DC activation raises the obvious question of what is doing the activating – the VLP or the antigen coupled to the VLP. All three batches of VLP utilised throughout these experiments showed varying levels of stimulation of DC (and T cells) and we remain unsure whether this was due to the VLP or due to issues with the DCs or T cells

themselves (See section 4.4.1 for S. Hook's unpublished observations on random OT-I T cell activation).

Broadly speaking each treatment was able to generate an expansion in the degree of each marker quantified, however substantial fluctuations were observed across experiments in terms of the ability of each treatment to generate a response and this made it difficult to reach solid conclusions about VLP-TL's ability to enhance the DC response versus TL or VLP alone. It remains to be established whether these fluctuations were due to possible protein degradation or differing levels of actual proteins delivered to the DC between experiments. Protein degradation is one suspect due to the fact that the original batches of TL were not aliquotted out, but rather the entire batch was thawed and re-frozen between experiments. However, all the VLP-conjugates (VLP, VLP-TL, VLP-SNu, VLP-SNc, VLP-SN+OVA) as well as the SNc were all stored in 50% glycerol, and thus were never actually frozen *per se* and therefore protein degradation should have been minimal. The SNu was aliquotted out and not re-frozen between batches and variations were still seen in the response which points more toward a dosing issue. Future experiments would need to address this issue and steps would ideally need to be taken to improve quantification of protein in each dose received by the DC. Having said that, in order to retain clinical relevance, it is important to do this without going down the protein fractionation and antigen identification route which would defeat the purpose of generating a 'generic' unidentified antigen coupled to VLP approach, which was the overall aim of this project.

Methods to improve the quality of tumour cell lysate included resuspending B16OVA cells in a protease inhibitor cocktail prior to lysing the cells (from the second batch of tumour lysate) in an effort to minimise protein degradation by proteases released upon lysis. From the second batch TL preparation was carried out on ice, instead of at room temperature. The tumour lysate used in this study was created by the slightly inferior method of four

cycles of freezing the cells in the -80°C freezer and thawing them at 37°C. It is possible that the very rapid freezing of the cells in liquid nitrogen or dry ice in methanol, followed by a rapid thaw in the 37°C waterbath would have resulted in a superior lysate product. Future experiments will use lysate prepared by the dry ice and methanol method which may result in more standardised results. Trypsinisation is a common method employed for detaching and harvesting adherent tumour cells from tissue culture flasks. A recent publication reported that inconsistent results were observed when DCs were pulsed with tumour lysates made post trypsinisation - trypsinisation generated DCs did not satisfactorily express the maturation markers CD40, CD80 or CD86⁹⁰. Replacing trypsin with EDTA, an ion chelator that divests cells of the critical adherence factor calcium, allows cells to be released from the plastic ware without trypsin's protease activity. In addition to cleaving cell surface proteins needed for adherence trypsin most likely cleaves other antigenic cell proteins, which may interfere with the lysate's ability to activate DCs. The authors specifically caution against the use of trypsin for DC-based vaccination against tumour antigens. Given that the trypsin method was used in this study for releasing B16OVA tumour cells it is entirely possible that this is the source of our inconsistent DC activation, and consequently T cell activation, results. If the quality of the lysate being coupled onto the VLP is less than ideal then the inability of VLP-TL to generate a robust response may be a result of the lysate itself, rather than any deficiency on the part of the VLP.

4.4 T Cell Proliferation

4.4.1 OT-I T Cell Proliferation and IFN- γ Production

It has been shown that VLP alone have a poor ability at stimulating a CTL response but when combined with DC stimulators, such as CpG oligonucleotides or anti-CD40 monoclonal antibodies, they can stimulate cytotoxic T cells⁶⁴. Thus we now turned our attention to the ability of VLP-TL to generate a cytotoxic T cell response. After 72 hours of co-culture with DCs pulsed with the various treatments (UT, VLP, TL, VLP-TL, SNu, VLP-SNu,

SNc, VLP-SNc, VLP-SN+OVA or the OT-I OVA peptide SIINFEKL) OT-I splenocytes showed very mixed results with only one of six repeated experiments showing that VLP-TL was able to stimulate a clear increase in proliferation compared to the other treatments (Fig. 3.11). In one assay both SNc and SNu demonstrated superior proliferation stimulating activity to any of the VLP conjugates, but not to VLP alone. While in another test only SNc was able to compete with VLP and VLP-TL for top proliferation-generating ability (See Appendix 2, Supplementary Data, Table S2.4). Similar to what has been shown previously^{62, 51} the final assay showed VLP and TL having an inhibitory effect on proliferation, and VLP-TL was unable to restore proliferation even to the untreated baseline level (Appendix 2, Supplementary Data, Fig. S2.5). Given what is now understood about the effect of trypsinisation on tumour cells⁹⁰ these fluctuations may be more understandable. Or it may be simply that the tiny amounts of OVA available in each VLP-TL prep differed between experiments, which could also help explain the varying levels of proliferation. VLP-TL is statistically less likely to contain as much OVA as TL since OVA will be just one of many proteins in the lysate which could be coupled on to the VLP. Our Western Blot (Fig. 3.5) did not detect any OVA in the VLP-TL preparation, but the fact that OT-I T cells proliferated when exposed to this VLP-TL demonstrates the presence of at least small amounts of this protein as these transgenic T cells only recognise the OVA peptide, SIINFEKL, presented on MHC-I molecules. That being said, one experiment (Fig. 3.11) demonstrates clearly our problem with VLP causing non-specific proliferation of said T cells whose receptors should only respond to SIINFEKL. This problem of random, non-specific proliferation in OT-I male mice is not a new one (S. Hook, unpublished observations). In accordance with the experience of our colleague the problem did not occur *in vivo*, thus we had no problems in the *in vivo* cytotoxicity assay, but remains an issue for *in vitro* assays. At the time of writing no explanation has been found for the problem which is a confounding issue in terms of quantifying the real effect of VLP-TL on OT-I proliferation and cytotoxicity since it cannot be demonstrated whether the proliferation is being triggered by the VLP component of the VLP-TL.

One experiment (Data not shown) gave a clear indication that VLP-TL is able to effect a superior IFN- γ response however the caveat is that the cells in this assay were inadvertently labelled with CFSE and the level of IFN- γ produced was greatly elevated compared to all the other ELISA assays, possibly as a consequence of the CFSE labelling. Also, IFN- γ was secreted in high amounts by the negative controls so the stimulus for IFN- γ production in this assay could not be truly identified. However, the final two repeats of this test duplicated the trend of VLP-TL bringing about a better IFN- γ reaction than either VLP or TL alone (Fig. 3.13 and Appendix 2, Supplementary Data, Fig. S2.6), indicating that further investigations are warranted.

4.4.2 OT-II T Cell Proliferation and IFN- γ Production

As with previous experiments DCs pulsed with SNu, SNc, VLP-SNu or VLP-SNc produced seemingly random, variable results so we again focused on VLP, TL and VLP-TL. All four repeats of CFSE proliferation assays showed the same result: no CD4⁺ splenocyte proliferation, even in the face of a double dose of treatments (Fig. 3.12). The OVA-containing positive controls caused proliferation confirming that the assay had worked but apparently the treatments were not able to stimulate CD4⁺ T cell proliferation. These results are interesting in light of the 2002 study by Ruedl *et al* which showed that the key APC involved in VLP-cross-presentation are CD8⁻ DC which activate CD4⁺ T cells⁷³. This present study would indicate that CD8⁺ DC, which specialise in cross-presentation of exogenous soluble antigen to CD8⁺ T cells via MHC-I⁹⁷, may be more active than previously thought in the VLP presentation process than CD8⁻ DC, although more robust repeat experiments are required to confirm this.

Two of the three ELISA anti-IFN- γ assays returned the same result – no IFN- γ produced by OT-II splenocytes. However one ELISA showed some IFN- γ being stimulated which led us to

suspect that this may be a threshold issue and that the reason that cells had not proliferated or produced IFN- γ was because the treatment dose was below the threshold for stimulating IFN- γ production. It may be that less stimulus is required for IFN- γ production and once a certain level of IFN- γ is produced proliferation is initiated. In order to test this theory the OT-II proliferation and IFN- γ assays were repeated with double the dose of treatment (100 $\mu\text{g}/\text{mL}$ as opposed to 50 $\mu\text{g}/\text{mL}$). This assay again returned a nil result in terms of proliferation but showed a doubling in the amount of IFN- γ production being stimulated by VLP-TL (0.14 ng/mL) compared with VLP (0.073 ng/mL) or TL (0.034 ng/mL) (Fig. 3.13). However, in spite of the doubled dose, the actual amounts of IFN- γ produced were only about half of the amount that had been shown to be produced in two earlier assays (0.025 ng/mL) and a fraction of the IFN- γ produced in the fourth assay (0.88 ng/mL). Thus, while the trend of IFN- γ produced observed in Experiment 30 is gratifying, it may again prove to be a random anomaly and not a robust result.

4.5 *In Vivo* Cytotoxicity

It became apparent that less reliable results were generated with the use of supernatant, whether undiluted, concentrated or conjugated to VLP. There appeared no rhyme or reason to the vacillations observed between experiments with SNu/SNc/VLP-SNu/VLP-SNc causing activation in one experiment and not in the next. VLP conjugated to SNu/SNc would return a result of improved stimulation compared to SNu or SNc alone in one assay and in another would show an inhibitory effect compared to SNu or SNc alone. From the point of view of clinical application this would appear to be a fairly serendipitous result given the fact that we are currently unable to culture most human tumours as cell lines and therefore would be unable to generate cell supernatant for the majority of human tumours. For this reason we focused the *in vivo* cytotoxicity assay on VLP, TL, and VLP-TL alone and ignored the SN at that point.

RHDV VLP have been shown to induce an anti-tumour response ⁹⁵ and our *in vitro* results of proliferation and IFN- γ production suggested cytotoxic capabilities in the proliferating CD8+ T cells which had been exposed to DC pulsed with VLP-TL. To examine this further, four groups of four C57/BL6 mice were vaccinated with 50 μ g per mouse VP60, TL, VLP-TL or VLP-OVA and the *in vivo* cytotoxicity assay carried out as previously described (See Materials and Methods Section 2.17). As seen in Fig. 3.15 a statistically significant improvement in specific cell lysis was seen between VLP alone and VLP-TL. TL on its own was able to generate a numerically greater cytotoxic result than VLP-TL though the difference between TL and VLP-TL was not statistically significant. It is reasonable to assume that TL would contain much higher amounts of OVA than would be coupled onto the surface of the VLP, and given that no OVA was detected in the VLP-TL, the fact that we saw any result at all with VLP-TL is in fact a positive sign. Also, due to the fact that we wished to detect differential responses between vaccines and the effects of lysate alone had not been ascertained in mice, we chose to administer a suboptimal dose administering 50 μ g/mouse, as opposed to the usual 100 μ g/mouse. Repeating the experiment with increased doses of VLP-TL may render a higher cell lysis than that generated by TL.

4.6 Conclusions

A small trend was observed in this study which indicates that VLP coupled to TL may indeed have a beneficial effect on the ability of DCs to stimulate T cell proliferation and cytotoxicity. VLP generally had a stimulatory effect on OT-I T cell proliferation and IFN- γ production while TL's effect was inhibitory (Appendix 2, Supplementary Data Table S2.5.) and coupling VLP to TL was sometimes able to overcome the lysate's inhibition. It appears that VLP-TL is unable to stimulate CD4+ T cell proliferation but it remains to be seen whether this is a dosing issue, since non-proliferating CD4+ T cells did produce small amounts of IFN- γ . VLP coupled to TL is able to generate an *in vivo* cytotoxic response and further

investigations with increased dosages are warranted to ascertain whether or not the effect seen is dose-dependent. Thus VLP-TL can be observed to invoke a slightly improved immune response when compared with VLP or TL alone, however determining the full potential of this vaccine potential requires further optimisation of this study's protocols.

4.7 Future Directions for VLP-TL

In spite of the T cells of cancer patients recognising TAAs *in vivo*, these antigens are either ignored or the CTL response generated is weak and ineffective, and methods of circumventing this immune ignorance or augmenting the CTL response remain to be delineated. Functionally intact tumour-antigen-specific CTLs can be found in the peripheral circulation of cancer patients yet the function of these CTLs is impaired at the tumour site ²⁹. Something in the tumour environment is shutting down these tumour killers and stimulating a more robust CTL response by combining VLP with TL may be one way of overcoming the tumour's inhibitory effect.

If the decades of cancer treatment and clinical trials have taught us anything it is that when it comes to curing cancer there is no 'one size fits all' approach. A treatment that brings about an objective remission or cure in one patient will prove futile in the next. Part of this problem lies in the genetic and biological variation between human individuals and thus a fairly customized line of attack is required. However, made to order medical intervention lies outside the reach of all but the wealthy elite and drug companies are not likely to fund research which is not broadly applicable across the general populace. With the concept of VLP conjugated to lysate generated from patients whose tumour specific antigens are never likely to be identified there exists the possibility of a highly personalised, yet broad-spectrum approach to cancer treatment: a generic, immune-stimulating scaffold onto which can be linked the proteins of your very own tumour.

It is possible that the use of TL generated from a tumour bearing a known cell surface antigen, such as MART-1 in human melanoma, as opposed to a secreted antigen (OVA), could yield more promising results than those of this study. It may be that a secreted antigen is not the best model antigen for the VLP-TL system and as such repeating these assays with a model surface-bound antigen may yield a clearer, more robust result.

Many of the parameters which remain to be defined in tumour immunotherapy cannot be optimised in clinical trials, thus there remains a need for *in vitro* models, such as the one used in this study, to focus on these issues. Questions such as the most favourable source and subtype of DC to target with VLPs; methods of tumour antigen preparation; how to ensure antigens enter MHC class I and/or II processing pathways; and which DC activators or adjuvants to use all remain to be characterized⁵¹. The need still exists to formulate approaches capable of delivering both the tumour antigen and the DC stimulatory signal in a restricted manner to DCs *in vivo* and to target the desired antigen(s) to both MHC I and II processing compartments in DCs²⁹. A DC-specific 'scaffold antigen' such as VLP, coupled to tumour lysate and possibly also to an adjuvant may one day fulfil these requirements.

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Appendix 1: Recipes

Acrylamide Gels for Electrophoresis:

10% Resolving Gel 20 mL

7.9 mL H₂O
6.7 mL 40% acrylamide/Bis solution
5.0 mL 1.5 M Tris (pH 8.8)
200 µL 10 % SDS
200 µL 10 % Ammonium Persulfate
10 µL TEMED

5% Stacking Gel 5 mL

3.4 mL H₂O
0.83 mL 30% acrylamide/Bis solution
0.63 mL 1 M Tris (pH 6.8)
50 µL 10 % SDS
50 µL 10 % Ammonium Persulfate
8 µL TEMED

SDS-PAGE Sample Buffer

0.5 mL 10 % SDS
100 µL 2-Mercaptoethanol
200 µL glycerol
120 µL Tris (pH 6.8)
10 µL 1% bromophenol blue
70 µL H₂O

5 x Electrophoresis Buffer

72 g glycine
15 g Tris
5 g SDS
Add dH₂O to 1 L
Use at 1 x strength, diluted in dH₂O

Coomassie Brilliant Blue Stain

0.25% coomassie blue (1.25 g)
45% methanol (225 mL)
9% acetic acid (45 mL)
dH₂O (230 mL)

De-stain Solution

10% methanol
10% acetic acid
dH₂O

Coupling Buffer

5.2 g NaH₂PO₄·2H₂O
23.66 g Na₂HPO₄
17.54 g NaCl
2 L dH₂O
pH 7.25 – 7.3

10 X Conjugation Buffer

Coupling buffer
25 mM ethylenediaminetetra-acetic acid (EDTA)

Semi-Dry Western Blot Transfer Solutions

Anode buffer I: 0.3 M Tris, pH 10.4, 10% methanol

Anode buffer II: 25 mM Tris, pH 10.4, 10% methanol

Cathode buffer: 25 mM Tris, 40 mM glycine, 10% methanol, pH 9.4

Stack assembly:

Starting at the bottom

2 x filter papers soaked in anode buffer I

1 x filter paper soaked in anode buffer II

Membrane soaked in anode buffer II

Gel soaked in anode buffer II

3 x filter papers soaked in cathode buffer

Place lid on top

PBS for Western Blotting

8% NaCl

0.2% KCl

1.44% Na₂HPO₄

0.24% KH₂PO₄

Sodium Casein Alanate Solution

20 mL PBS

0.2 g sodium casein alanate (1%)

4 µL Tween-20 (0.02%)

PBS Tween

PBS

0.02% Tween-20

Trichloride Acetic Acid (TCA)

1 g TCA

9 mL mQ H₂O

Stripping Buffer

2% SDS

100 mM β-Mercaptoethanol

50 mM Tris

pH to 6.8

Alsevi's Solution

20.5 g dextrose (D-glucose)

4.2 g NaCl

8.0 g Na citrate

1 L milliQ H₂O

Fluorescence Activated Cell Sorting (FACS) Buffer

1 X PBS

0.1% Bovine Serum Albumin (BSA) (Gibco)

0.01% Sodium Azide (NaA₃)

Paraformaldehyde (PFA) Solution

8% paraformaldehyde (Sigma)

Diluted in FACS Buffer to 2% working solution

ELISA Carbonate Coating Buffer

0.1 M NaHCO₃ in dH₂O
pH 8.2

ELISA Wash Buffer

1 X PBS
0.5% Tween-20

ELISA Blocking Buffer

1 X PBS
1% Bovine Serum Albumin (BSA) (Gibco)
Use ice cold and store at -20°C between uses

4N H₂SO₄

Concentrated H₂SO₄ is 18.4 M
4N = 2 M therefore add 54 mL to 446 mL dH₂O
Working stock is 1N so dilute ¼

10 x Phosphate Buffered Saline (PBS)

160 g NaCl
4 g KCl
22.7 g Na₂HPO₄
4 g KH₂PO₄
2 L dH₂O
Use at 1 X strength, diluted in dH₂O

Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma)

Catalogue number: D5773
Made up in lab as per manufacturers
Filter sterilized
pH to 7.2

Complete DMEM (cDMEM)

To 500 mL of Dulbecco's Modified Eagle Medium (DMEM; Gibo) was added 5 mL each of

- filter sterilised L-arginine (11.6 g/L)
- L-asparagine (3.6 g/L)
- folic acid ((0.6 g/L)
- penicillin/streptomycin (Pen/Strep) containing 10,000 units of penicillin and 10,000 µg/mL of streptomycin

and

- 500 µL of 5.5x10⁻² M 2-mercaptoethanol (2-ME)

T Cell Culture Medium

cDMEM
5% Foetal Bovine Serum (FBS)

Dendritic Cell Medium

cDMEM
20 ng/mL granulocyte macrophage colony stimulating factor (GM-CSF)
5% Foetal Bovine Serum (FBS)

B16OVA Tumour Cell Medium

To a 500 mL bottle of Iscove's Modified Dulbecco's Medium (IMDM) add

- 5 mL PenStrep
- 500 μ L 2Me
- 5 mL L-Glut (only if not using Glutamax medium)
- 1 % Geneticin(G418)
- 5% FBS

Hanks Buffered Salt Solution (HBSS)

0.35 g NaHCO₂

Make up to 1 L in dH₂O

pH 7.2 - 7.4

Filter sterilise to help stop cells clumping

Foetal Bovine Serum (FBS)

Heat inactivate 500 mL bottle at 56°C for 45 minutes

Store at -18°C

Freezing Mix

For cryopreserving cells when freezing into liquid nitrogen storage

90% FBS

10% DMSO (Sigma)

Filter sterilise

Resuspend cells to be frozen at $5 \times 10^6 - 4 \times 10^7$ cells/mL

Keep freezing mix, cryotubes, 'frosty boy' and cells on ice

Change isopropyl alcohol (IPA) in 'Frosty Boy' every 5th time used

Trypan Blue (TB)

0.25% TB in PBS

Filter sterilise

Serum-free media for B16OVA tumour cells

25 mL DMEM

75 mL RPMI 1640

1 mL 10% BSA in PBS (sterile) – final concentration 0.1%

1 mL Penicillin Streptomycin (Gibco)

1 mL Geneticin

100 μ L 2-Mercaptoethanol

100 μ L 5 μ g/mL Transferrin

100 μ L 5 μ g/mL insulin

16.83 μ L 5pM Triiodothyronine

10 μ L 10 nM Sodium selenite

10 μ L 50 nM Hydrocortisone

1 mL 1.5 mM L-Glut (only if not using GlutaMax DMEM or RPMI)

Carboxyfluorescein diacetate N-succinimidyl ester (CFSE)

Make up at 10 mM in DMSO

10 mM = 0.025g CFSE + 4.49 mL DMSO

Aliquot 50 μ L quantities into ependorf tubes

Store at -20°C

Can be thawed and re-frozen up to four times

Ammonium Chloride (ACK) Red Blood Cell Lysis Buffer

NH₄Cl 4.15 g

KHCO₃ 0.5 g

Disodium ethylenediaminetetra-acetic acid (EDTA) 0.0186 g

Make up to 500 mL in milli-Q water

pH 7.2 – 7.4

Filter sterilise

Brefeldin A (BFA)

Stock is at 5 mg/mL

Use at 10 µg/mL (1 in 500 dilution)

Appendix 2: Supplementary Data

Table S2.1. Concentrations of OVA (ng/mL) in undiluted supernatant of B16OVA cells cultured for 3 days in serum-free medium detected by direct anti-OVA ELISA.

Unknowns	Mean (OD)	Concentration ng/mL	Dilution
1	0.0615	10.24	1.0
2	0.0595	19.78	2.0
3	0.0470	30.1	4.0
4	0.0490	64.66	8.0
5	0.0465	122.4	16.0
6	0.0325	167.6	32.0
7	0.0395	412.5	64.0
8	0.0330	681.5	128.0
9	0.0250	1010.0	256.0
10	0.0195	1535.0	512.0

Table S2.2. Concentrations of OVA (ng/mL) in undiluted supernatant of B16OVA cells cultured for 3 days in serum-free medium detected by indirect anti-OVA ELISA.

Unknowns	Mean (OD)	Concentration ng/mL	Dilution
1	0.0628	0.128	1.0
2	0.0443	-5.412 ¹	2.0
3	0.0503	-7.418 ¹	4.0
4	0.0528	-11.232 ¹	8.0
5	0.0548	-17.561 ¹	16.0
6	0.0493	-62.085 ¹	32.0
7	0.0488	-129.083 ¹	64.0
8	0.0503	-228.732 ¹	128.0
9	0.0468	-594.732 ¹	256.0
10	0.0178	-3463.196 ¹	512.0
11	0.0063	-8732.489 ¹	1024.0

¹ Extrapolating below lowest standard

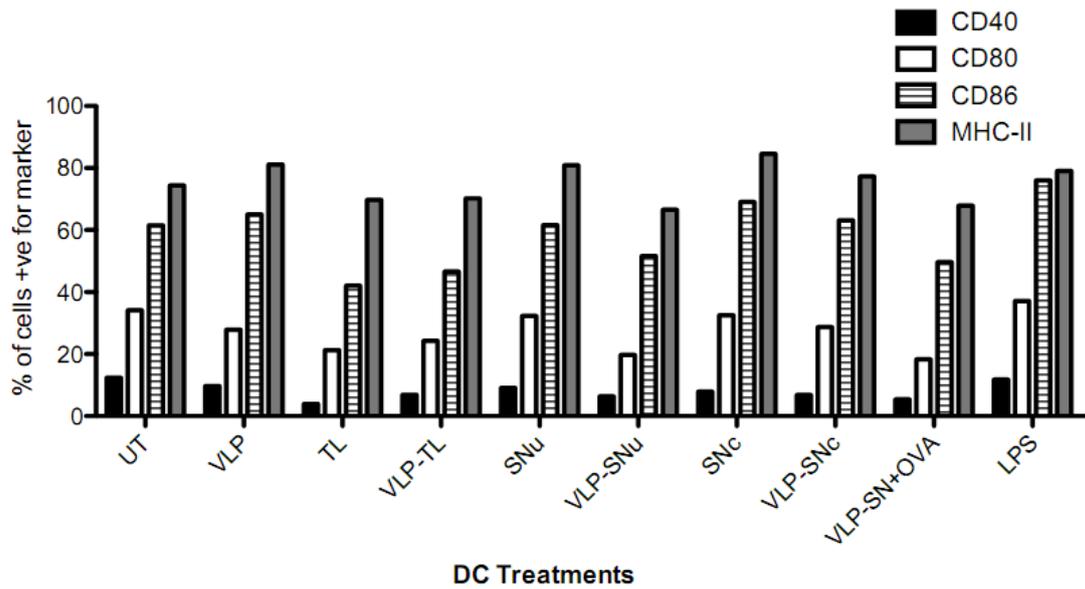


FIGURE S2.1. Tumour lysate has an inhibitory effect on the percentage of cells positive for DC activation markers. Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the percentage of cells positive for the markers analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNu: undiluted supernatant; VLP-SNu: VLP coupled to SNu; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

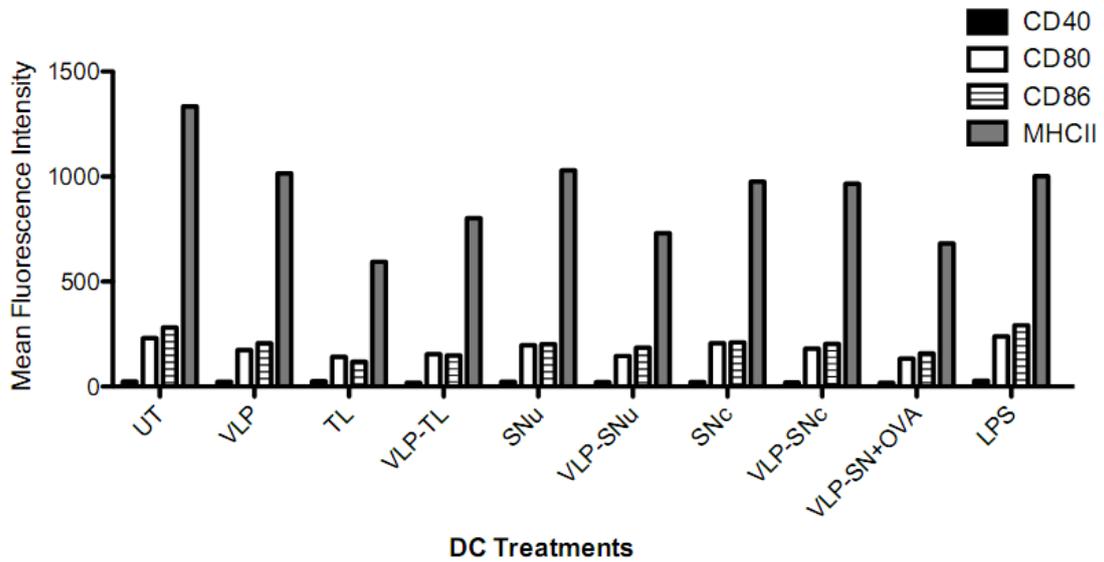


FIGURE S2.2. Tumour lysate has an inhibitory effect on DC activation marker MFI. Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the MFI analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNU: undiluted supernatant; VLP-SNU: VLP coupled to SNU; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

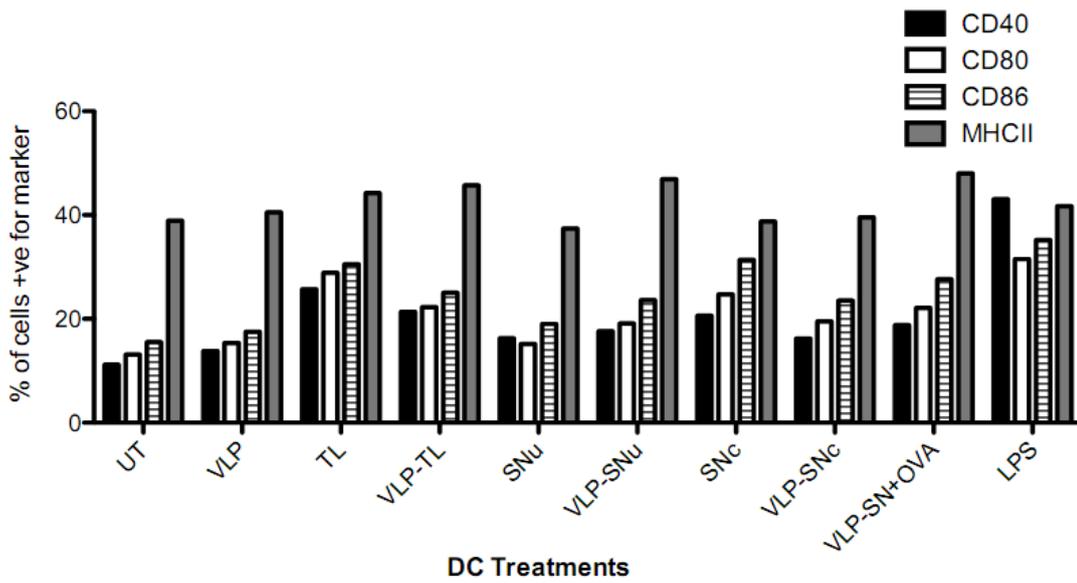


FIGURE S2.3. **Coupling SNu or SNc to VLP results in little change or inhibition of cells positive for DC activation markers.** Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the percentage of cells positive for the markers analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNu: undiluted supernatant; VLP-SNu: VLP coupled to SNu; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

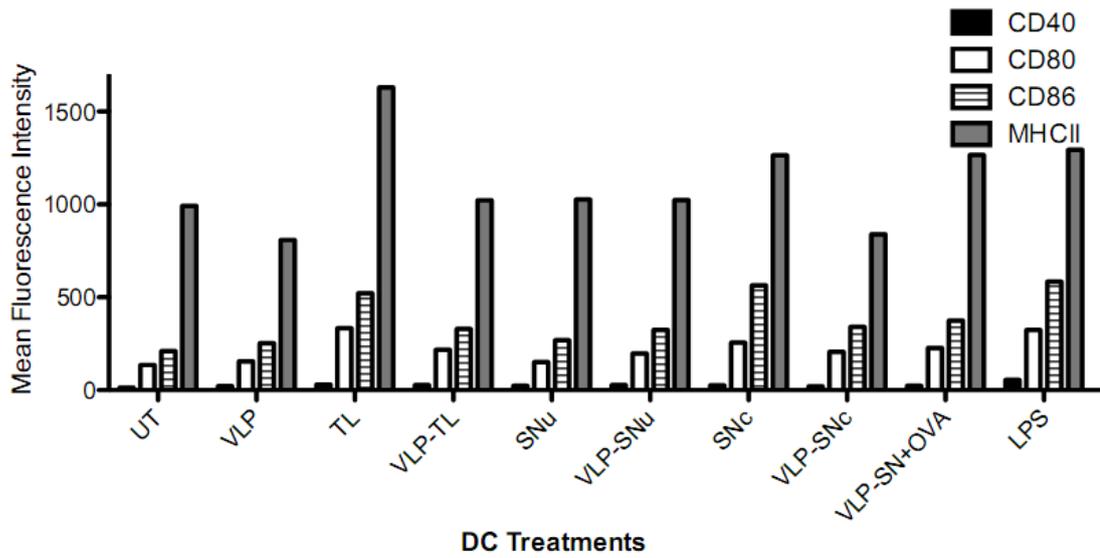


FIGURE S2.4. **Coupling SNU or SNc to VLP results in little change or inhibition of DC activation marker MFI.** Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the MFI analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNU: undiluted supernatant; VLP-SNU: VLP coupled to SNU; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

Table S2.3. Raw data of OT-I CFSE proliferation (percentage of max) showing that coupling SNU or SNc to VLP most often results in little change or an inhibitory effect on OT-I T cell proliferation.

Experiment Number	UT	VLP	TL	VLP-TL	VLP-SN/TL		Spl+ media	Spl+ OVA	SNU	VLP-SNU		SNc	VLP-SNc
					+OVA	OVA				SNU	SNc		
15	22	59.2	15.8	59.8	82.2	87.9	43.7	68.2	28.8	25.9	24.4	29.3	
17	56.6	88.2	18.6	17.7	50.8	19.8	80.7	71.9	87.4	59.3	89.2	66.8	
19	54.4	91.9	15.4	96	97.1	88.7	5.26	12.2	32.6	97.4	92.3	63	

Table S2.4. Raw data of OT-I CFSE proliferation (percentage of max) showing that SNc or VLP-SNU were sometimes able to stimulate similar or better OT-I T cell proliferation than VLP or VLP-TL.

Experiment Number	UT	VLP	TL	VLP-TL	VLP-SN/TL		Spl+ media	Spl+ OVA	SNU	VLP-SNU		SNc	VLP-SNc
					+OVA	OVA				SNU	SNc		
15	22	59.2	15.8	59.8	82.2	87.9	43.7	68.2	28.8	25.9	24.4	29.3	
17	56.6	88.2	18.6	17.7	50.8	19.8	80.7	71.9	87.4	59.3	89.2	66.8	
19	54.4	91.9	15.4	96	97.1	88.7	5.26	12.2	32.6	97.4	92.3	63	

Table S2.5. Raw data of OT-I IFN- γ production (ng/mL) showing that VLP can have a stimulatory effect on IFN- γ production; TL's effect is usually less than that of VLP; and that VLP-TL is sometimes able to overcome this inhibition.

Experiment Number	UT	VLP	TL	VLP-TL	VLP-SN/TL		Spl+ media	Spl+ OVA	SNU	VLP-SNU		SNc	VLP-SNc
					+OVA	OVA				SNU	SNc		
13	0.07	0.056	0.2	0.148	0.467	n/a	n/a	0.867	0.08	0.095	0.214	0.087	
17	7.92	3.13	2.48	4.13	7.78	6.89	6.3	7.82	2.22	5.64	0.7	2.07	
19	0.245	4.382	0.21	4.05	4.33	4.33	0.01	0.435	0.05	4.37	1.74	0.029	
24	0.17	0.33	0.35	0.60	1.77	2.23	0.075	1.39	0.59	0.18	n/a	0.39	
29	0	0.05	0.04	0.06	0.72	1.22	0	0	n/a	n/a	n/a	n/a	

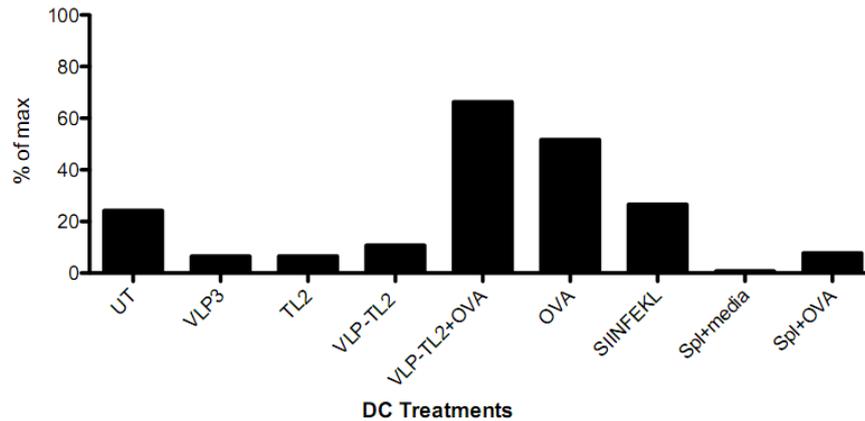


FIGURE S2.5. VLP-TL is sometimes unable to restore OT-I T cell proliferation to the level of those cells exposed to untreated DC. Immature (UT) or mature (treatment-pulsed) DC were co-cultured with CFSE-labelled OT-I splenocytes for 72 hours and proliferation was analysed. Quantification of FACS Calibur flow cytometric results is shown. UT: untreated; VLP3: unconjugated VP60 virus-like particles, 3rd batch; TL2: tumour lysate, 2nd batch; VLP-TL2: VLP coupled to 2nd batch of tumour lysate; VLP-TL2+OVA: VLP coupled to 2nd batch of TL which was spiked with OVA; OVA: whole OVA protein; SIINFEKL: OT-I-specific peptide of OVA protein; Spl+media: splenocytes alone, no DCs; Spl+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC. Representative data from one of six experiments.

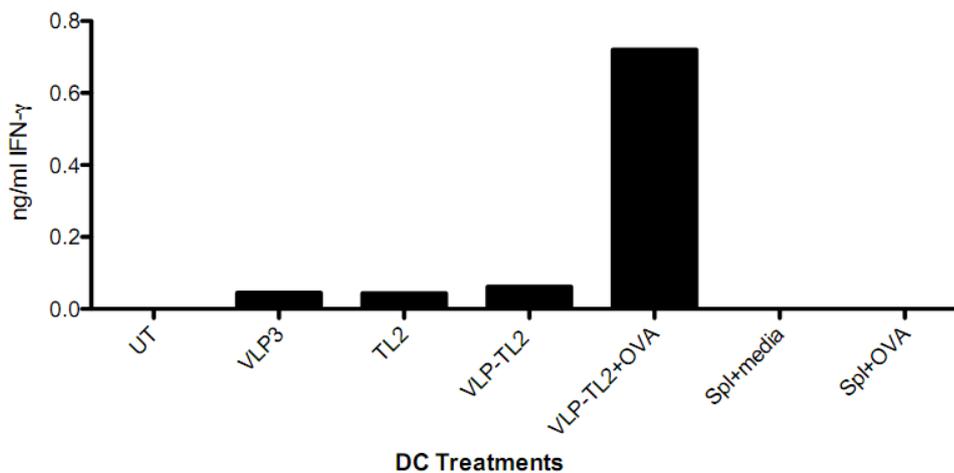


FIGURE S2.6 Production of IFN- γ by OT-I splenocytes can be improved by VLP-TL. Immature (UT) or mature (treatment-pulsed) DC were co-cultured with CFSE-labelled OT-I splenocytes for 72 hours and IFN- γ levels in the supernatant were analysed by direct anti-IFN- γ ELISA. UT: untreated; VLP3: unconjugated VP60 virus-like particles, batch 3; TL2: tumour lysate, batch 2; VLP-TL2: VLP coupled to 2nd batch of tumour lysate; VLP-TL2+OVA: VLP coupled to 2nd batch of TL that was spiked with OVA; Spl+media: splenocytes alone, no DCs; Spl+OVA: splenocytes which had whole OVA protein added to the media in the absence of DC. Representative graph showing quantification of one of four ELISA results.

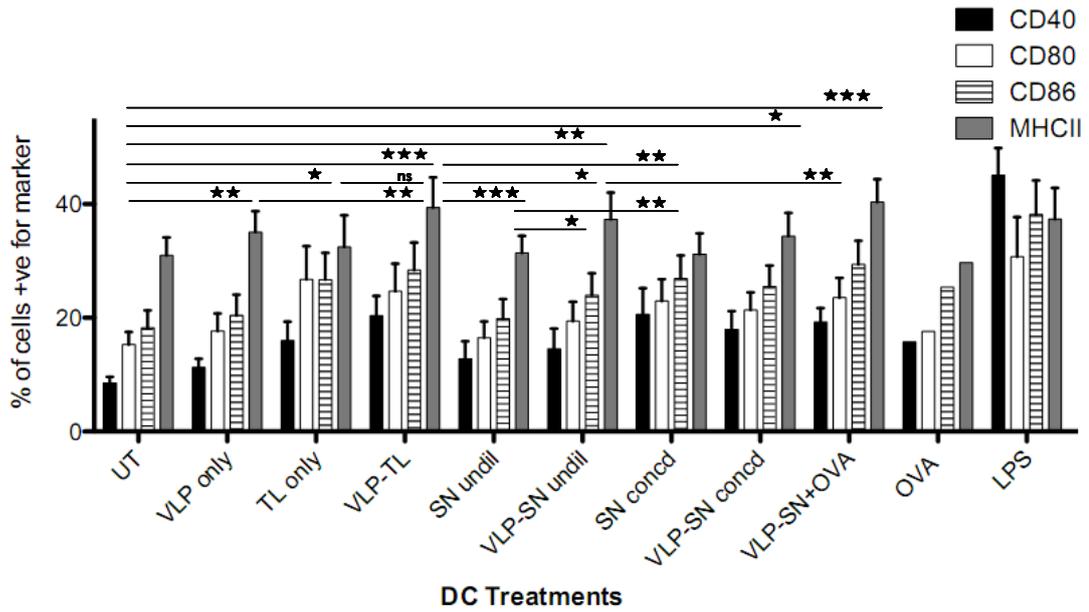


FIGURE S2.7. **VLP-TL is superior to either VLP or TL at upregulation of DC activation markers.** Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the percentage of cells positive for the markers analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNU: undiluted supernatant; VLP-SNU: VLP coupled to SNU; SNC: concentrated supernatant; VLP-SNC: VLP coupled to SNC; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Collated data from five flow cytometric analyses. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$ using paired Student's *t* test before Bonferroni correction was applied.

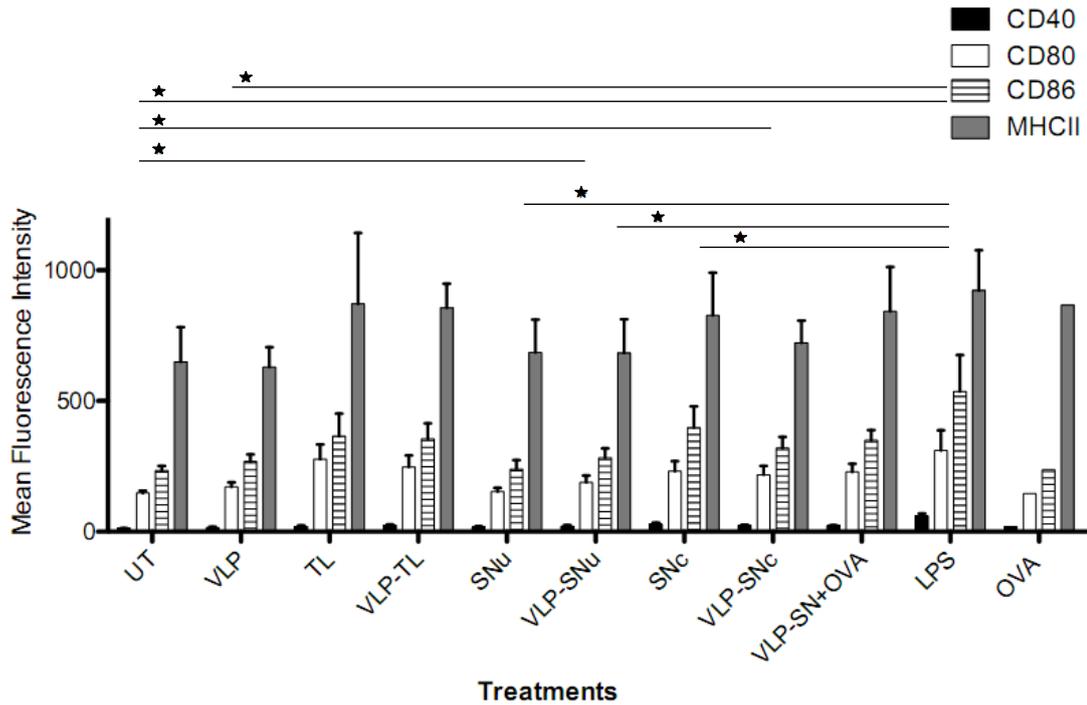


FIGURE S2.8. **VLP-TL is superior to VLP but not to TL at upregulation of DC activation markers' MFI.** Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the MFI of cells positive for the markers analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNu: undiluted supernatant; VLP-SNu: VLP coupled to SNu; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.

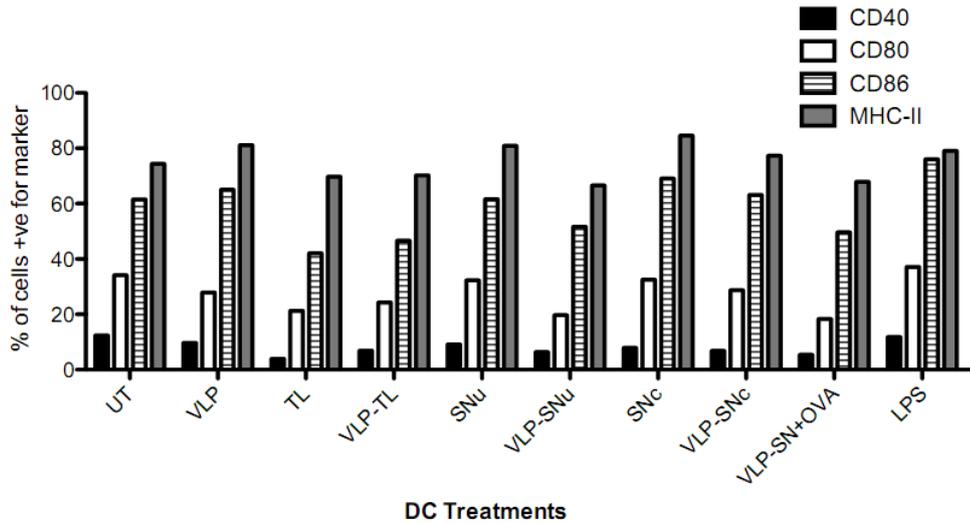


FIGURE S2.9 VLP, TL and VLP-TL can cause inhibition of upregulation of DC activation markers. Cultured bone marrow dendritic cells (BMDC) were pulsed with the indicated treatments on Day 6 and harvested on Day 7. BMDC were labelled with antibodies for the cell surface proteins CD40, CD80, CD86 and MHC-II and the percentage of cells positive for the markers analysed by Flow Cytometry. UT: untreated; VLP: unconjugated VP60 virus-like particles; TL: tumour lysate; VLP-TL: VLP coupled to tumour lysate; SNu: undiluted supernatant; VLP-SNu: VLP coupled to SNu; SNc: concentrated supernatant; VLP-SNc: VLP coupled to SNc; VLP-SN+OVA: VLP coupled to undiluted supernatant that was spiked with OVA; LPS: lipopolysaccharide. Representative quantitative example of five flow cytometric analyses.