Investigating Cytotoxic T cell Responses to RHDV Virus-like particles

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Abstract

Immunotherapies prime cells of the immune system to generate an appropriate response against a specific target. One area of immunotherapeutic research is the development of cancer vaccines such as the vaccines based on Virus-like particles (VLP). VLP derived from Rabbit Hemorrhagic Disease Virus (RHDV) have been shown to be effective in the induction of the cytotoxic responses to specific tumour antigens. The cytotoxic response of CD8\(^+\) T cells generated in response to a tumour is important for the clearance of tumour cells and the generation of memory T cells. Previous studies have shown that RHDV VLP were taken up by dendritic cells (DC) and cross presented, utilising the MHC-I recycling pathway but the cytotoxic response generated was not 100% effective against tumour challenge, in particular where the specific activation of CD4\(^+\) T cells was not activated. The aims of this research were firstly to produce RHDV VLP incorporating tumour epitopes and to determine the dendritic cell subset with the capacity to cross-present VLP to induce cytotoxic responses \textit{in vivo}. Following on from this was an investigation into the role of CD4\(^+\) help to optimise cytotoxic responses and determine when is help required in relation to CD8\(^+\) activation. The addition of adjuvant was investigated in its capacity to improve the cytotoxic responses seen \textit{in vivo}. Finally the production of antibody against VLP protein VP60 was investigated and the impact this antibody has on VLP uptake. To carry out these aims recombinant VLP expressing different immunogenic antigens; gp33 derived from LCMV and OTI, an ovalbumin derived MHC-I peptide, were generated using a baculovirus expression system (VLP.gp33 and VLP.OTI). Some VLP were also chemically coupled with ovalbumin derived MHC-II peptide (VLP.OTII and VLP.OTI.OTII). The importance of DC subsets expressing langerin on cross-presentation for the generation of cytotoxicity was investigated by depleting langerin positive DC using LangDTREGFP mice and vaccinating with VLP.gp33. The requirement for CD4 cells in the induction of in vivo cytotoxicity was assessed by determining the impact of vaccinating mice with VLP.OTI following the adoptive transfer of antigen specific OTI CD4 T cells. The timing of CD4 activation and whether CpG adjuvant could improve in vivo cytotoxic responses were carried out utilising the different VLP previously generated. The presence of antibody was assessed from the serum of animals that had previously been vaccinated with
VLP.OTI and the impact on VLP uptake was determined utilising an *in vitro* uptake assay. These experiments showed that when depletion of the langerin\(^+\) DC was maintained, the in vivo specific lysis of gp33-labelled target cells was significantly reduced. The adoptive transfer of OTII CD4 T cells showed that when the transferred cells were activated by the presence of OTII peptide the cytotoxic responses were improved. The improved cytotoxic response was also observed when animals were vaccinated with VLP that were able to activate both CD4 and CD8 T cells (VLP.OTI.OTII). Regardless of when CD4 help was given in relation to CD8 activation there was no significant improvement to the cytotoxic response. Experiments with the addition of adjuvant to the VLP vaccination were able to show an improved cytotoxic response indicating the requirement of an adjuvant. Finally antibody was detected in high quantities against the VP60 protein and the presence of these antibodies was able to improve the uptake of VLP early on during the uptake process. Overall these results indicate that cross-presentation of peptides by langerin\(^+\) DC is important for the generation of cytotoxic responses of CD8 T cells and are improved when both CD4 and CD8 T cells are activated.
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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria Toxin</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>I.D</td>
<td>Intradermal</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>I.P</td>
<td>Intraperitoneal vaccination</td>
</tr>
<tr>
<td>I.V</td>
<td>Intravenous vaccination</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans Cell</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis virus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>moi</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RHDV</td>
<td>Rabbit Hemorrhagic disease virus</td>
</tr>
<tr>
<td>S.C</td>
<td>Subcutaneous vaccination</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th₁</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th₂</td>
<td>T helper 2</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
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Chapter 1

Introduction

Cancer is the leading cause of death worldwide\(^1\). The burden of cancer is far reaching and the implementation of many strategies has been introduced to try to reduce this burden. The use of vaccinations such as those for cervical cancer\(^2\), screening programmes for early detection\(^3\) and new therapies to improve the treatment of patients are all being developed with the aim to improve patient survival.

The ability of tumours to evade the immune response makes it difficult for the immune system to control the growth of the tumour\(^4\). This ability of immune evasion seems to be a major contributing factor in cancer progression and by perhaps improving the immune responses the treatment and prevention of cancer can be greatly improved\(^5\). This idea of immunotherapy is not a new one with developments in the field dating back to the advent of vaccination\(^6\). By priming the immune system against a pathogen, the patients’ own immune system can recognise and eliminate a threat upon re-exposure\(^7\). For a cancer vaccine immunotherapy the immune system can be primed either through the introduction of tumour antigens after a tumour has established itself as an immunotherapeutic strategy or as a prophylactic vaccine to deliver tumour antigens prior to a tumour challenge. Utilising this idea of a cancer vaccine, this research aims to use tumour antigens to induce optimal cytotoxic T cell responses capable of clearing a tumour.

1.1 Cancer

The global impact of cancer continues to rise with incidence and mortality rates increasing every year, even with the advances that have been made in the screening and treatment of cancer. Based on GLOBOCAN 2008 data, 12.7 million cases of
cancer and 7.6 million cancer deaths were estimated in 2008\textsuperscript{1}. Lung cancer accounts for the highest mortality rates in males each year, while among females breast cancer accounts for the highest mortality rates\textsuperscript{8}. The variation in cancers and the associated rates can be due to many different factors such as sex, age, genetic and environmental factors\textsuperscript{9}, all of which make cancer more difficult to detect, treat and prevent. Current screening for cancers such as breast and cervical cancer and developments in screenings for colorectal cancer can be carried out, however, screening programmes are very much limited by the tissue and the type of cancer being screened\textsuperscript{3}. Current established treatment of cancer involves surgery, chemotherapy and/or radiotherapy. Surgery aims to remove the tumour mass depending on size and accessibility\textsuperscript{10}. Chemotherapy uses drugs that target rapidly dividing cells to eliminate the tumour although these drugs typically kill other immune cells such as stem cells resulting in immunocompromised patients\textsuperscript{11}. Radiotherapy uses radiation to kill tumour cells. This can be targeted specifically by focal radiation, or be targeted to the whole body\textsuperscript{12}. Total body irradiation can result in side effects similar to chemotherapy, and has also been shown to reduce the immune cell population, and therefore patients become immunocompromised\textsuperscript{13}. Although these therapies are well established, there is still the possibility of improvement with the aim to increase the survival of patients. Immunotherapies have been researched for some time\textsuperscript{14}, with the aim of developing a way to use the patients own immune system to fight the cancer. Several advances have been made in the field of immunotherapy and are becoming well established in cancer therapy\textsuperscript{7}. Therapies such as monoclonal antibodies, adoptive cell transfer and recombinant cytokines have been approved for use. Development of monoclonal antibodies to target cancer-associated proteins has resulted in the approval of treatments such as Trastuzumab (Herceptin), an antibody against the HER2/neu receptor\textsuperscript{15} and Ipilimumab, a monoclonal antibody against CTLA4\textsuperscript{16}. These antibodies function to block their respective targets and their downstream effects resulting in the interruption of the oncogenic pathways involved. For example, Ipilimumab acts by blocking CTLA4, a molecule present on activated T cells. CTLA4 provides inhibitory signals to T cells by disrupting the co-stimulatory signal provided by the interaction of CD80/CD86 on DC cells and CD28 on T cells\textsuperscript{17}. The
blockade of CTLA4 results in enhanced T cell stimulation, therefore eliciting a better anti-tumour response. Studies on the functions of Ipilimumab have also demonstrated that there is a negative effect on T reg cell function resulting in a better anti-tumour response.\(^1\)

Adoptive cell transfer\(^1\),\(^2\) and dendritic cell therapy\(^2\) aim to improve the patients quantity and quality of lymphocytes. This can be carried out in a variety of ways such as removing tumour infiltrating lymphocytes (TIL) from the patient’s tumour and expanding them \textit{ex vivo}. The cells are returned to the patient where they ultimately induce a stronger anti-tumour response.\(^1\) The response in clinical trials has been variable and improvements have been made in adoptive T cell transfer techniques.\(^2\) For example the advances in the development of chimeric T cell receptors improve the specificity of the T cells to give better responses \textit{in vivo}.\(^2\),\(^3\)

Cytokines have also been approved for use along side other therapies. Recombinant cytokines such as IL-2 (Proleukin) and Interferon-\(\alpha\)\(^2\) have been licensed for use for melanoma and renal cell cancer patients.\(^2\),\(^3\) These immunostimulatory cytokines act to improve the T cell growth and survival. The efficacy of recombinant cytokines is not great and there can be problems with toxicity, hence these are used in combination with other established treatments.\(^2\) Another area of immunotherapy research has been in the development of cancer vaccines. The idea of a cancer vaccine began to develop from the discovery that patients can harbour CD4\(^+\) and CD8\(^+\) tumour specific T cells.\(^2\) Utilising the presence of tumour specific T cells a vaccine could improve the quality and the quantity of these cells, however the development of a cancer vaccine has had limited success. This is area of research still requires further investigation.

### 1.2 Immune response to Tumour

With advances in the treatment of cancer, death rates would be expected to fall, however tumour cells have their own ways of avoiding and manipulating the immune system allowing for evasion and survival of the tumour cells.\(^4\) (Fig 1.1). Tumour cells can directly escape immune regulation by reduction of the cell surface molecules such as Major Histocompatibility Complex class I (MHC-I) or adhesion molecules
Figure 1.1: Mechanisms for immune evasion by tumours. 

a) Tumours down-regulate MHC and co-stimulation molecules resulting in low immunogenicity of the tumour.

b) Tumour antigens expressed and presented to DC without co-stimulation molecules resulting in T cell anergy.

c) Tumours can create immune privileged sites by producing molecules, preventing immune cell access.

d) Secretion of factors which act to down-regulate immune responses.
(Fig 1.1a). The loss or down regulation of MHC-I molecules on the tumour cells results in the ineffective recognition by cytotoxic T cells\(^{29}\). The down regulation of MHC-I has been detected among various different cancers including, but not limited to, lung cancer\(^ {29}\) and prostate carcinoma\(^ {30}\).

Tumours can also down regulate adhesion molecules such as E-selectin, resulting in the impairment of T cell recruitment to the tumour site\(^ {31}\). These mechanisms result in low tumour cell immunogenicity and a reduced ability to stimulate T cells.

Down regulation of co-stimulation molecules has an effect on the stimulation of T cells and also has an effect in the recognition of tumour as self-antigen. For full activation T cells require not only signals from the MHC-I:peptide complex, but they also require a second signal from co-stimulation molecules such as CD80 and CD86\(^ {32}\) (Fig 1.1b). In a situation where T cells are stimulated in the absence of co-stimulation, for example in a tumour environment, T cell anergy can take place\(^ {33}\). Anergy results in tolerance of T cells against tumour antigen, preventing effector immune responses against the tumour\(^ {33}\). Investigations into this have shown that the induction of the co-stimulatory molecules on tumour cells was able to sufficiently activate CD8\(^ +\) T cells therefore abrogating the tolerance caused by T cell anergy\(^ {34}\).

Tumours can also create an immune privileged area, whereby factors such as collagen and fibrin provide a physical barrier to prevent recognition by the immune system\(^ {35}\) (Fig 1.1c). By creating an immune privileged site the tumour is able to avoid immune surveillance long enough for the tumour mass to grow to a point where it becomes too difficult for the immune system to control\(^ {35}\).

Tumours can secrete various factors, which act on the immune system to dampen down effector responses and recruit regulatory T cells (Fig 1.1d). Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is one of the factors that a tumour cell may secrete leading to the suppression of CD8\(^ +\) T cell responses. This also enhances the regulatory T cell development, another benefit for the tumour\(^ {36}\). Another example is the expression of immunosuppressive cytokine IL-10. The effect of IL-10 is to reduce dendritic cell activity and directly inhibit T-cell activation\(^ {37}\).

A critical function of the immune system is thought to be immune surveillance\(^ 5\) and the ability to deal with any changes in cell homeostasis, however tumour cells are able to avoid and manipulate the surveillance and response of the immune system.
When developing immunotherapies these factors need to be taken into consideration, whether it is the breaking of tolerance caused by T cell anergy or eliciting an immune response where the tumour has escaped recognition. With this in mind a cancer immunotherapy should be able to elicit a strong immune response overcoming the issues of immune evasion. The therapy should also be non-toxic and non-invasive to the patient, minimise the hospitalisation time and reduce the number of procedures that patients have to go through.

1.3 **Virus-Like particles**

Virus-like particles are particles derived from the structural proteins of viruses\(^{39}\). Using protein expression systems, the over-expression of recombinant viral structural proteins results in the self-assembly of proteins into particles. These particles are generally safe to use as vaccines as they lack the viral genomic material essential for replication and infection. They have been shown to successfully prime the immune system due to the similarities in structure and antigenicity to the viruses from which they are derived\(^{39}\) (Fig 1.2). The particles also resemble the size of virus from which they are derived from which makes them ideal for uptake by DC.

The development of VLP as a vaccine has resulted in the approval of VLP based vaccines such as those against Human Papillomaviruses (HPV): Gardasil\(^{®}\) (Merck) and Cervarix\(^{®}\) (GlaxoSmithKline)\(^{40,41}\). These vaccines use VLP derived from the structural L1 protein of HPV allowing the immune system to recognise infection by certain HPV types\(^{42}\), which can develop into cervical cancer\(^{43}\). These vaccines confer good protection against HPV infection and measurements of serum antibody against the L1 protein indicate good humoral responses\(^{40,44}\). Virus-like particles have been developed for use in vaccinations against Influenza. Conventional Influenza vaccines target the major envelope proteins Hemagglutinin (HA) and Neuraminidase (NA). Incorporation of these viral proteins as well as the M1 matrix protein with VLP as vaccines against seasonal influenza strains have been investigated\(^{45}\).

VLP can be modified by recombinant expression or chemical coupling of heterologous antigens\(^{46}\). Antigens such as gp33 peptide (MSAVYFATMGGS) from Lymphocytic Choriomeningitis Virus (LCMV) and Ovalbumin derived MHC-I...
Figure 1.2: RHDV VLP cryoEM reconstruction image: Dr Tom Smith, Donald Danforth Centre, St Louis, MO, USA.
epitope recognised by CD8 T cells (SIINFEKL; OTI) and MHC-II epitope recognised by CD4\(^+\) T cells (CISQAVHAAHAEINEAGR; OTII) have been added successfully either through recombinant technology\(^{47}\) or chemical coupling\(^{46}\) to VLP. The addition of antigens to VLP has been shown to improve the immunogenicity of the antigens\(^{48}\) and provides protection against this antigen expressed on target cells\(^{47}\). VLP demonstrate a versatile platform for the delivery of tumour antigens in the development of cancer vaccines.

1.4 Rabbit Hemorrhagic Disease Virus

The VLP used in this research are derived from Rabbit Hemorrhagic Disease Virus (RHDV). It is a single-stranded, positive-sense RNA virus of the *Caliciviridae* family, *Lagovirus* genus and is the cause of hemorrhagic disease in rabbits\(^{49}\). RHDV has a 7.5 kb genome, which contains two open reading frames. The first open reading frame encodes for non-structural proteins and the VP60 capsid protein. The VP60 gene produces a 60 kDa protein that forms dimers to make up the structure of the virus capsid\(^{50}\). The viral capsid of RHDV is made up of 90 of these dimeric units\(^{51}\) and the resulting structure is around 40 nm in diameter\(^{49}\). The VP60 gene has been expressed in protein expression systems and shown to self-assemble\(^{50}\). The use of these particles for the vaccination of rabbits against RHDV has been successful in protecting rabbits against lethal challenges of RHDV\(^{52,53}\). These particles have been successfully modified to include heterologous peptides either by recombination or chemical coupling. The internal facing N-terminus domain can easily incorporate antigens of an appropriate size without disruption of the particle structure. The particles also allow for the incorporation of epitopes at the C-terminus without disruption to the particle assembly\(^{54,55}\). RHDV does not naturally infect humans or mice as it exclusively infects rabbits. This means that there is no pre-existing immunity in humans or mice, allowing for the vaccination to elicit immune response without the interference of antibody, which may eliminate the particle before it is able to be effective\(^{56}\). The presence of pre-existing immunity may affect the efficacy of the immune response to the vaccine, particularly where a prime-boost vaccination strategy is required\(^{57}\). Taken together RHDV VLP provide an ideal system for the
development of a cancer vaccine as both a prophylactic and immunotherapeutic vaccine.

1.5 Virus-like particle production

There are various methods used for the production of VLP, such as those based on yeast, mammalian or bacterial cell systems. Yeast-based expression systems use *Saccharomyces cerevisiae* cells for the production of recombinant proteins\(^5^8\) whereas mammalian systems use mammalian cell lines such as CHO-K1 from Chinese Hamster ovaries\(^5^9\) and bacterial systems most commonly use *Escherichia coli*\(^6^0\). Work carried out in our laboratory uses a baculovirus insect cell system\(^6^1\) (Fig 1.3) based on the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNV). The VP60 gene is cloned into a transfer vector, which contains a baculovirus promoter and flanked by baculovirus derived sequences. Following transfection the gene of interest is incorporated into the genome of AcMNV by homologous recombination. Infection of the insect cells with the recombinant baculovirus results in the over expression of the proteins and the self-assembly into particles\(^6^1\). The particles are purified through differential centrifugation steps. Once purified and checked, the VLP can also have peptide coupled to the outside of the particle\(^4^6\). Peptides have been successfully associated both by recombination and chemical coupling without disruption to the self-assembly of the particles, however there are limitations to the size of the peptide included. The addition of peptides by recombination means that the antigen of interest is cloned into the transfer vector at the N-terminus of VP60\(^5^4\). The two genes are expressed at the same time and the resulting proteins assemble together with the antigen present inside the particle. Chemical coupling requires the addition of a chemical linker prior to the addition of the antigen. The chemical linker is able to bind to free amines on the surface of the VLP. Following the addition of the linker the antigen of interest is bound to the particle. These additions occur following the purification of the VLP. The techniques used are well established and have been shown to be very successful for the generation and modification of VLP.
Figure 1.3: Virus-like particle production by baculovirus expression. (Adapted from Roy et al\textsuperscript{62}) a) Baculovirus acts as a vehicle to deliver recombinant DNA. b) Viral DNA replication. c) Recombinant protein expression is driven by strong very-late viral promoters. d) Viral mRNA is used for the synthesis of recombinant proteins. e) VLPs are assembled by the interaction of proteins within the cytoplasm.
1.6 Immune response to VLP

VLP have been shown to elicit both humoral and cellular immune responses. The highly ordered and repetitive arrangement of antigens has been shown to induce humoral immune responses with the activation of B cells and the production of protective antibodies\textsuperscript{56}. The particles also resemble the size of virus from which they are derived, which allows for effective uptake by DC and therefore activation of cellular immunity\textsuperscript{63} (Fig 1.4).

The uptake of RHDV VLP has been shown for various subsets of DC, in particular CD8α\textsuperscript{+}, CD103\textsuperscript{+} and CD11b\textsuperscript{+} DC (Li et al submitted for publication). These subsets of DC are able to process and present the antigens on both MHC-I and MHC-II complexes, vital to induce the observed cytotoxic responses. VLP are exogenous antigens and are taken up and processed by the DC by the endogenous pathway, but this is not the only pathway of antigen processing and presentation that DC are able to utilise. The ability of DC to cross present exogenous antigens to allow the activation of CD8 as well as CD4 T cells means that the immune response generated is more effective\textsuperscript{64}. Following the uptake of the VLP, DC migrate to the draining lymph node. Once in the lymph node the DC encounter CD8 and CD4 T cells to activate them. The activation of the T cells results in the proliferation of these cells and differentiation into cytotoxic T cells (CD8) and helper T cells (CD4).

Modified RHDV VLP with model tumour antigens, have been shown to elicit cytotoxic immune responses required to combat tumours. Animals that were vaccinated with VLP coupled with OVA or OTI peptide were able to specifically kill target OTI peptide-pulsed cells that had been adoptively transferred\textsuperscript{48}. Vaccination with VLP coupled with OVA or both OTI and OTII peptides were also shown to induce a therapeutic anti-tumour response against the B16 OVA melanoma cell line. This was not observed with VLP coupled with the OTI peptide alone demonstrating the need for CD4 help\textsuperscript{48}. Mice that had been challenged with B16-OVA tumour cells after receiving VLP coupled with OVA peptide showed reduced growth of the tumours providing evidence of modified VLP as a prophylatic vaccine\textsuperscript{48}.
Figure 1.4: Virus-like particle processing and presentation. VLP taken up by DC, processed and presented by exogenous presentation pathway to CD4 cells and cross-presentation pathway to CD8 cells.
1.7 Cross-presentation

The cross-presentation of peptides, typically exogenously derived, has been demonstrated by antigen presenting cells (APC) through various proposed pathways\(^\text{35}\).

The cytosolic pathways are proteasome dependent and utilise the MHC-I loading machinery\(^\text{65}\). In the phagosome-to-cytosol pathway the antigen is taken up into the phagosome and translocated into the cytosol where it is degraded by the proteosome (Fig 1.5a). Once degraded the antigen is transported by the TAP transporter into the endoplasmic reticulum (ER) where it is loaded onto MHC-I\(^\text{66}\). This pathway has been shown to be dependent on the transport of peptide loaded MHC-I by the Golgi apparatus to the cell surface. Blocking this transport with brefeldin A has shown evidence of this\(^\text{67}\).

The second pathway involves the fusion of the phagosome and ER vesicles\(^\text{68}\) (Fig 1.5b). Evidence of ER-Phagosome fusion has been shown by the presence of proteins involved in the MHC-I loading machinery originating in the ER\(^\text{69}\). This pathway is also dependent on cytosolic proteasome degradation and TAP transport whereby the protein is degraded in the cytosol\(^\text{68}\). Once the peptide has been degraded, the antigen is loaded onto MHC-I within the phagosome due to the presence of the ER associated MHC-I loading machinery\(^\text{66}\). This pathway is not dependent on the Golgi for transport of MHC-I complexes as the transport has been shown to be partially inhibited in the presence of brefeldin A\(^\text{70}\).

A third pathway has been shown to be proteasome and TAP transporter independent\(^\text{71}\). The vacuolar pathway or MHC-I recycling pathway degrades the antigens within the phagosome by proteases and loads the peptides onto MHC-I recycled from the cell surface\(^\text{65, 72}\) (Fig 1.6).

Various DC subsets and other APC such as Macrophages have been shown to cross present antigens\(^\text{65, 73}\). DC subsets such as CD8\(^\text{a}^+\), CD103\(^+\), CD11b\(^+\) DC and langerin positive cells\(^\text{74,75}\) have been shown to be capable of presenting exogenous peptides on MHC-I, utilising cross-presentation pathways (Li et al submitted for publication). It has also been shown that cross-presentation of RHDV VLP occurs via the MHC-I recycling pathway. The uptake of RHDV VLP has been demonstrated to occur by phagocytosis or macropinocytosis, and is not mediated by specific receptors\(^\text{64}\). The
Figure 1.5: Cytosolic pathways of cross-presentation\textsuperscript{65} a) Phagosome-to-cytosol pathway c) ER-phagosome fusion pathway.
Figure 1.6: MHC-I recycling pathway of cross-presentation\textsuperscript{65}: Antigens loaded onto recycled MHC-I molecules
cross-presentation of RHDV VLP is independent of proteosome degradation and TAP transport as shown by Win et al\textsuperscript{64}. Blocking the proteosome and TAP transporter had no significant impact on the cross-presentation of RHDV VLP and its associated antigen SIINFEKL, whereas blocking acidification of the lysosome or the trafficking of recycling molecules showed a decrease in cross-presentation of peptide\textsuperscript{64}.

1.8 CD\textsuperscript{8} Cytotoxic T cells

T cells are important for cell-mediated immune responses, crucial for the activation of other cells and effector functions. T cells differentiate into various different subsets, each with their own specific functions. During the development of T cells, T cell precursors from the bone marrow undergo various selection processes in the thymus. Positive selection is the selection of double positive cells that recognise self peptide:self MHC expressed on thymic endothelial cells. These cells mature into single positive CD4 or CD8. Following this, negative selection takes place where those cells that have a high affinity for self-antigen are deleted. Following thymic selection the single positive CD\textsuperscript{8} T cells make up 30-40\% of the T cells that circulate in the blood and secondary lymphoid organs while the remaining 60-70\% are CD4\textsuperscript{+} T cells\textsuperscript{35}. CD\textsuperscript{8} T cells are the cells responsible for the targeted killing of cells infected with virus or intracellular pathogens. They also provide protection against tumours through detection of antigenic changes on tumour cells.

1.8.1 Activation of CD8\textsuperscript{+} T cells

Activation of the CD8\textsuperscript{+} T cells requires naïve CD8\textsuperscript{+} T cells to recognise peptide presented on MHC-I molecules by a number of receptor-ligand interactions: unique T cell receptors (TCR) that interact with MHC-I:peptide, the interaction of the CD8 molecule and the MHC-I:peptide ligand and the ligation of co-stimulatory receptors i.e. CD28 interacting with its corresponding ligand CD80/CD86 on DC\textsuperscript{76} (Fig 1.7). The first signal is provided by the interaction of TCR and CD8 on the T cell with the MHC-I:peptide on the DC (Fig 1.7). The interaction of the T cell with DC is
Figure 1.7: Activation of Cytotoxic CD8 T cells. Signal 1 is provided by peptide loaded onto MHC-I on DC and is recognised by TCR and CD8 molecules on the CD8 T cell. Signal 2 co-stimulation is provided by CD86 on DC interacting with CD28 on CD8 cells. Cytokines produced by CD4 T deliver signal 3.
dominated by the TCR and MHC-I:peptide binding. The interaction of these two complexes has a relatively low affinity (Reviewed in 77). The interaction of the CD8 receptor and MHC-I:peptide binding, which has a lower affinity than TCR and a faster kinetic interaction, has been shown to enhance the TCR and MHC-I interaction\textsuperscript{78}. The second signal (Fig 1.7) required is provided from the interaction of co-stimulation molecules such as CD28 on the CD8\textsuperscript{+} T cell and their corresponding receptor CD80/CD86 on DC. The signal provided by DC through this interaction is important for the clonal expansion of the T cells. By signalling through these co-stimulation signals the number of MHC:peptide-TCR interactions required for activation are thought to be reduced. Without the co-stimulation signals T cells are not properly activated and results in T cell anergy. This is important for self-tolerance, as the T cell is unable to be activated upon subsequent exposure to the antigen on APC, even in the presence of co-stimulatory molecules.

Co-stimulation can also be helped by a third signal, provided by cytokines present in the environment, in particular from CD4\textsuperscript{+} T cells (Fig 1.7). For example the production of IL-2 by has been shown to be important for the activation and the proliferation of T cells. The importance of CD4 T cell help has also been implicated in memory CD8 T cell survival\textsuperscript{79}.

The interaction of these complexes results in the downstream signalling which causes the activation and proliferation of these cells.

1.8.2 Function

The pathways involved in the function of cytotoxic T cells include direct cell-to-cell contact and indirect contact pathways. Cytotoxic T cells produce proteins perforin and granzyme once the naïve CD8\textsuperscript{+} T cell has become activated\textsuperscript{80}. These are highly cytotoxic and the CD8\textsuperscript{+} T cells have mechanisms in place to ensure that it and bystander cells are not targeted while causing apoptosis of the target cells. These proteins are contained within membrane-bound organelles, or secretory lysosomes\textsuperscript{81}. Perforin is thought to act as a pore-forming protein, creating pores in the cell membrane to allow for granzyme release\textsuperscript{82} from the cytotoxic T cell and has also been hypothesised to be involved in the release of granzyme into target cells that have taken up the proteins by endocytosis\textsuperscript{83}. Studies on mice depleted of perforin
have demonstrated its importance for granzyme-dependent killing. These mice also shown an increase in their susceptibility to pathogens and tumour development. The second family of proteins produced by cytotoxic T cells are the granzymes. Granzyme B is among this family of serine proteases and is the most well known. It has been shown to function by the induction of cell death by apoptosis. The presence of granzymes within the target cell causes the activation of apoptotic pathways initiated through the cleavage of caspases. The result is a targeted death of infected cells or of tumour cells expressing the target antigen.

1.8.3 CD8 T cell phases
The Cytotoxic T lymphocyte (CTL) response can be divided into four phases: expansion, contraction, memory maintenance and rapid recall response phases (Fig 1.8). The expansion phase includes the activation of the CD8 cells and rapid clonal proliferation of the antigen specific cells. During normal cell homeostasis the precursor frequency of antigen specific CD8+ T cells that respond to one particular antigen is around 1 in 100,000. Upon the clonal expansion of the antigen specific T cells the number of these cells can increase up to 50,000 fold. The increase in cell number is required to clear the pathogen infected or tumour cells. The cytokines and release of perforin and granzyme facilitate the cell killing during this phase. The expansion phase is thought to last for 1-2 weeks until the pathogen has been cleared. The contraction phase occurs once the target cells have been cleared, thought to be around 4-5 weeks after clearance of the pathogen. During this phase the majority of the activated CD8+ T cells die, with around 5-10% of the antigen-specific cells remaining as long-lived memory cells. The mechanisms behind the differentiation of effector and memory cell is unclear, however there are several models that have been proposed. The linear differentiation model proposes that a naive cell can differentiate into an effector cell followed by differentiation into a memory CD8+ T cell. The second model is the asymmetrical division model. This model proposes that upon stimulation a naïve CD8+ T cell asymmetrically divides into two distinct cell types; effector or memory cells. The memory cells are crucial for the ongoing rapid recall responses. If the antigen is encountered again by the memory cells they are able to rapidly proliferate and act on the target cells.
Figure 1.8: Phases of Cytotoxic T cell activity. Expansion of antigen specific CD8 T cells. Contraction phase of antigen specific CD8 T cell population. Long-lived memory CD8 population Rapid expansion upon re-exposure to target antigen.
1.9 CD4 Helper T cells

CD4\(^+\) T cells make up the majority of the T cells circulating in the blood and lymphatic system in the body. These cells are vital players in the adaptive immune system providing help to other immune cells including CD8\(^+\) T cells, B cells and macrophages.

1.9.1 Activation

The activation of CD4\(^+\) T cells is similar to that of CD8\(^+\) T cells. The first signal is provided by the recognition of specific antigen through the interaction of the peptide bound to the MHC-II complex and the TCR. The interaction is complemented by the interaction of the CD4 molecule, which acts in the same way as the CD8 molecule, as described above\(^35\). Co-stimulatory molecules on DC provide the second signal to CD4 cells, through the CD80/CD86 and CD28 interaction. The third signal provided by cytokines causes CD4\(^+\) T cells to differentiate into different effector phenotypes depending on the cytokines present in the cellular environment. For example the presence of cytokines such as IL-12 and IFN-\(\gamma\) favour the differentiation towards a T helper 1 (T\(_h\)1) phenotype, whereas the presence of IL-4 favours a T\(_h\)2 phenotype\(^35\).

Once activated the CD4 T cells produce various cytokines, which can act in an autocrine manner to induce further proliferation. IL-2 is one of the cytokines released by CD4\(^+\) T cells, which is important for T cell growth and function. This IL-2 provided by CD4\(^+\) T cells has been shown to be important not only for CD4\(^+\) cells and in the early activation of CD8\(^+\) T cells\(^92\), but also for memory CD8\(^+\) responses to inflammatory pathogens\(^93\).

1.9.2 Function

A crucial role of CD4\(^+\) T cells is through the provision of help in the initial activation and the differentiation of CD8\(^+\) T cells and survival of memory T cells\(^94\). T\(_h\)1 CD4 T cells provide help to CD8 T cells during in viral infections, particular where the co-stimulation provided by DC is inadequate. CD4 T cells express the CD40L, which is able to bind to the CD40 molecule induced on DC. This interaction causes the up regulation of CD80/CD86 molecules. Help from CD4 T cells also prevents tolerance and promotes the survival of effector and memory CD8 T cells\(^95\). In an anti-tumour
setting CD4 help is less understood, as tumours tend to be MHC-I positive resulting in poor activation of CD4 T cells\textsuperscript{96}. The role that CD4\textsuperscript{+} T cells play in providing help to CD8\textsuperscript{+} T cells is important for the development of a cancer vaccine. By activating CD4\textsuperscript{+} cells the cytotoxic and memory responses of the CD8\textsuperscript{+} T cells would be expected to be greater, therefore providing a more potent and long-lasting vaccine.

1.10 Antibody

The uptake of antigens by DC can be improved by opsonisation, the binding of antibodies to the antigen. For VLP vaccination, the generation of antibodies against the VLP may enhance the uptake of the VLP with a boost vaccination. Alternatively, the presence of antibody may cause the vaccine to be mopped up before the vaccine is able to induce protective immunity.

Antibodies are an important part of the humoral immune response. Produced by B cells, the antibodies are able to bind to antigens. Antibodies act in several ways to neutralise pathogens, activate the complement pathway and tag pathogens for optimal phagocytosis\textsuperscript{35}.

Once B cells have been activated and produce antibodies a process called class switching occurs. Initial production of antibody is of the IgM or IgD isotype. The different isoforms perform different functions, however, the variable region of the antibody remains the same allowing the antigen specificity of the antibody to remain unchanged. The process of isotype switching depends on the interaction of CD40 on B cells and CD40L on CD4\textsuperscript{+} T cells and cytokines produced by the CD4\textsuperscript{+} T cells\textsuperscript{97}.

The Fragment crystallisable region (Fc region) of the antibody molecule binds to receptors on the cell surface and stimulates the uptake of the antigen\textsuperscript{98}. The Fc\gamma R1 receptor (CD64) on DC is an example of an Fc receptor that recognises the Fc region on IgG1 and IgG3 isotypes. Upon binding to the DC there is enhanced uptake of the bound antigen. The result of antibody binding is enhanced phagocytosis and enhanced inflammatory responses, both beneficial for anti-tumour responses.
1.11 Aims

The aims of this work were to:

• produce RHDV VLP incorporating the epitopes gp33, OTI and/or OTII, using techniques established in our laboratory
• investigate the capacity of VLP to induce CD8 cytotoxic responses in vivo
• investigate the requirement for CD4 helper T cells to optimise CD8 cytotoxic responses in vivo
• determine when CD4 help is required, in relation to CD8 activation in vivo
• investigate whether adjuvant can improve cytotoxic responses in vivo
• investigate the production of antibody against the VP60 proteins of VLP and its effect on VLP uptake.
Chapter 2

Methods

2.1 Virus inoculum stock
Viral inoculum stocks for the production of RHDV VLP and RHDV VLP containing the gp33 epitope (VLP.gp33) were produced by recombinant baculovirus in insect cells. A recombinant AcMNV baculovirus containing the gp33 epitope MSAVYFATMGGS at the 5’ end of the RHDV VP60 gene and under the control of the AcMNV p10 promoter was supplied by the Ward Laboratory. Stocks of recombinant baculoviruses were generated by infecting 200 ml suspension cultures of Sf21 cells at 1 x 10^6 per ml in Sf900-III medium (Gibco, Life Technologies, CA, USA) at a multiplicity of infection (moi) of 1 and incubating at 125 rpm at 25°C for 3 days. The culture was clarified at 500 g for 5 min and the viral titre in the clarified supernatant was determined by plaque assay. Viral stocks for the production of RHDV VLP with the OT-I peptide SIINFEKL were provided by the Ward Laboratory.

2.2 Plaque Assay
Three millilitres of Sf21 cells at 5x10^5 cells/ml in Sf900-III serum free medium was added to each well of a 6 well plate and incubated for 1 hour to allow cell adherence. The medium was removed from the wells and 100 µl of baculovirus inoculum diluted from 10^-3 to 10^-8 in Sf900-III medium was added. The 6 well plate was incubated at room temperature for 1 hour with rocking every 20 min to prevent the cells from drying out. Seven millilitres of SF900-III medium + 10% FBS was prewarmed to 43°C in a water bath and an equal volume of molten 3% sea plaque agarose (Lonza, Basel, Switzerland) was added, mixed and maintained at 43°C. After
the plate had been incubated for 1 hour the inoculum was removed and 2 ml of FBS/agarose mixture was added and left to set for 20 min. The agarose was overlayed with 1.5 ml of Sf900-III medium, which contained 1 µg/ml pen/strep (stock concentration of 500x, 50,000 units/ml penicillin, 50 mg/ml streptomycin). The plate was incubated at 27°C for 4 to 5 days. The wells were stained with 10 µl of 10 mg/ml neutral red stain. Wells were then incubated for 2 hours at 27°C then the stain and medium was removed. The plate was incubated overnight at room temperature to allow the plaques to clear, the plaques were counted and the titre of virus was determined.

2.3 Generation of recombinant RHDV VLP.gp33 and VLP.OTI

Sf21 insect cell suspension cultures of 400 ml were infected with the recombinant virus at a moi of 1. To the suspension culture 800 µl of pen/strep was added and the cultures were incubated for 3 days at 27°C with agitation at 120 rpm. After 3 days the cultures were treated with 0.5% Triton X 100 and incubated for 30 min at room temperature to release VLP from the unlysed cells. The cell debris was removed by centrifugation at 10,000 g for 20 min. The supernatant was collected and centrifuged at 30,000 rpm (Fiberlite FL40 rotor) for 1.5 hours to collect the VLP in the pellet. One millilitre of insect PBS was added to each pellet and left overnight at 4°C to resuspend. The pellet was resuspended further by pipette and was transferred to microfuge tubes. The resuspended VLP was centrifuged at 10,000 g for 20 min. Without disturbing the pellet the supernatant was taken from the microfuge tubes and loaded on to the top of a Caesium chloride (CsCl) gradient of 1.2 g/cm³ and 1.4 g/cm³. The gradient was topped up with insect PBS and centrifuged for 18 hr at 23,400 rpm (SW32.1 Beckman-Coulter rotor). The VLP bands were harvested from the gradient and stored in CsCl at 4°C. The expression of VP60 was confirmed by 10% SDS-PAGE gel and Western Blot. SDS-PAGE gels were stained with Coomassie Brilliant blue stain. Western blots were performed using Trans-Blot SD semi-dry Transfer cell (Bio-Rad, CA, USA) to transfer the protein to a nitro immobilon membrane (Millipore, Merck, Darmstadt, Germany). The membrane was
blocked with 1% casine alanate then stained with anti-RHDV primary antibody and anti-rabbit HRP as the secondary antibody. Both the SDS-PAGE and Western blot were imaged on a ChemiDoc (Bio-Rad). The presence of gp33 or OTI peptide was confirmed by Mass Spectrometry, which was carried out at the Centre for Protein Research, University of Otago. Dialysis was carried out to remove the CsCl. Dialysis tubing (Spectrum Laboratories, Auckland, NZ) (50 kDa MWCO) was loaded with 1 ml of VLP and placed in a 400 ml beaker with sodium phosphate buffer and placed at 4°C with stirring. Buffer was changed after 6 hr, overnight and 4 hr respectively. The purified VLP were removed from the dialysis tube and the concentration was determined using a nanodrop spectrophotometer with an extinction coefficient of 78,000 and molecular weight 60 kDa. Samples were placed in an equal volume of sterile glycerol and stored at -20°C.

2.4 Electron Microscopy
Assembly of the VP60 into VLP particles was confirmed by Electron Microscopy and was carried out at the Otago Centre for Electron Microscopy, University of Otago. Plasma-glowing grids was carried out in the Edwards E306A vacuum coater. Grids were placed onto a piece of filter paper and placed in the middle of the stage to be coated. Negative staining was then carried out where the plasma coated carbon grid had 10 µl of the VLP suspension added on top of the grid and left for 60 sec. The grid was then blotted dry and allowed to air dry before adding 10 µl of phosphotungstic acid stain. The stain was immediately blotted away and the grid was completely dried. Images were taken on the Phillips CM100 transmission electron microscope.

2.5 Coupling OT-II peptide
VLP.OTI and VP60 preparations were dialysed in coupling buffer with a buffer exchange occurring at 4 hr, overnight and 2 hr respectively. The concentration of the samples was determined on a nanodrop spectrophotometer and 10x molar excess of Sulfo-SMCC (Pierce, IL, USA) was added for 30 min with rotation. Dialysis of the
VLP + Sulfo-SMCC was carried out with a buffer exchange at the time points above. OT-II peptide CISQAVHAAHAIEINEAGR (JPT Peptide Technologies, Berlin, Germany) was then added to the VLP preparations at 5x molar excess for 1 hour with rotation. A final dialysis step was carried out as above. The VLP.OTI.OTII and VLP.OTII were stored in an equal volume of sterile glycerol at -20°C.

2.6 Mice

Specific pathogen free C57BL/6 (MHC haplotype H-2b) mice were obtained from Animal Resource Centre, Western Australia and housed in the animal facility within the Department of Microbiology and Immunology. All experimental protocols using these mice as recipients for in vivo experiments were approved under AEC 1/11 and donors for the in vivo cytotoxicity experiments were covered by ET 39/10 and ET 20/11 approvals. Transgenic OT-II mice expressing T cell receptors recognising the ovalbumin peptide sequence ISQAVHAAHAIEINEAGR complexed to MHCII were obtained from the Hercus Taieri Research Unit (HTRU), University of Otago and used for the adoptive transfer experiment (ET 39/10). Genetically modified mice expressing the human Diphtheria Toxin Receptor fused to Enhanced Green Fluorescent Protein under the control of the langerin gene (langDTREGFP) were obtained from Dr Ian Hermans at the Malaghan Institute of Medical Research with a material transfer agreement from Dr. Bernard Malissen and bred within the departmental animal facility. Protocols using these mice were approved under AEC 42/10. The langDTREGFP mice express the diphtheria toxin receptor and the EGFP protein along with the langerin protein such as in langerin+ DC and Langerhans cells (LC). As mice do not normally express diphtheria toxin receptors this specific subset of cells can be depleted. The depletion is only temporary with langerin+ DC repopulating after 2-3 days and LC repopulating up to 4 weeks after the depletion.

2.7 Genotyping

Around 10 – 12 drops of blood was collected from the lateral tail vein of transgenic OT-II mice into 1 ml Alseviers solution. The cells were spun down at 300 g for 6 mins and lysed in 2 ml of warm RBC lysis buffer. Cells were washed and then
stained with 2 µl of CD4-APC, Vα2-PE (antibody against TCR variable region alpha chain 2), Vβ5.1-biotin (antibody against TCR variable region beta chain 5.1) (BioLegend, CA, USA) and incubated for 30 min on ice. Excess antibody was washed off and 2 µl of Streptavidin-PerCP antibody was added to all tubes that had previously been stained with Vβ5.1-biotin. Cells were incubated for a further 30 min on ice, washed again and resuspended in 100-200 µl FACS buffer. Cells were analysed by flow cytometry, as described in 2.14.

2.8 AutoMACS sorting

Spleens were collected from OT-II positive mice. A single cell suspension was generated by passing the splenocytes through a 70 µm cell strainer with DPBS + 5% FCS. Red blood cells were lysed in 2 ml of warm RBC lysis buffer for 3 min at 37°C. The cells were washed twice in DPBS + 5% FCS before being counted in trypan blue. Thirty microlitres of cold MACS buffer and 3.3 µl of CD4 MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany) were added per 10^7 cells. Cells were incubated with CD4 beads for 15 min at 4°C. The cells were washed in 1-2 ml of MACS buffer per 10^7 cells and were centrifuged for 10 mins at 300 g and 4°C. Cells were resuspended in 500 µl of MACS buffer per 10^8 cells. A positive selection (Possel) was selected on the AutoMACS separator, with the negative fraction passing through the magnetic column and then washing the positive fraction from the column. The CD4 positive fraction was collected and washed twice in DPBS and counted. CD4 cells were resuspended at 1x10^8/ml in DPBS and injected intravenously into mice.

2.9 Adoptive Cell Transfers, langerin^+ DC depletions and Vaccinations

For the adoptive transfer of OT-II cells, each C57BL/6 mouse was injected intravenously with 1x10^7 OT-II CD4 AutoMACS (Miltenyi Biotech) sorted cells in 100 µl of DBPS.
To deplete langerin positive DC, each langDTREGFP mouse received a 100 µl injection of 500 ng diphtheria toxin in DPBS or a PBS mock injection intraperitoneally twice or four times. Each injection was 2-3 days apart. On day 3 each animal received a 25 µl intradermal injection of 50 µg VLP gp33 in DPBS to both flanks.

For VLP vaccinations, animals were immunised with 100 µg of the appropriate VLP or PBS, delivered subcutaneously in 100 µl volumes. Experiments where a boost vaccination was required, the second vaccinations were administered 2 weeks after the first vaccination. Where adjuvant was required, 25 µg per mouse of CpG ODN 1826 adjuvant (GeneWorks, SA, Australia) was administered with the VLP.

For the in vivo cytotoxicity assay mice received intravenous injections of 200 µl volumes of the CFSE (Carboxyfluorescein succinimidyl ester (Sigma, MO, USA)) labelled cells, as described in 2.13 receiving 2x10⁷ cells total. All animals were monitored several hours after injection to ensure that there were no adverse reactions.

2.10 Serum preparation

Mice were bled from the lateral tail vein to collect 150-200 µl of blood from mice previously vaccinated with VLP.OTI or PBS followed by a boost vaccination of VLP.OTI or PBS two weeks later. Blood was collected two weeks after the boost vaccination. Blood was kept at room temperature overnight to allow blood to clot. Serum was collected and stored in 30 µl aliquots at -80°C.

2.11 Antibody detection ELISA

ELISA 96 well plates were coated, allowing for blank controls, with 1 mg of VLP per well in 50 µl of coating buffer (Appendix 2) overnight at 4°C. Plates were washed 6 times in ELISA wash buffer (Appendix 2) and blocked using 200 µl/well blocking buffer (pH 9.62) (Appendix 2) for 1 hour at 37°C. Plates were washed as above in wash buffer. A serial dilution of sera in blocking buffer from 1/10 to 1/10⁵
was added 100 µl/well, including a ‘no serum’ control, and incubated overnight at 4°C. The wash step was repeated. Peroxidase goat anti-mouse Ig (Zymed, Life Technologies) was added at a 1/5000 dilution and incubated for 30 min at 37°C. The plate was washed again and 100 µl/well of TMB substrate was added. The colour was allowed to develop at room temperature and the reaction was stopped using 1N H₂SO₄. The optical density was measured on a microplate reader (Bio-Rad) at 450 nm.

2.12 VLP uptake assay
Bone marrow dendritic cells (DC) were generated from the hind legs of C57BL/6, washing the cells out with DPBS + 5% FCS. Cells were counted and resuspended at 2x10⁶ cells/ml in complete DMEM containing granulocyte macrophage colony stimulating factor (GM-CSF) at 20ng/ml. Five millilitre per well was added to a six well plate and incubated at 37°C, 10% CO₂. After 4 days incubation the top 3 ml of media was removed and replaced with equal volume of new media. Non-adherent cells were harvested after 6 days of incubation by removing the media and washing the well out using DPBS + 5% FCS. Harvested DC were resuspended at 1x10⁷ cells/ml in cDMEM. In a 24 well plate 50 µl of cell suspension was incubated with 15 µg of VLP.gp33.dylight 633 (provided by the Ward laboratory) and with or without 2%, 1% or 0.1% serum at 37°C or 4°C for 30 min, 2 hr or 4 hr. Cells were harvested and washed at the specified time point and stained with a DC cell marker, CD11c-PE antibody. Cells were incubated with 2 µg of CD11c-PE antibody (BioLegend) at 4°C for 30 min. Following incubation cells were washed and resuspended in 200 µl FACS Buffer. Cells were analysed by flow cytometry as described in 2.14.

2.13 In vivo Cytotoxicity Assay
Single cell suspensions were prepared from the spleens of the C57BL/6 donor mice as described previously (para 2.8) and cells were resuspended at 2x10⁷ in DMEM + 5% FCS. Cells were either pulsed with peptide (gp33 or OTI) at various
concentrations or remained unpulsed and incubated for 3hrs at 37°C, 10% CO₂. Cells were washed in DPBS three times and resuspended at 4x10⁷ per ml. Pulsed cells were then labelled with an equal volume of CFSE diluted 1/2500 from a stock concentration of 10 mM, (CFSE<sub>hi</sub>) while the unpulsed cells were labelled with CFSE diluted 1/25,000 (CFSE<sub>lo</sub>), for 8 mins. An equal volume of FCS was then added to the CFSE cell suspension. Cells were washed thoroughly three times and resuspended at 1x10⁸ in DPBS. CFSE<sub>lo</sub> and CFSE<sub>hi</sub> cells were mixed together at a ratio of 1:1, and injected intravenously into the vaccinated mice. In langerin depletion experiments 100 µl of blood was taken from the tail vein of mice at various time points post CFSE target cell transfer. The blood samples were collected in 1 ml of Alseviers solution. The cells were washed after the red blood cells were lysed and resuspended in 100 µl FACS buffer. For all experiments mice were euthanized 48 hours after the transfer of target cells and splenocytes were prepared as previously described (para 2.8). Splenocytes were resuspended between 1x10⁶ and 1x10⁷ and analysed by flow cytometry as described in 2.14. The ratio of CFSE<sub>lo</sub> to CFSE<sub>hi</sub> cells was used to determine the specific lysis.

\[
\text{% Lysis} = 1 - \left\{ \frac{(%\text{CFSE}^{\text{hi}}/\%\text{CFSE}^{\text{lo}} \quad \text{vaccinated})}{(%\text{CFSE}^{\text{hi}}/\%\text{CFSE}^{\text{lo}} \quad \text{PBS})} \right\} \times 100
\]

2.14  FACS Analysis

Samples were analysed using BD FACSCalibur Flow cytometer (BD Biosciences, California, USA), BD Cell Quest Pro (BD Biosciences) and FlowJo version 8.8.6 (Stanford University, Tree Star, Inc. OR, USA)

2.15  Statistical analysis

Statistical analysis was carried out using Prism version 5 (GraphPad Software, Inc, CA, USA). Statistical significance was calculated by two-way ANOVA, one-way ANOVA or paired Student’s t test.
Chapter 3

Results

3.1 Recombinant VLP.gp33 production.

The techniques used to produce and purify RHDV VLP have been established in the Ward laboratory and used to great success\(^{46}\). Using these protocols and carrying out SDS-PAGE and Western blot analysis, VLP.gp33 was successfully produced of a sufficient quality to allow \textit{in vivo} and \textit{in vitro} work in mice. Infection of Sf21 insect cell suspension cultures with the viral inoculum stocks resulted in the expression of the recombinant RHDV VP60.gp33 protein (Fig 3.1a, b). The predominant band seen in lanes 2 and 3 in both SDS-PAGE gel and Western blot, corresponds to that of the VP60 protein, which is 60 kDa in size. When the band of 60 kDa was compared between the total cell suspension sample (lane 1) and the samples after the various purification steps (lane 2-3) an increase in the purity of the VLP preparation was observed. The purification steps sufficiently excluded the unwanted proteins while retaining a high quantity of the desired VP60.gp33 protein. Although SDS-PAGE and Western blot are able to confirm the production and the purity of the purified peptide, the self- assembly of these proteins into particles requires visualisation by transmission electron microscopy (Fig 3.1c, d). The characteristic cog-like structures are indicative of the VLP particle structure and confirm the self-assembly of the 35 – 40 nm particles\(^{46}\). This work demonstrates the successful production of VLP.gp33 of a sufficient quality.
Figure 3.1: Analysis of RHDV VLP.gp33. a) 10% SDS-PAGE of recombinant VLP.gp33 fractions from CsCl gradient (Lanes 2-3) with total cell suspension sample (Lane 1). Marker bands are indicated in kDa. A total of approximately 4 µg of protein was loaded in a volume of 10 µl per lane. b) Corresponding Western blot developed with anti-RHDV and anti-rabbit HRP. c) Transmission electron micrograph of VLP.gp33. Scale of 100 nm. d) Transmission electron micrograph of VLP.gp33, scale 200 nm.
3.2 Langerin\(^+\) DC cross-presentation of VLP peptide initiates \textit{in vivo} cytotoxic T cell responses.

The ability of VLP to initiate anti-tumour responses has been established\(^{48}\) and this has been shown to rely on the ability of DC to cross present the antigens to CD8 T cells\(^{64}\). To assess the importance of langerin\(^+\) DC cross-presentation of VLP on the \textit{in vivo} cytotoxic response of CD8 T cells, the langerin DTREGFP mouse model\(^{100}\) was utilized.

LangDTREGFP mice received two Diphtheria toxin injections administered two days apart allowing langerin\(^+\) cells to be temporarily depleted. Following langerin\(^+\) DC depletion, mice were immunised with VLPgp33 or gp33 peptide only (Fig 3.2a). \textit{In vivo} cytotoxicity assays were initiated 1 week after vaccination to determine the cytotoxic response in the absence of the langerin\(^+\) DC. Antigen (gp33) pulsed target cells were adoptively transferred into these mice and specific lysis was determined at various time points by analysing the ratio of peptide pulsed target cells to unpulsed cells. Where the langerin positive cells were depleted over the first 6 days of the experiment (Fig 3.2a), there was a significant reduction in the cytotoxicity response 24 hours post target cell transfer. However, at 48 hours, there is no difference between the various groups of animals either with or without langerin\(^+\) cell depletion or with or without the vaccination. The lack of difference between the groups was probably because the langerin\(^+\) DCs had begun to repopulate because they have been shown to repopulate as early as 2 days following depletion\(^{101}\). To determine the impact of continual depletion DT was administered throughout the time course of the experiment (Fig 3.3a). When the cells were continually depleted (Fig 3.3b), the depletion significantly reduced the generation of cytotoxic responses against the target peptide. Without depletion of the langerin\(^+\) DC (Fig 3.3b, blue line), VLP.gp33 was able to elicit cytotoxic responses between 20\% and 30\% by 16 hours and increased to an average of 35\% by 48 hours. In comparison animals that were continually depleted of langerin\(^+\) DC (Fig 3.3b, red line), the specific lysis of target cells was no greater than 15\%.

Using this mouse model we were able to show that by depleting the langerin\(^+\) DC population the cytotoxic responses against the target peptide gp33 was significantly
Figure 3.2: Langerin⁺ DC is important for cross-presentation. a) Schematic for the depletion of langerin⁺ DC. Diphtheria toxin was administered on days 0 and 2. Intradermal injections on day 3 with VLP.gp33 or gp33 peptide were administered to both flanks of the animals. A total of 50 µg of vaccine was injected. CFSE target cells were pulsed with 10 µg gp33 peptide. Blood samples were analysed 4, 24, 48 and 72 hrs after CFSE target cell transfer. b) Specific lysis following depletion of langerin⁺ DC. Individual animals plotted, lines are plotted to the mean value of each group. Statistical significance calculated with two way ANOVA with Bonferroni posttests, * p<0.05.
Figure 3.3: Continual depletion of Langerin\(^+\) DC reduces \textit{in vivo} cytotoxicity responses of CD8 T cells. \textbf{a)} Schematic for the continual depletion of langerin\(^+\) DC on days 0, 3, 6 and 9. Intradermal injections were administered to both flanks of the animals on day 3 with VLP.gp33 or PBS. A total of 50 µg of vaccine was injected. CFSE target cells pulsed with 10 g gp33 peptide. Blood samples analysed at 4, 16, 24 and 48 hr post CFSE target cell transfer. \textbf{b)} Specific lysis with continual depletion of langerin\(^+\) DC as described above. Individual animals plotted, lines are plotted to the mean value of each group. Statistical significance calculated with two way ANOVA with Bonferroni posttests. , ** p<0.001.
reduced. This indicates the importance of these cells, enabling cross-presentation of VLP associated peptide to CD8 T cells.

### 3.3 Recombinant VLP.OTI preparation and Chemical coupling of OTII peptide

The help provided by CD4 T cells has been shown to be important for the activation of CD8 cells as well as in the memory development of long-lived CD8 cells\(^9\). The role of CD4 T cells in amplifying the CD8 T cell response was therefore investigated. In order to study this, cells from transgenic animals needed to be utilized. As there is no CD4 T cell transgenic mouse model of LCMV, the model investigated was changed to the Ovalbumin model. The new model changed peptides from gp33 (derived from LCMV) to OTI and OTII peptides derived from Ovalbumin, which bind to CD8 and CD4 cells respectively. The preparation of VLP.OTI was carried out using the same protocols as the VLP.gp33 preparation using recombinant viral inoculum stocks provided by the Ward laboratory (para 2.3). Infection of Sf21 insect cell suspension cultures with the recombinant VP60.OTI baculovirus resulted in the expression of the recombinant RHDV VP60.OTI protein in lane 2 (Fig 3.4a).

SDS-PAGE gels indicate a band of approximately 60 kDa (lane 1) corresponding to the presence of the VP60 protein only. The larger band in lane 2 corresponds to the size shift of the VP60 protein caused by the addition of the OTI peptide, which is 0.963 kDa in size. In lane 3 the largest protein band corresponds to the addition of the OTII peptide of 1.88 kDa to the VP60 and OTI peptides resulting in a total molecular weight of around 63 kDa. The transmission electron microscopy image (Fig 3.4b) shows that with the recombinant expression of the OTI peptide along with the VP60, particles are still able to form. Mass spectrometry was also carried out on a sample of VLP.OTI to ensure the expression of both the VP60 protein and the OTI peptide (data not shown). Using these checks we were able to guarantee the presence of the heterologous epitopes.
Figure 3.4: Analysis of RHDV VLP.OTI and peptide coupling. a) SDS-PAGE of recombinant VLP.OTI and coupled VLP.OTI.OTII. Gel was loaded with VLP (lane 1), VLP.OTI (Lane 2) and VLP.OTI.OTII (Lane 3). Marker bands are indicated in kDa. A total of 5-10 µg of protein was loaded in a volume of 10 µl per lane. b) Transmission electron micrograph of VLP.OTI, scale 200 nm.
3.4 **Adoptively transferred CD4 cells are able to improve cytotoxic T cell responses.**

To investigate the requirement of CD4 help on the *in vivo* cytotoxic responses of CD8 cells, CD4 cells from transgenic OTII mice were transferred to C57BL/6 mice prior to VLP vaccination. Initial experiments were performed to develop the assays that would be used to test the efficacy of the VLP. These assays only used peptide to vaccinate animals, rather than the VLP. Cells were obtained from OT-II mice and sorted by AutoMACS magnetic separation. Pre-sorted cells were 17.6% for CD4 (Fig 3.5a) whereas Positive selection sorts using CD4 magnetic beads gave yields of 96.5% purity (Fig 3.5b). These sorted cells were then intravenously injected into recipient mice. In preliminary work (Fig 3.6b) female C57BL/6 mice received a transfer of CD4 cells from female OTII transgenic mice or PBS alone. One day after transfer, mice were vaccinated with OTI peptide or PBS. One week following vaccination CFSE target cells were pulsed with OTI peptide and transferred as previously described. The result shows that in the absence of OTII peptide, the transferred CD4 cells were not specifically activated. VLP was then utilized in these experiments. Mice vaccinated with VLP.OTI (Fig 3.6c) with or without the addition of CD4 cells induced a strong cytotoxic response, with 75% of target cells destroyed. In this experiment the adoptively transferred CD4 T cells were not activated first with specific antigen and this suggests that VLP without OT-II peptide do not provide substantial ‘bystander help’. In the next experiments OT-II peptide was included: mice were vaccinated on day 1 with VLP.OTI only, VLP.OTI and OTII peptide or VLP.OTII and OTII peptide following the same protocol with the exception that male C57BL/6 and OTII transgenic mice were used instead of females, due to availability. One week after vaccination with VLP.OTI and in the presence of OTII peptide, the *in vivo* cytotoxicity was significantly improved (Fig 3.7). Assessing the *in vivo* cytotoxicity 2 months after vaccination shows similar trends with the activation of the CD4 cells with OTII peptide and activation of the CD8 cells by VLP.OTI giving the best *in vivo* cytotoxic response (Fig 3.8b). By providing CD4 cells and activating them with the appropriate OTII peptide, the *in vivo* cytotoxic responses are significantly improved demonstrating the importance of activating both CD4 as well as CD8 T cells in vaccinations against tumour antigens.
Figure 3.5: Analysis of AutoMACS CD4 positive sort. a) Representative Flow cytometry FSC, SCS plot prior to sort. Lymphocytes gated on size and granularity. b) Histogram of CD4 positive cells pre-sort. c) Histogram of CD4 positive cells post-sort. Gates indicates percentage of CD4 positive cells, gated against isotype control (red).
Figure 3.6: Adoptive cell transfer of OTII CD4 T cells has no effect on in vivo cytotoxicity induced to VLP.OTI in the absence of OTII peptide. a) Schematic of experimental protocol with cell transfer on day 0 and vaccination on day 1. CFSE target cells pulsed with 10 µg OTI peptide and transferred day 8. Analysis carried out 48 hr after CFSE transfer. b) In vivo cytotoxicity 1 week following adoptive transfer of CD4 OTII sorted cells and vaccination with OTI peptide. c) In vivo cytotoxicity 1 week following adoptive transfer of CD4 OTII sorted cells and vaccination with VLP.OTI. Individual animals plotted with standard error.
Figure 3.7: Adoptively transferred OTII CD4 cells improve cytotoxicity of VLP.OTI in the presence of OTII peptide. As per the schematic in figure 6b, *In vivo* cytotoxicity 8 days after adoptive transfer of CD4 OTII sorted cells followed 24hr later by vaccination with VLP.OTI or VLP.OTII +OTII peptide or VLP.OTI + OTII peptide. CFSE target cells were pulsed with 0.01 µg OTI peptide. Male OTII and C57BL/6 mice used. Spleens were analysed 48 hours after CFSE transfer. Individual animals plotted with standard error. Statistical significance calculated by paired Student’s t test, * p>0.05.
Figure 3.8: Adoptively transferred OTII CD4 cells improve cytotoxicity 2 months after vaccination with VLP.OTI in the presence of OTII peptide. a) Schematic of experimental protocol with cell transfer on day 0 and vaccination on day 1. CFSE target cells pulsed with 0.01 µg OTI peptide and transferred day 56. Analysis carried out 48 hr after CFSE transfer. b) In vivo cytotoxicity 56 days after adoptive transfer of CD4 sorted cells and vaccinations. Male OTII and C57BL/6 mice used. Spleens were analysed 48 hours after CFSE transfer. Individual animals plotted with standard error. Statistical significance calculated by paired Student’s t test, * p<0.05.
3.5 Optimisation of the target peptide for *in vivo* cytotoxicity assays.

In the determination of specific lysis, preliminary experiments demonstrated high levels of cytotoxic activity following vaccination with VLP.OTI. To allow differences between the vaccination groups to be observed the amount of peptide used to pulse the target cells was titrated. Reducing the amount of peptide by 10 fold resulted in specific lysis of around 70%. The reduction of peptide to a 1 in 100 dilution or 1 in 1000 dilution resulted in specific lysis of around 40%. (Fig 3.9) A cytotoxic response of around 40% would mean that differences between the vaccination groups would be able to be identified. As a result of this experiment the amount of peptide used in the pulsing of target cells was reduced from 10 µg down to 0.01 µg.

3.6 VLP.OTI.OTII is able to elicit better cytotoxic T cell responses than VLP.OTI.

Having established the requirement of CD4 cells using the adoptive cell transfer, we wanted to investigate the ability of VLP to deliver both the OTII and OTI peptides required for the activation of CD4 and CD8 T cells respectively. Vaccination of mice with recombinant VLP.OTI or the recombinant VLP.OTI with the addition of the OTII peptide coupled, the *in vivo* cytotoxicity was determined 2 weeks and 4 weeks after the boost vaccination (Fig 3.10a and Fig 3.12a). In preliminary experiments the presence of both the OTI and the OTII peptides on the VLP were able to demonstrate a trend towards an increase in the specific lysis of target cells (Fig 3.10b). This was also observed in repeat experiments where the target cells were pulsed with 0.01 µg of target peptide (Fig 3.11). At 2 weeks the differences between VLP.OTI and VLP with both OTI and OTII peptides is significant, whereby the presence of both peptides has increased the cytotoxicity response from around 20% up to around 40%. This response was also noted 4 weeks after vaccination (Fig 3.12b), however this difference was not significant, indicating that there is a trend towards improvement of the function of memory CD8 population. These results demonstrate that the VLP
Figure 3.9: Peptide dilution for target cell coating reduces *in vivo* cytotoxicity. As per the schematic in figure 10a, *In vivo* cytotoxicity 1 week after vaccination with 100 µg VLP.OTI. Target cells were pulsed with 1 µg/ml (1/10), 0.1 µg/ml (1/100), 0.01 µg/ml (1/1000), 0.001 µg/ml (1/10,000). Individual animals plotted, lines are plotted to the mean value of each group.
Figure 3.10: VLP.OTI.OTII induced higher in vivo cytotoxicity than VLP.OTI with high dose peptide on target cells. a) Schematic of the experimental protocol, with vaccination on day 0 and boost vaccination on day 14. CFSE targets pulsed with 10 µg OTI peptide and were transferred on day 28. b) In vivo cytotoxicity 2 weeks after boost vaccination with VLP.OTI or VLP.OTI.OTII. Individual animals plotted with standard error. Statistical significance was calculated by paired Student’s t test, * p< 0.05
Figure 3.11: VLP.OTI.OTII tend to generate better cytotoxicity than VLP.OTI with low dose peptide on target cells. As per the schematic in figure 10a, *In vivo* cytotoxicity 2 weeks after boost vaccination with VLP.OTI or VLP.OTI.OTII. CFSE target cells pulsed with 0.01 µg OTI peptide. Spleens were analysed 48 hours after CFSE transfer. Individual animals plotted with standard error.
Figure 3.12: VLP.OTI.OTII tends to generate better cytotoxicity 1 month after boost vaccination than VLP.OTI. a) Schematic of the experimental protocol, with vaccination on day 0 and boost vaccination on day 14. CFSE targets pulsed with 0.01 µg OTI peptide and were transferred on day 42. Spleens were analysed 48 hours after CFSE transfer. b) In vivo cytotoxicity 4 weeks after boost vaccination with VLP.OTI or VLP.OTI.OTII. Individual animals plotted with standard error.
is able to deliver both peptides effectively to activate both CD4 and CD8 T cells resulting in the improvement of specific lysis. These results also demonstrate the trend towards improvement of CD8 memory function when CD4 help has been provided.

3.7 Timing of CD4 help does not improve CD8 cytotoxicity.
Previous results have indicated that CD4 T cell help significantly improves the in vivo cytotoxic responses during the effector phase of the CD8 T cells and a trend towards the improvement of the functional memory responses. Following on from this we wanted to investigate whether CD4 help was more important during the effector phase or at the memory phase of the CD8 T cell response. To carry out this work mice were vaccinated with VLP to activate CD4 (VLP.OTII) or CD8 (VLP.OTI) or to activate both at the same time (VLP.OTI.OTII). Two weeks after this first vaccination animals that had been vaccinated with VLP.OTII were then vaccinated with VLP.OTI to activate CD8 cells after CD4 activation. Conversely, animals vaccinated with VLP.OTI first were then vaccinated with VLP.OTII i.e. CD4 cells were activated after CD8 activation. Two weeks following the second vaccination all groups were assessed by in vivo cytotoxicity assay (Fig 3.13a). Across the three different VLP vaccination groups no significant difference was identified indicating that in the presence of CD4 help, regardless of when it is activated relative to CD8 activation, is important (Fig 3.13b). In the repeat of this experiment (Fig 3.14b) the time after the second vaccination was extended to 4 weeks. Again no significant difference can be seen across the three vaccination groups. These results indicate that while CD4 help is important for the improvement of CD8 cytotoxic responses, the timing in the activation of CD4 T cells either before, after or at the same time as CD8 T cell activation has no significant effect on the degree of cytotoxic response generated.
Figure 3.13: The timing of CD4 activation does not affect the in vivo cytotoxic response. a) Schematic of the experimental time protocol, with vaccination on day 0 and alternative vaccination on day 14. CFSE targets pulsed with 0.01 µg OTI peptide and were transferred on day 28. Analysis carried out 48 hrs after CFSE transfer. b) In vivo cytotoxicity following vaccination with VLP.OTIOTII or VLP.OTII followed by vaccination. Individual animals plotted, with standard errors.
Figure 3.14: The timing of CD4 activation does not affect the in vivo cytotoxicity response 1 month after the final vaccination. 

a) Schematic of the experimental time protocol, with vaccination on day 0 and alternative vaccination on day 14. CFSE targets were transferred on day 28. Analysis carried out 48 hrs after CFSE transfer. 

b) In vivo cytotoxicity after vaccination on day 0 of VLP.OTI, VLP.OTII or VLP.OTI.OTII. Mice received the alternate vaccination on day 14. Other mice were did not receive a second vaccination. Individual animals plotted with standard error.
3.8 **CpG adjuvant and VLP are able to generate the optimal *in vivo* cytotoxic responses.**

Adjuvants are included in vaccination strategies to increase the efficacy of the vaccine. Here we wanted to determine what vaccination strategy provides a better cytotoxic immune response; VLP.OTI.OTII or VLP.OTI with the addition of CpG adjuvant vaccination.

Groups of C57BL/6 mice were vaccinated with VLP.OTI with or without CpG or VLP.OTI.OTII (Fig 3.15a) and were assessed for cytotoxic responses 4 weeks (Fig 3.15b) or 8 weeks (Fig 3.16) following the vaccination.

In the presence of CpG the VLP.OTI vaccination group demonstrated the highest specific lysis of between 92 and 98% specific lysis, indicating an improved cytotoxic response when CpG adjuvant is administered alongside the VLP. This result was seen at both 4 weeks and at 8 weeks after the vaccination. Comparison between this and the presence of both the OTI and the OTII peptide on the VLP showed that CpG was able to provide the better response. These results indicate that the presence of adjuvant such as CpG in the presence of the VLP is ideal to get the best cytotoxic responses and is important in early responses such as those seen at 4 weeks and in later responses seen at 8 weeks following vaccination.

3.9 **Serum antibody against VLP increases uptake of VLP by Dendritic cells.**

Vaccines typically initiate antibody responses against the antigen that is being presented. In the case to RHDV VLP the particle itself is considered an antigen, therefore antibody can be produced against it as well as the antigenic peptide, it carries. Pre-existing antibody to vaccines can inhibit boosting whilst it has also been shown that pre-existing antibody to vaccines are beneficial and target them to antigen presenting cells\(^{102}\). The aim of this experiment was to determine whether or not antibody is present against the VP60 protein and if this is beneficial to the uptake of VLP. To carry out this work an indirect ELISA against the VP60 protein was used to determine the presence of antibody. VLP uptake assays were also used to investigate
Figure 3.15: CpG adjuvant increases VLP.OTI generated in vivo cytotoxicity against OTI peptide coated target cells. a) Schematic of the experimental time protocol, with vaccination on day 0 with or without CpG. CFSE targets pulsed with 0.01 µg OTI peptide, were transferred on day 28. Analysis carried out 48 hrs after CFSE transfer. b) In vivo cytotoxicity 4 weeks after vaccination. Individual animals plotted with standard error. Statistical significance calculated by paired Student’s t test. *** p<0.0001. ** p<0.0032.
Figure 3.16: CpG adjuvant increases memory recall of VLP.OTI generated in vivo cytotoxicity against OTI peptide. 

a) Schematic of the experimental time protocol, with vaccination on day 0 with or without CpG. CFSE targets pulsed with 0.01 µg OTI peptide, were transferred on day 28. Analysis carried out 48 hrs after CFSE transfer. 

b) In vivo cytotoxicity 8 weeks after vaccination. Individual animals plotted with standard error. Statistical significance calculated by paired Student’s t test. *** p<0.0001.
whether or not the uptake of VLP was improved in the presence of the antibody against VP60.

Blood serum samples were collected from mice that had previously received a prime-boost vaccination with VLP.OTI. The vaccinations were administered 2 weeks apart and the blood samples were collected 2 weeks after the last injection (Fig 3.17a). Indirect ELISA was used to determine the presence of antibody in dilutions of the serum using VP60 as the coating antigen and an antibody against mouse IgG as the detector antibody. ELISA was able to detect high levels of anti VP60 antibody in serum dilusions from 1/10 and 1/100 (Fig 3.17b). A titration effect was seen through 10 fold dilutions 1/1000 to 100000. The levels of antibody detected in the serum of mice vaccinated with PBS were minimal showing the sensitivity of the assay.

Using serum from one of the animals which antibody against the VP60 peptide was present, VLP uptake was determined. Bone marrow-derived dendritic cells were incubated with VLP labelled with a fluorescent dye – dylight633 (provided by the Ward laboratory) and various percentages of serum. After incubation for 30 min, 2 hours or 4 hours the cells were analysed for VLP uptake by flow cytometry (Fig 3.18a). In the presence of serum antibodies the uptake was significantly improved after 30 min and less so at 2 hours, however by 4 hours the levels of VLP uptake were all very similar (Fig 3.18b). This shows the importance of the antibody responses in the early uptake of VLP by DC.
Figure 3.17: Antibody against VP60 peptide detected in serum samples. 

a) Schematic of the experimental protocol for indirect ELISA against VP60 proteins. Mice were vaccinated on day 0 followed by a boost vaccination on day 14 with VLP.OTI or PBS. Blood was collected 2 weeks after boost vaccination. Serum samples were diluted for ELISA.

b) Ten fold dilutions of serum from VLP (black) or PBS (blue) vaccinated mice. Antibody levels determined by optical density (OD). Individual animals plotted.
Figure 3.18: Serum VLP antibody enhances uptake by VLP. a) Schematic of experimental protocol. BMDC from hind legs C57Bl/6 mice were cultured for 6 days before stimulation with serum and 15 µg VLP-gp33-dylight633. Cells were harvested at 30 min, 2 or 4 hr after incubation. Cells were stained with CD11c-APC and analysed by flow cytometry. b) VLP uptake assay. Uptake measured by percentage of DC positive for VLP-dylight633. Bars plotted with standard error. Statistical significance was calculated between the PBS vaccinated serum versus the VLP vaccinated serum at the different concentrations and different time points by Two-way ANOVA with Bonferroni posttests. ** p<0.001, * p<0.01
Chapter 4

Discussion

Immunotherapies prime cells of the immune system to generate an appropriate response against a specific target and establish a memory response, to give protection to an individual over a prolonged period of time. One of these therapies is the development of cancer vaccines to be used both therapeutically and prophylactically. RHDV VLP has been shown to act as a platform to effectively deliver heterologous tumour antigens to the immune system. These particles are effective in the induction of the cytotoxic responses to specific tumour antigens. In previous studies the cytotoxic response generated was not 100% effective against tumour challenge, in particular where the specific activation of CD4 cells was not achieved. Therefore this work described in this thesis was carried out to address the role of CD4 helper T cells in the cytotoxic response and to investigate how the cytotoxic response following vaccination with RDHV VLP could be improved.

4.1 Virus-like Particle production

The production and purification of RHDV VLP by recombinant baculovirus protein expression has been shown to be effective for generating high quantities and high quality VLP. Using established techniques the VLP prepared as part of this work was generated with yields similar to yields shown by other research. The baculovirus expression system has been shown to be a fast and simple way to effectively produce VLP following various modifications of VP60. The purification steps are essential for the removal of any impurities derived from baculovirus material, which have been shown to induce immune responses, which are independent of the VLP.
Inoculation of animals with baculovirus has been shown to induce the production of pro-inflammatory cytokines including IFN, TNF and IL-6 in a nonspecific way\textsuperscript{103,104}. The presence of impurities derived from baculoviruses is able to cause danger signals that are detected by toll-like receptors causing the production of these cytokines and thus activate the innate immune response\textsuperscript{103}. To reduce the contaminants that can be produced by this method that would be necessary for use in the clinic, serum-free medium can be utilised to reduce the risk of contamination by minimising the animal proteins that are associated with the medium without affecting the cell growth dramatically\textsuperscript{105}. Downstream processing involving the inactivation of the baculovirus and removal of baculovirus and cellular debris by techniques such as caesium chloride gradient centrifugation or ion exchange is used extensively to purify VLP\textsuperscript{106}. The purification process used for this research, as shown by Peacey et al\textsuperscript{46}, is effective in the removal of cellular debris with up to 99% purity of the VLP and results in a high quality, purified product which does not cause the upregulation of co-stimulatory or activation markers such as CD80/CD86. The purity of the VLP is an important factor to be aware of for the reliability of results. Delivery of VLP free from contaminants means that the cytotoxic response seen are specific to the antigen and not generated by the presence of contaminating proteins.

RHDV VLP have been successfully modified to induce cytotoxic T cell responses against target peptides. Previous work has demonstrated the ease of chemical modification and recombinant expression of antigenic peptides\textsuperscript{46,61}. Recombinant expression of antigenic peptides takes advantage of the capsid proteins ability to self assemble when a peptide is at the N-terminus of the VP60 protein. This region of the VP60 protein includes a flexible linker region, which is important for the formation the particle and is found on the internal face of the particle\textsuperscript{55}. The recombinant expression of short antigenic peptides at this N-terminus is well-tolerated\textsuperscript{107}. The addition of peptides such as gp33 or OTI (SIINFEKL), are of a suitable size for recombinant expression. Generation of recombinant VLP with these peptides associated was successfully carried out (Fig 3.1c, Fig 3.4b). The limitations of recombinant modification places limits on the size of the peptides added and any conformational changes that may be caused by the addition of the peptides. For the
inclusion of larger peptides or whole proteins such as the OT-II peptide or whole OVA protein, which may adversely affect the assembly of the particles, chemical coupling can be carried out.

The successful addition of various antigens to the outside of VLP by chemical coupling has been shown\textsuperscript{46,61,108}. The coupling of antigens to the outside of the particle, rather than the inclusion of the antigen within the particle, means that the antigens are optimally exposed for recognition by immune cells as the antigen is present on the outside of the VLP\textsuperscript{2}. The addition of peptides by chemical linking is a more time consuming method, as extra steps are required. This will have an impact on the scalability of VLP production; aspects such as the ease of manipulation of the VLP will need to be considered to allow for the optimal production.

The modification of VLP by both recombinant expression and chemical coupling can also work well together as has been shown in this work. Using peptides that are MHC restricted is important for determining the involvement of CD4 and CD8 T cells and their impacts on each other. As both the OTI and OTII peptides are too large to be able to be included in a single VLP by recombination using both techniques allowed us to engineer the VLP to include both peptides without compromising the structural integrity of the particle. However by using both methods, the accessibility of both peptides will differ. The OTII peptide coupled to the outside of the particle will be more easily accessible for recognition than the internal OTI peptide. The impact this may have on the activation of T cells is also impacted by the ability of VLP to be presented by both the endogenous antigen presentation pathway and the MHC-I recycling cross-presentation pathway. The result of this is that VLP with both peptides associated are able to activate both CD4 and CD8 cells\textsuperscript{64}.

Although there are limitations with both of the methods of modification, the ease of which both recombinant and chemical coupling can be carried out is beneficial for modification of the particles.
4.2 Target peptide titration and in vivo cytotoxicity assay

In establishing the in vivo cytotoxicity assay the amount of peptide used to pulse the target cells was chosen from the literature and based on previous work in our laboratory. However, work carried out by Peacey et al. had shown that VLP with OTI peptide chemically coupled were unable to elicit anti-tumour responses against B16-OVA tumours. This may be due to B16-OVA tumour cells secreting the antigen rather than express the peptide on the cell surface which may contribute to the lack of cytotoxic response seen. Tumours also down regulate the expression of antigens on the cell surface to reduce immunogenicity, contributing to the reduction in the antigen seen by the immune system. Therefore to generate results that are closer to the biological system that these experiments are based on, the amount of peptide used to coat the target cells was lowered. The aim of this was to reduce the specific lysis down to around 40%. The results of this work lead to the reduction in the amount of peptide used for the in vivo cytotoxicity assays. Using 0.01 µg/ml means that the cytotoxic responses are closer to that seen in the tumour model. The reduction of the target peptide means that the target cells more closely resemble what may be seen by the immune system when a tumour begins to develop.

The in vivo cytotoxicity assay is an effective way to be able to determine the function of cytotoxic cell responses to the vaccination. This method of determining the specific lysis of CTL relies on the ability of CD8 T cells to be primed against a target antigen and to kill a labelled target cell that it later encounters. The use of labelled target cells does not mimic what is seen in a tumour environment as the cells may have higher expression of MHC molecules, the antigen is present on the cells, rather than secreted and the cells do not produce immunosuppressive cytokines like tumours do. The differences between peptide pulsed target cells rather that tumour cells means that the sensitivity of the target cells to CTL-mediated killing is much greater than that of tumour cells. Previous work has also show that the kinetics of the cytotoxic response can also be observed by taking multiple blood samples from animals that had received CFSE-labelled cells (Appendix 3). Analysis of the spleen, lymph nodes or the blood gave similar specific lysis results with no significant difference seen between the different tissues. This highlights the sensitivity of the assay.
4.3 Cross-presentation

VLP are able to induce cytotoxic responses and have been shown to rely on the ability of antigen presenting cells to cross present the peptides to activate CD8 T cells\textsuperscript{48}. There are a large number of DC subsets all with an important function to play in the development of immune responses. The evidence for the role of DC in cross-presentation has been shown across the various subsets, although the different subsets have been shown to cross present to different degrees\textsuperscript{65,110}. For example, CD8α\textsuperscript{+} DC has been shown to cross present antigens more strongly than CD8\textsuperscript{−} DC\textsuperscript{111}. Migratory CD103\textsuperscript{+} DC from the skin has been identified in the cross-presentation of antigen to CD8\textsuperscript{+} T cells in the lymph nodes\textsuperscript{112,74}. Another DC subset called Langerhans cells, which are resident in the skin are also able to cross present antigen to T cells\textsuperscript{113}. In the work carried out by Win et al\textsuperscript{64}, it was shown that the CD8α\textsuperscript{+} DC were responsible for the cross-presentation of RHDV VLP-OVA. The work carried out by Li et al (submitted for publication) has shown the importance of CD8α\textsuperscript{+}, CD103\textsuperscript{+} and CD11b\textsuperscript{+} for the uptake of VLP. Understanding that there are various different DC subsets that take up and cross-present VLP can allow for vaccination to be targeted towards these cells. Among these cells, there are various ways in which cross-presentation can be carried out. Investigation into the pathways of uptake and presentation of RHDV VLP demonstrated the utilisation of the MHC-I recycling pathway by DC\textsuperscript{64}. These studies showed that by blocking the pathways of phagocytosis and macropinocytosis there was a reduction in the uptake of VLP\textsuperscript{64}. The processing of antigens was shown to be TAP and protease independent, whereby blocking these pathways do not affect the processing and presentation of antigen. The importance of cross-presentation of VLP antigens has been shown to be vital for the cytotoxic responses that have been generated to the recombinant antigen. The CD8α\textsuperscript{+} DC has been shown by others to be one of the major cell subsets that are involved in cross-presentation. One subset of CD8α\textsuperscript{+} DC that resides in the skin is the langerin\textsuperscript{+} CD8α\textsuperscript{+} dermal DC\textsuperscript{114}. The langDTREGFP mouse model allows for the depletion of those cells that express langerin, namely the langerin\textsuperscript{+} DC and Langerhans cells. In this work the depletion of the langerin\textsuperscript{+} CD8α\textsuperscript{+} DC was carried out to further determine their role in the cross-presentation of antigen to activate cytotoxic T cells. The transgenic mouse model langDTREGFP developed by
Kissenpfennig et al\textsuperscript{100} was utilised to deplete the langerin\textsuperscript{+} cells including Langerhans cells (LC) and CD8α\textsuperscript{+} dermal DC. The depletion using diphtheria toxin was carried out using 500 ng i.p. which differs from that published by others, however we selected this dose to minimise adverse effects to the animals and to allow for safe delivery of multiple injections\textsuperscript{115}. Although the degree of depletion of cells was not assayed directly in this research, the evidence from the literature shows that this model is effective in the depletion of LC and other langerin\textsuperscript{+} DC following the administration of DT\textsuperscript{100, 101}. Had time allowed for more in depth investigation, this would have ideally been carried along with possible phenotype analysis of the repopulating cells that seemed to have an affect on the generation of cytotoxic T cells. The langerin\textsuperscript{+} DC have been shown to repopulate at a different rate to LC. Following a single injection of DT cells expressing langerin are depleted 24 hours later\textsuperscript{100}. The repopulation of LC does not occur until two weeks following this injection, however the repopulation of CD8α\textsuperscript{+} langerin\textsuperscript{+} DC has been shown to occur less than 5 days after injection\textsuperscript{101}. The effect of this can be seen in Figure 3.2b, where the significant difference seen at 24 hours is lost at 48 hours. The toxicity of DT and number of injections given needed to be considered here although in several studies multiple injections of DT was shown to be administered safely with minimum of harm to the animals\textsuperscript{115}. Investigations into the role of LC, particularly their role in cross-presentation showed that with depletion of these cells cross-presentation still occurred, which suggests an overlapping function of various DC subsets\textsuperscript{116}. This can be seen where the continual depletion has shown a greater negative effect on cross-presentation. Overall the results have shown that by depleting langerin\textsuperscript{+} cells the cytotoxic responses of CD8 T cells is significantly reduced following intradermal vaccination of VLP.gp33.

4.4 Requirement of CD4 Help

Previous work has shown that vaccination with coupled VLP.OTI was unable to protect mice from a tumour challenge whereas mice vaccinated with cVLP.OVA showed an increase in survival\textsuperscript{48}. These results indicate a role for CD4 cells in the activation of cytotoxic T cells. The importance of CD4 T cells for the help that they
provide to other cells, particular cytotoxic T cells means that the activation of these cells along side CD8 T cells should also be investigated. To determine the importance of activating CD4 cell by VLP vaccination, adoptive cell transfer of OTII specific CD4 T cells was carried out. In preliminary experiments the aim was to develop the assay using only the OVA peptide recognised by CD4 cells rather than incorporated into VLP so the efficacy of the VLP could then be determined. The results of this showed that while there is a trend towards the improvement of cytotoxic T cell generation in the presence of CD4 help, in the absence of specific help this was not significant. When the transferred CD4 cells had been activated by the presence of the OTII peptide alongside the VLP vaccination, this trend was significant. As others have demonstrated when there is CD4 help that is specific to the response being generated, there is improvement in degree of the CTL generation and B cell activation leading to improved antibody responses\textsuperscript{117}. The help provided by CD4 T cells has been shown to provide enhanced co-stimulation signals\textsuperscript{94} either by the CD4 cell production of IL-2 or inducing the upregulation of co-stimulatory molecules on DC through the interaction of CD40/CD40L enhancing the activation and of CD8\textsuperscript{+} T cells\textsuperscript{118,119}. This ability of CD4 T cells to provide help through various different effector functions highlights their importance in the immune response.

The generation of long-lived memory cells is thought to occur 4-5 weeks after the clearance of pathogen\textsuperscript{89}, or in this case vaccination. The in vivo cytotoxic responses generated 8 weeks after vaccination indicates that VLP are able to elicit a memory response in the presence of CD4 help. This means that for the CD8 T cells, the signals required for optimal activation are assisted by CD4 help and the cytotoxic responses and memory recall responses are stronger benefiting the efficacy of the vaccine strategy. Based on the assumption that these cells have moved from the effector phase to a memory maintenance phase, the recall response is generated upon re-exposure of the target peptide on the CFSE target cells. These cells were not phenotyped as memory cells in this project, and further investigation into the details of this memory response will need to be followed up in the future.

The requirement of CD4 help has also been demonstrated in experiments where both the CD4 and CD8 T cells were activated by the OTI and OTII peptides associated on
the same VLP. Activating CD4 and CD8 by the same DC has been demonstrated to be important for the co-stimulatory signal\textsuperscript{118}; therefore activating both CD4 and CD8 cells with the same VLP is beneficial to the vaccination strategy.

4.5 Timing of CD4 help

The importance of CD4 T cells has been shown to be in their provision of help at various critical stages of cytotoxic T cell development. The co-stimulation and cytokine help provided by CD4 cells is important for the initial activation and proliferation of CD8 T cells\textsuperscript{120}. CD4 cells activated prior to CD8 T cells can also cause DC to upregulate their co-stimulator markers, providing strong activation signals, reducing the likelihood that T cell anergy occurs\textsuperscript{94}. The benefit of CD4 help following the clearance of the pathogen has also been shown to improve the survival of memory CD8 cells\textsuperscript{121}. The production of cytokines such as IL-7 are important for the survival of these cells and allowing for their rapid proliferation should they become re-exposed to the specific antigen\textsuperscript{122}. This is of importance for prime-boost regimes, for determining when would be the best time to activate CD4 cells in regards to CD8 T cells. These experiments utilised a time course that was based on that of prime boost regimes, however the kinetics of the effector cell response means that the second injection was given during the contraction phase of the cells. This could mean that rather than providing help at a critical stage the help may have been delivered at a non-crucial point in the response\textsuperscript{123}. The activation of CD4 cells early on in the response can also result in CD4 memory cells. These cells may be able to provide help to CD8 cells in the effector phase and also during the memory phase, whereas those activated after may only provide help at the memory phase\textsuperscript{123}. The timing of the vaccinations and experimental design requires more investigation to determine the impact of when CD4 help is required. From this work we have shown that in the presence of help, there is a benefit for CTL responses. With further investigation the question of timing may have a clearer answer.
4.6 Adjuvant

As has been previously shown, vaccination with VLP with the addition of an adjuvant can improve immune responses in vivo\textsuperscript{48,124}. Adjuvants are widely used in vaccines to improve the immune response primed against the antigen of interest\textsuperscript{125} and is becoming common in immunotherapy\textsuperscript{126,127}. Previous work using RHDV VLP has shown that the cytotoxic responses can be improved with the addition of CpG\textsuperscript{48}. Other work carried out has shown that adjuvant can be conjugated to the RHDV VLP particle itself, allowing for the delivery of the adjuvant and antigen to the same APC and demonstrating an improved cytotoxic T cell response\textsuperscript{124}. CpG is a strong inducer of Th1 responses and is a toll-like receptor antagonist shown to improve DC responses, therefore better activation of CD8 T cells\textsuperscript{128}. Results from experiments vaccinating animals with tumour peptides in the presence of CpG showed CD8 T cell responses were able to elicit anti-tumour functions\textsuperscript{129,130}. These experiments also demonstrated that the presence of IL-2 acted in a synergistic way\textsuperscript{129}, indicating the requirement of CD4 cells producing IL-2 to enhance the adjuvant effects. Alternatively it has been shown that CpG is able to bypass the requirement of CD4 help by the interaction of the pathogen pattern recognition receptors resulting in the upregulation of co-stimulatory molecules\textsuperscript{127}. It is proposed that these pathways activate signalling cascades resulting in maturation of DC, therefore making a MHC-I restricted peptide more antigenic in the absence of CD4 help\textsuperscript{131}. These results indicate the importance of CpG adjuvant. The results here are in agreement with the previous work carried out with RHDV VLP\textsuperscript{48}, demonstrating that in the presence of CpG, VLP.OTI was able to elicit significantly better cytotoxic responses to the OTI target cells than vaccination with the VLP.OTI alone (Fig 3.15b, 3.16b).

4.7 Antibody response to VLP

RHDV VLP have been shown to generate both a cellular and humoral immune response, which are beneficial for an anti-tumour response. The humoral response generated to VLP vaccination results in the activation of B cells and the production of antibodies\textsuperscript{56}, particularly with the addition of an adjuvant\textsuperscript{132}. The presence of antibodies against tumour antigens is important for complement-dependent
cytotoxicity, another defence against tumour. The presence of antibodies prior to vaccination can cause issues, particularly for prime-boost strategies, if the antibodies produced are neutralising causing T cell induction can be reduced. The effect of this is the binding of the vaccination components by antibody before they are able to activate the immune system. However, the presence of antibody could also result in more effective uptake by opsonisation, making the antigen more recognisable for phagocytosis. Evidence of improved uptake of VLP by Fc receptor binding, has been shown to enhance cross-presentation of tumour-associated antigens. This receptor binding may then lead to the utilisation of alternative cross-presentation pathways, which may be more effective in the presentation of the tumour antigens. The cross-presentation of VLP has been shown to be via the MHC-I recycling pathway, independent of protease and TAP transporter processing. Altering the uptake of VLP from phagocytosis and macropinocytosis, to a receptor-mediated uptake, may influence the pathways of cross-presentation used.

As RHDV do not naturally infect humans or mice, upon the initial vaccination with VLP there is no antibody present that recognise the particles. Upon the administration of boost vaccination however, IgG antibodies have been shown to be present specific to the VLP. Total antibody levels were found to be high in mice vaccinated with two doses of VLP.OTI (Fig 3.17b). The high levels of antibody identified by ELISA were able to confirm, consistent with others, that antibodies are generated to the VLP structural protein VP60. The identification of the isotypes present was not carried out, and as a result conclusions to the involvement of CD4 help, an important part of isotype switching, cannot be drawn. Had the isotypes present been identified, information into the type of antibody response could also be discussed. For example, the presence of IgM, would indicate that isotype switching has not occurred, however the presence of IgG would indicate that CD4 help has allowed for isotype switching to occur and that there is possibly a role of these antibodies in the opsonisation of the VLP, as IgG has a strong role in opsonisation. The presence of antibody and the effect that it has on the uptake of the VLP vaccination was investigated to determine whether the antibodies present were instigating a neutralising effect or enhancing the uptake of particles by opsonisation. Using an in vivo uptake assay as carried out by Peers-Adams (manuscript submitted
for publication) the uptake of VLP was determined in the presence of serum antibodies. The binding of VP60 specific antibodies allowed for the targeted uptake by Fc receptor binding on DC and seen by the enhancement of uptake at 30 min and 2 hours following the addition of VLP and serum. Although in this work we were able to identify a benefit for the presence of VLP specific antibodies, others have shown that the presence of VLP specific antibodies has little affect on the antigen presentation by DC\textsuperscript{56}. The results indicate that the presence of antibody may be beneficial but this response requires further investigation into whether the improved uptake of VLP by DC improves the responses of T cells.

4.8 Future directions

Much of the work carried out was influenced by time constrains and availability of animals. Investigation into the memory responses by phenotypic analysis would allow insight into the kinetics of the responses that have been indicated by experiments thus far. The memory responses generated by this vaccine would also be of interest for the application of this as a cancer vaccine in the clinic. A hallmark of a good vaccine is the ability to generate long-term memory. Further investigation into the timing of CD4 help is also required to fully determine the importance of CD4 help during CD8 effector phase and memory maintenance. The indications are that help is important during both phases but further investigations would allow the issues that have been discussed to be resolved. Repeats of the adjuvant work, altering the time course and addition of a VLP.OTI.OTII + CpG group would improve on the conclusions already established. Antibody work involving identification of the isotype, kinetics of the response, and further work into the uptake work looking at other time points would also elucidate some of the conclusions that have been indicated as part of this work.
4.9 Conclusions

The overall conclusions from this work is that the *in vivo* cytotoxic T cell response generated against RHDV VLP and the associated tumour antigens can be improved by CD4 help. The ability of VLP to cross-present peptides as demonstrated by Win et al\textsuperscript{64} has flow on effects in the CTL responses generated. This work demonstrates the importance of langerin\textsuperscript{+} DC for cross-presentation of the antigen to activate CD8 T cells. The effect of depleting the langerin\textsuperscript{+} DC results in the ineffective activation of CD8 T cells when vaccinating via the intradermal route and therefore the lack of a cytotoxic response.

The CD4 T cells have been demonstrated to provide help at important stages such as co-stimulation\textsuperscript{120} and the memory maintenance of CD8 memory cells\textsuperscript{121}. In the presence of CD4 help the cytotoxic response have shown improvement during effector phase and memory maintenance phases of CD8 T cell activation. The cytotoxic response was also heightened by the addition CpG adjuvant to the vaccination strategy. This work has shown that *in vivo* cytotoxic responses can be enhanced by the manipulation of VLP to deliver OTII and OTI peptides to CD4 and CD8 T cells respectively and the inclusion of an adjuvant improves the immunogenicity of the vaccination.

VLP have also been shown to be able to generate antibody against VP60 protein. The production of antibody leads to a more rapid uptake of VLP by opsonisation that may benefit the subsequent response. This would mean that for a prime-boost regime the antibodies may not be detrimental to the immune response, but actually may be beneficial.

This work has demonstrated a wide variety of possibilities for further investigation of RHDV VLP as cancer vaccines.
References


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88. **Butz EA, Bevan MJ.** 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 8: 167-75


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Appendix

A.1 VLP Preparation and peptide coupling

Insect Phosphate buffered saline (Insect PBS)

1 nM \( \text{Na}_2\text{HPO}_4 \)
10.5 mM \( \text{KH}_2\text{PO}_4 \)
140 mM \( \text{NaCl} \)
40 mM \( \text{KCl} \)
Make up to 1 L with distilled water. Autoclave

Phosphate Buffered Saline (PBS)

11.5 g \( \text{Na}_2\text{HPO}_4 \)
2 g \( \text{KH}_2\text{PO}_4 \)
2 g \( \text{KCl} \)
80 g \( \text{NaCl} \)
Make up to 1 L in distilled water

Cesium Chloride gradients

1.2 g/cm^3 = 2.85 g \( \text{CsCl} \) + 10 ml milli-Q \( \text{H}_2\text{O} \)
1.4 g/cm^3 = 6.15 g \( \text{CsCl} \) + 10 ml milli-Q \( \text{H}_2\text{O} \)

Coupling Buffer

5.2 g \( \text{NaH}_2\text{PO}_4.2\text{H}_2\text{O} \)
23.66 g \( \text{Na}_2\text{HPO}_4 \)
17.54 g \( \text{NaCl} \)
Make up to 2 L with distilled water, pH = 7.35, Autoclave.
**SDS-PAGE Gel**

**Resolving Buffer**

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<th>Quantity</th>
<th>Description</th>
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<tr>
<td>Tris</td>
<td>18.2 g</td>
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<tr>
<td>H₂O</td>
<td>70 ml</td>
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pH = 8.8 (adjust with HCl)

make up to 100 ml with distilled water

**Stacking Buffer**

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pH= 6.8 (adjust with HCl)

make up to 100 ml with distilled water

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<td>TEMED</td>
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<tr>
<td>10% APS</td>
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<tr>
<td>TEMED</td>
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**10x Electrophoresis Buffer**

144 g  Glycine
30 g  Tris
10 g  SDS
make up to 1 L with distilled water

**SDS Sample Buffer**

0.5 ml  10% SDS
200 µl  glycerol (sterile)
120 µl  Tris (1 M pH= 6.8)
10 µl  1% Bromophenol Blue
70 µl  H₂O
100 µl  2-mercaptoethanol

**Coomassie Blue Stain**

1.25 g  Coomassie Brilliant Blue G-250 (BD-Bioscience)
225 ml  Methanol
45 ml  Acetic acid
250 ml  distilled water

**Destain Solution**

100 ml  methanol
100 ml  acetic acid
800 ml  distilled water

**Western Blot**

**PBS/Tween**

1000 ml  1x PBS
200 µl  Tween
**Casine alanate**

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<tr>
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**Sodium Phosphate buffer**

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<td>Na$_2$HPO$_4$</td>
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<tr>
<td>17.54 g</td>
<td>NaCl</td>
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make up to 2 L, pH= 7.3
A.2 Cell Culture, FACS, Cell sorting and ELISA

DPBS (Dulbecco’s phosphate buffered saline) (Sigma)
Made up according to manufacturer’s instructions
pH adjusted to 7.0

DMEM + 5% FCS (Dulbecco’s modified eagle medium)
200 ml DMEM (Gibco, Invitrogen)
10 ml Fetal Calf Serum

Complete DMEM (cDMEM)
3.75 ml L-Glutamine
5 ml L-asparagine
5 ml L-arginine
5 ml Folic acid
500 ml DMEM

Ammonium Chloride (RBC Lysis buffer)
4.15 g NH₄Cl
0.5 g KHCO₃
0.0186 g EDTA
500 ml deionised water
pH = 7.2

Trypan Blue
0.5 g Trypan Blue
10 ml distilled water

FACS Buffer
1x PBS
0.01% Sodium azide
0.1% Bovine serum albumin (BSA)
Paraformaldehyde 4%
100 ml distilled H₂O
4 g Paraformaldehyde

Alseviers Solution
20.5 g Dextrose
4.2 g NaCl
8.0 g Sodium citrate
1 L milliQ H₂O

AutoMACS buffer
1x PBS
0.5% BSA
2 mM EDTA

AutoMACS running Buffer
1x PBS
0.5% BSA
2 mM EDTA

AutoMACS rinsing buffer
1x PBS
2 mM EDTA

AutoMACS cleaning solution
70% Ethanol

ELISA coating buffer
0.42 g NaHCO₃
make up to 50 ml with distilled water and adjust pH to 9.36
ELISA Wash Buffer
2x PBS
1 ml Tween20

ELISA blocking Buffer
1x PBS
1% BSA
pH = 7.2
A.3 Result not included in chapter 3

**Specific lysis across different samples:** Samples collected 72 hours after transfer of gp33-peptide pulsed target cells. Individual animals plotted with standard error.